Secreted amyloid precursor protein alpha as a therapeutic for insulin signaling dysfunction in the nervous system

By

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ABSTRACT

Background: The amyloid precursor protein (APP) cleavage product secreted amyloid precursor protein alpha (sAPPα) is a neurotrophic factor demonstrated to be protective in animal models of neurodegeneration. Despite evidence that sAPPα activates the insulin signaling pathway the effects of sAPPα on diabetes-induced pathology in the nervous system are unknown.

Hypothesis: Studies by us and others strongly suggest that sAPPα is an activating ligand for neuronal insulin receptors (IRs). Therefore, we hypothesized that sAPPα could inhibit neuronal dysfunction in an animal model of diabetes. This hypothesis was tested in 3 aims.

AIM 1: Because diabetes is a risk factor for Alzheimer's disease (AD) and the two diseases share similar biochemical indices, we examined if sAPPα could inhibit the development of Alzheimer's-like pathology in diabetic brain tissue.

AIM 2: AD brain and diabetic peripheral tissues display a similar pathological profile and many experimental treatments that are successful at reducing pathology in AD mice are also beneficial in the diabetic PNS. Therefore, in this aim, we analyzed if sAPPα could slow the development of diabetes-induced peripheral neuropathy.

AIM 3: In our final aim, we examined the effects of sAPPα overexpressing neural stem cells (sAPPα-NSCs) engrafted into the hippocampi on Morris water maze (MWM) performance of healthy mice.

Results: Analysis of brain tissue from diabetic sAPPα mice revealed that sAPPα blocked the development of Alzheimer's-like pathology in the form of aberrant tau phosphorylation. Additionally, sAPPα decreased diabetes-induced activation of the unfolded protein response

(UPR), a sign that diabetic sAPPα mice maintained better overall brain health compared to diabetic controls. Interestingly, despite *in vitro* evidence that both insulin and sAPPα require activation of the survival kinase AKT for their survival effects, AKT activation was paradoxically increased in wild-type (Wt) diabetic brain tissue and normalized by sAPPα.

We found that sAPPα slowed the development of diabetes-induced thermal hypoalgesia, an indicator of sensory neuropathy, in our model. Cell culture experiments demonstrated that the neurotrophic effects of sAPPα in the PNS are associated with up-regulation of the neuroprotective transcription factor NFκB and increased expression of the mitochondrial antioxidant MnSOD.

In the final set of experiments, we found that hippocampal injections of sAPPα-NSCs altered Morris water maze (MWM) performance of healthy SAMR1 mice. Although future studies are required to determine the effects of sAPPα-NSCs on cognition, these preliminary results nevertheless warrant future studies investigating the therapeutic potential of sAPPα-NSCs.

Conclusion: In total, the results presented in this thesis demonstrate that sAPPα can inhibit pathology in the diabetic nervous system. Therefore, the data generated from these studies has provided a foundation for the development of sAPPα based therapeutics, potentially in the form of sAPPα-NSCs, as a treatment option for diabetes and AD.

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DEDICATION

I would like to dedicate this thesis to my incredible wife Courtney because if not for her love and support this would not have been possible.

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ABBREVIATIONS

- ACSF - artificial cerebral spinal fluid
- AD – Alzheimer's disease
- APOE - apolipoprotein E
- APP – amyloid precursor protein
- Aβ – amyloid beta
- Aβo - oligomeric amyloid beta
- Co-IP - Co - immunoprecipitation
- Cu/ZnSOD – copper zinc super oxide dismutase
- DRG - dorsal root ganglia
- eGFP - enhanced green fluorescent protein
- FoxO - forkhead box class o
- FTD - frontotempoal lobe dementia
- HFD - high fat diet
- IB - immunoblot
- IDE - insulin degrading enzyme
- IGF1R – Insulin-like growth factor 1 receptor
- IHC - immunohistochemistry
- IKK - IκB kinase
- IP - immunoprecipitation
- IR – insulin receptor
- IRS - insulin receptor substrate
- KO - knockout

- LTP – long term potentiation
- MWM - Morris water maze
- NFTs - neurofibrillary tangles
- NFκB – nuclear factor kappa b
- NSC – neural stem cell
- OD - optical density
- ORF - open reading frame
- PI3K - phosphoinositide-3 kinase
- ROS - reactive oxygen species
- RT-PCR - reverse transcriptase polymerase chain reaction
- sAPPα - secreted amyloid precursor protein alpha
- sAPPβ - secreted amyloid precursor protein beta
- STZ – streptozotocin
- T1DM – type 1 diabetes mellitus
- T2DM – type 2 diabetes mellitus
- TBI - traumatic brain injury
- Tg - transgenic
- TGN - trans golgi network
- UPR – unfolded protein response
- WB - western blot
- Wt - wild-type
- β-CTF - beta c-terminal fragment

CHAPTER 1: LITERATURE REVIEW AND HYPOTHESIS[1]

1.1: INTRODUCTION

AD was first identified in 1901 by Dr. Alois Alzheimer after he was presented with 51-year-old Auguste Deter who was suffering from "mental incompetence, aphasia, disorientation, paranoia, and unprovoked bursts of anger". Although symptoms such as these were considered a natural part of aging, Dr. Alzheimer correctly deduced that because Deter was middle age her mental deterioration was the result of neurodegenerative brain disease. Over the next four and half years Deter became increasingly demented until her death at the age of 55. Upon examination of Deter's brain Dr. Alzheimer found microscopic strands of protein which he described as "tangled bundles of fibrils" (neurofibrillary tangles) in addition to "miliary foci" (amyloid plaques). In 1906, at the 37th Conference of South-West German Psychiatrists in Tübingen Alois Alzheimer presented Deter's case as, "a peculiar disease of the cerebral cortex" thus giving birth to the field of Alzheimer's disease research.

AD is the most common form of dementia (Alzheimer's 2016) and by 2050 it's estimated that over 100 million people worldwide will have the disease (Brookmeyer et al. 2007) and the annual cost of treating AD is predicted to exceed $1 trillion in the United States alone (Vellas et al. 2007). AD is characterized by severe cognitive impairment, neuronal loss and by the presence of amyloid plaques, composed of aggregated amyloid beta (Aβ), and neurofibrillary tangles (NFTs), composed of aggregated, hyperphosphorylated tau proteins, in the brain. Confirmation of an AD diagnosis can only take place post-mortem when the presence of plaques and tangles can be documented. In the clinic, probable-AD is diagnosed by deficits in episodic memory (Albert et al. 2011) while procedural memory is spared even during the late stages (Kawai et al.

[1] Sections of chapter 1, were created by Brent Aulston and published in © 2013 Aulston BD, Odero GL, Aboud Z, Glazner GW. "Understanding Alzheimer's Disease" - see permissions section

2002). With advancements in PET imaging amyloid (Rinne et al. 2010) and tau (Chiotis et al. 2016) pathology can now be measured in living patients, therefore diagnosing guidelines may soon move away from the post-mortem paradigm as soon as these new imaging techniques become widely available.

Mounting evidence suggests that impairment of the brain's insulin signaling pathway may be pathogenic in AD (Frolich et al. 1999, Cheng et al. 2012, Talbot et al. 2012). Type 2 diabetes (T2DM) a risk factor for AD (Ott et al. 1996, Cheng et al. 2012) and insulin signaling dysfunction is present in the AD brain even in the absence of systemic diabetes (Talbot et al. 2012). Insulin is critical to brain health and provides protection by stimulating anti-oxidant and anti-apoptotic gene transcription (Duarte et al. 2005, Apostolatos et al. 2012) and deactivating pro-apoptotic machinery (Brunet et al. 1999, Barber et al. 2001, de la Monte et al. 2002). Insulin also negatively influences Aβ accumulation and tau hyperphosphorylation which are the defining characteristics of AD leading to the hypothesis that AD is a brain-specific form of diabetes or "type-3 diabetes" (Steen et al. 2005, Kandimalla et al. 2017).

Interestingly, APP mice develop a pattern of peripheral neuropathy that is similar to that of type 1 diabetic animals such as thermal hypoalgesia, loss of tactile sensitivity, and reduced intraepidermal nerve fibers in the hind paws (Jolivalt et al. 2012). Moreover, presence of the ε4 isoform of the apolipoprotein E (APOE) is a genetic risk factor for both AD and peripheral neuropathy (Corder et al. 1993, Monastiriotis et al. 2013, Hatami et al. 2017) and mutations to APP cause familial AD and familial sensory neuropathy (Shin et al. 2012). Therapeutic strategies that have been successful at treating cognitive deficits in experimental AD mice have also been successful at reversing experimental diabetic sensory neuropathy such as enhancement of the transcriptional co-activator PGC1α by resveratrol (Roy Chowdhury et al. 2012, Gong et al. 2013)

and overexpression of the antioxidant MnSOD (Vincent et al. 2007, Dumont et al. 2009). In total, AD and sensory neuropathy are connected biochemically and may be treatable by similar therapeutics.

Work by us and others demonstrates that the APP cleavage product sAPPα activates neuronal IRs (Jimenez et al. 2011) and therefore sAPPα may be protective in the diabetic nervous system. sAPPα is protective against cognitive impairment in AD mice (Fol et al. 2016) and against trophic depletion in dorsal root ganglia (DRG) (Nishimura et al. 2003) neuronal cultures suggesting that sAPPα has neurotrophic activity in both the CNS and PNS . With this in mind, we designed a series of experiments to determine if sAPPα could replace the effects of insulin in the diabetic nervous system by examining the effects of sAPPα on Alzheimer's-like pathology in the brain and on diabetes-induced peripheral neuropathy.

1.2: THE AMYLOID HYPOTHESIS OF ALZHEIMER'S DISEASE

Overview - Aβ is a small peptide 38-43 amino acids long that is believed to have a major role in neurodegeneration and pathology of AD (Thinakaran et al. 2008). Aβ monomers are neuroprotective in culture and amyloid toxicity is mediated by Aβ oligomers (Cleary et al. 2005, Giuffrida et al. 2009). Nevertheless, over-production of Aβ is the centerpiece of AD research since all known familial forms of AD feature enhanced Aβ production (Rocchi et al. 2003, Hatami et al. 2017).

Aβ is produced by successive cleavages of the cell surface protein APP by β- and γ-secretases (Zhang et al. 2011b). APP can also be processed by α- secretases, which both prevents amyloid production and produces the neuroprotective product sAPPα (De Strooper et al. 2000).

Therefore, dysregulation of protective α-secretase processing in favor of enhanced amyloidogenic cleavages of APP may play a central role in AD pathogenesis and progression.

APP belongs to a family of transmembrane proteins that includes APP-like protein 1 and 2 (APPLP1/APPLP2). All APP family members are processed in a similar fashion by α, β, and γ secretases (Wasco et al. 1992, Wasco et al. 1993, Coulson et al. 2000), however the Aβ domain is unique to APP. Three isoforms of APP have been identified consisting of 695, 751, or 770 amino acids that arise from alternative splicing of the same gene located on chromosome 21 (Goate et al. 1991). APP 751 and APP 770 are expressed in most tissues and contain a 56 amino acid Kunitz protease inhibitor domain not found in the neuron specific 695 isoform (Kang et al. 1990, Rohan de Silva et al. 1997). Increased neuronal expression of 770 and 751 isoforms has been linked to increased Aβ production and their expression is elevated in the AD brain (Menendez-Gonzalez et al. 2005).

While the physiological role of APP remains unknown, APP likely plays a role in neurite outgrowth, synaptogenesis, neuronal trafficking along the axon, transmembrane signal transduction, cell adhesion and calcium metabolism (Zheng et al. 2006, Muller et al. 2017). APP concentrations are elevated in the brain during the prenatal period in mice which implies a role of APP in brain development (Loffler et al. 1992, Sosa et al. 2017). In the adult brain, APP is expressed in regions of synaptic modification (Loffler et al. 1992, Caldwell et al. 2013) and increases hippocampal neuronal response to glutamate (Tominaga-Yoshino et al. 2001).

Alpha-secretase processing of APP: APP is synthesized and transported through the Golgi apparatus to the trans Golgi network (TGN) (Hartmann et al. 1997, Xu et al. 1997, Greenfield et al. 1999). From there, APP can be transported in secretory vesicles to the cell surface where α-secretases reside (Parvathy et al. 1999). APP processing by α-secretase

precludes and prevents Aβ production, and while many candidates have been suggested, ADAM10 has been acknowledged as the physiologically relevant α-secretase enzyme (Kuhn et al. 2010). The physiological role of APP may require α-secretase processing as abnormalities in APP knockout (KO) mice can be reversed by sAPPα treatment (Ring et al. 2007).

Beta-secretase processing of APP: If not cleaved by α-secretase, APP can be alternatively processed by β and γ secretase enzymes to produce Aβ. BACE1 is an aspartyl protease that is universally considered the major β-secretase enzyme (Cai et al. 2001). Because of the cellular localization of BACE1, it is hypothesized that the majority of Aβ production occurs in the golgi or endosomes (Koo et al. 1994, Thinakaran et al. 1996). BACE1 cleavage of APP produces a β-carboxyl terminal fragment (βCTF), that can be further processed into Aβ, and the secreted amyloid precursor protein beta (sAPPβ) (Zhang et al. 2011b). sAPPβ differs from sAPPα by only 16 amino acids yet produces minimal neurotrophic effects (Furukawa et al. 1996). sAPPβ has been identified as a ligand for Death Receptor 6 which mediates axonal pruning and neuronal death (Nikolaev et al. 2009). Familial AD APP mutations, which are present in 5-10% of AD cases (Bekris et al. 2010), may cause Aβ overproduction by decreasing α-secretase processing of APP (Haass et al. 1994) or by increasing the susceptibility of APP towards β-secretase cleavage (Zhou et al. 2011).

Gamma-secretase processing of APP: Generation of Aβ requires additional processing of the βCTF by γ-secretase. The γ-secretase complex consists of PS1 or PS2, nicastrin, APH-1 and PEN2 (De Strooper 2003, Kimberly et al. 2003). Outside of the APOE ε4 allele (Corder et al. 1993) all genetic predispositions to AD involve mutations to APP or presenilin 1 and presenilin 2 (Price et al. 1998, Li et al. 2000, Selkoe 2001, Janssen et al. 2003). Presenilin mutations may increase Aβ production by increasing γ-secretase activity and altering APP

trafficking (Naruse et al. 1998, Kim et al. 2001, Wirths et al. 2002, Kimberly et al. 2003). Like BACE1 processing, γ-secretase cleavages occur in the TGN (Xu et al. 1997, Kimberly et al. 2003) where Aβ peptides of various lengths are generated including Aβ40, which composes the majority of the Aβ produced (Burdick et al. 1992), and Aβ42, the more toxic and aggregate-prone species (Roher et al. 1993, Mann et al. 1996). A decreased Aβ40/Aβ42 ratio has been implicated in AD pathogenesis (Fryer et al. 2005, Wiltfang et al. 2007).

Figure 1: Mechanisms of Aβ overproduction in Alzheimer's disease

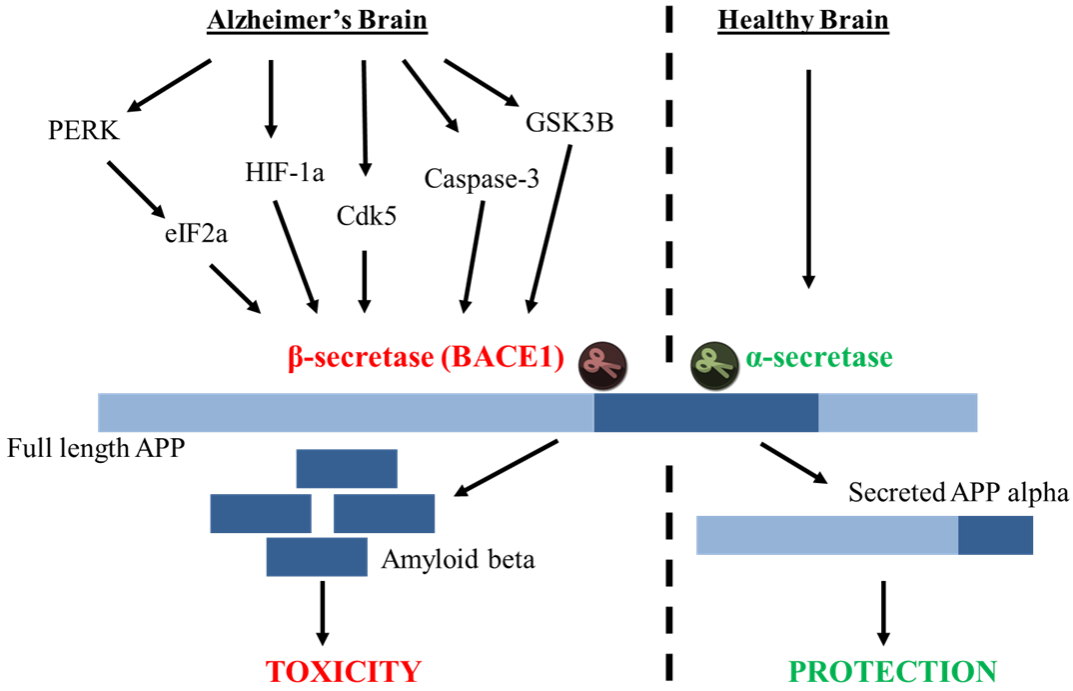


Figure 1: Mechanisms of Aβ overproduction in Alzheimer's disease *- The histological hallmark of AD is deposition of amyloid within the brain, yet the mechanisms that underlie Aβ accumulation remain poorly understood. In healthy individuals, the majority of APP is cleaved by alpha secretase enzymes to produce the neurotrophic peptide sAPPα. However, increased*

expression of BACE1, in AD indicates that there is a disease-induced shift in favor of amyloidogenic APP processing that underlies Aβ overproduction. Experimentally, several elements have been identified that may contribute to AD-associated BACE1 overexpression, including the unfolded protein response mediators PERK/eIF2α, the transcription factor HIF-1α, the kinases CDK5 and GSK3β, and the protease caspase-3. Therefore, multiple pathways may contribute to Aβ accumulation and AD pathogenesis.

Mechanisms of Aβ toxicity: Although amyloid plaques are a striking histopathological finding in AD, their presence likely has little effect on cognition as antibody-mediated clearance of amyloid plaques failed to improve ADAS-cog scores in AD patients (Holmes et al. 2008) . Instead, the detrimental effects of Aβ have been attributed to oligomeric Aβ (Aβo), which is formed by elevated concentrations of Aβ monomers (Stine et al. 2003, Nag et al. 2011). Aβo levels more strongly correlate with cognitive decline than does plaque load (Lesne et al. 2006, Lesne et al. 2008, Tomic et al. 2009) and Aβo negatively impacts learning and memory in animal models (Walsh et al. 2002). Oligomers come in a variety of sizes from low molecular weight dimers/trimers to high molecular spheres that aggregate into plaque forming fibrils (Glabe 2008). Intermediate-sized spherical Aβo is the most toxic oligomeric species (Glabe 2006, Glabe et al. 2006, Meier et al. 2006).

Aβo at the cell surface: Aβ oligomers likely contribute to neuronal death through both direct cytotoxicity and impairment of neurotrophic signaling pathways. Both sAPPα and insulin signaling effects are susceptible to Aβo-mediated inhibition of PI3K/AKT which may due to direct binding of oligomers to IR/IGF1R (Zhao et al. 2008, Jimenez et al. 2011). In addition to blocking IR/IFG1R, extracellular Aβo at the cell surface triggers apoptotic signaling via an interaction with the NGF receptor p75 (Tsukamoto et al. 2003), inhibits neuroprotective Wnt/β-

catenin signaling (Magdesian et al. 2008) and aberrantly activates NMDA receptors (NMDARs) resulting in excitotoxic cell death (De Felice et al. 2007) The association between Aβo and receptors such as NDMARs may underlie Aβo accumulation at, and disruption of, neuronal synapses (Shankar et al. 2007, Reddy et al. 2008).

Aβo channel and permeability hypothesis: In addition to cell surface interactions, Aβo toxicity is associated with permeabilization of the plasma membrane. This occurs by the formation of Aβo into cation specific channels (Arispe et al. 1993a, Arispe et al. 1993b) and channel-independent disruption of internal and external lipid bi-layers (Demuro et al. 2005). In both cases, permeabilization causes cellular toxicity by elevating intracellular calcium concentrations resulting in free radical production, bioenergetic inefficiency and activation of cell death signaling (Rodrigues et al. 2000, Eckert et al. 2003, Resende et al. 2008).

Mechanisms of AD amyloidosis; upregulation of BACE1: Regions of the brain affected by AD have elevated BACE1 activity and expression levels (Sinha et al. 1999, Vassar et al. 1999). Once identified, BACE1 became a popular therapeutic target for AD treatment however, BACE1 KO mice have reduced survivability and body size (Dominguez et al. 2005). BACE1 KO mice also present with hyperactive behavior (Dominguez et al. 2005) and other abnormalities such as hypomyelination of peripheral nerves, reduced grip strength, and elevated pain sensitivity (Hu et al. 2006).

The unfolded protein response: Activation of the UPR inhibits protein synthesis during cellular insult and also stimulates the expression of BACE1. There are multiple indices of UPR activation in the AD brain including increased chaperone expression and enhanced PERK phosphorylation (Hoozemans et al. 2005). PERK is a threonine/serine kinase that suppresses translation by phosphorylating eIF2α (Wu et al. 2006) that along with eIF2β, eIF2B and eIF2γ,

form a translation initiation complex (Kimball 1999). There are increased levels of phosphorylated eIF2α (peIF2α) in the brains of AD patients that positively correlates with BACE1 and amyloid loads (O'Connor et al. 2008). Furthermore, the APOE4 allele, a major risk factor for AD development (Corder et al. 1993, Chartier-Harlin et al. 1994) increases peIF2α levels in transgenic mice (Segev et al. 2013). At the cellular level, increased eIF2α phosphorylation occurs in response to energy depletion (O'Connor et al. 2008), Aβ (Lee do et al. 2010) and hypoxia (Kimball 1999, Althausen et al. 2001, Fahling 2009).

Under normal conditions, eIF2B, which catalyzes the exchange of GDP for GTP on the eIF2γ subunit, is free to bind and dissociate from the complex. The exchange of GDP for GTP allows for methionine-tRNAs to be incorporated into the initiator complex resulting in the translation of AUG start codons on mRNA transcripts. However, eIF2B does not readily dissociate from peIF2α that results in decreased GDP being exchanged for GTP, reduced incorporation of met-tRNAs into initiator complexes and an overall reduction in translation. Paradoxically, increased peIF2α is associated with increased translation of BACE1 transcripts (O'Connor et al. 2008) revealing a unique regulatory mechanism for translation of BACE1 mRNA.

The BACE1 transcript contains 3 open reading frames. Interestingly, a stem loop structure is present after the second ORF (O'Connor et al. 2008). When peIF2α levels are low, and initiator complexes can be readily formed, both ORFs on the BACE1 transcript are translated and that the stem loop structure following ORF2 causes dissociation of 40s and 60s ribosomal subunits before the third ORF can be translated. However, during cellular stress and UPR activation, high levels of phosphorylated eIF2α result in inefficient initiator complex formation and decreased formation of ribosomal complexes. Therefore, because of decreased

translation of the second ORF, fewer 40S scanning ribosomal subunits are dissociated at the stem loop structure. Instead, 40S subunits are allowed to scan past the first 2 ORFs and reach the third ORF resulting in initiator complexes being formed at the BACE1 start codon resulting in translation of BACE1 mRNA (O'Connor et al. 2008).

GSK3β, JNK, and CDK5: In addition to PERK/eIF2a, GSK3β may contribute to BACE1 overexpression by indirectly enhancing BACE1 transcription (Ly et al. 2013) causing increased Aβ production *in vitro* (Yu et al. 2012). Enhanced BACE1 expression may be also be caused by elevated intracellular calcium levels leading to calpain induced activation of the serine/threonine kinase CDK5 (Pierrot et al. 2006). Increased intracellular calcium activates calpain which cleaves p35 substrates to p25, an activator of CDK5 (Cruz et al. 2006). Active CDK5 enhances BACE1 expression by phosphorylating the transcription factor STAT3 resulting in STAT3-mediated stimulation of the BACE1 promoter (Wen et al. 2008).

Vascular insufficiency and HIF-1α: Vascular insufficiency is a major risk factor for AD (Helzner et al. 2009, Li et al. 2011) and cerebrovascular dysfunction is associated with neurodegeneration in AD patients and APP models (de la Torre 2004, Bell et al. 2009). Experimental hypoperfusion increases both Aβ and BACE1 (Zhiyou et al. 2009). In addition to activating GSK3β (Elali et al. 2013) and PERK (Kumar et al. 2001), vascular insufficiency may increase BACE1 expression by activating HIF-1α (Bernaudin et al. 2002). HIF-1α is a transcription factor (Semenza 2000, Baranova et al. 2007) that, under normoxic conditions, is ubiquitinated and targeted for degradation, but is stabilized and active during oxygen depletion (Chun et al. 2002, Dery et al. 2005, Semenza 2007). Stable HIF-1α forms an active transcriptional complex with HIF-1β that initiates the transcription of several survival genes (Minet et al. 2001, Greijer et al. 2005, Harms et al. 2010) as well as BACE1 (Sun et al. 2006,

Zhang et al. 2007, Guglielmotto et al. 2009), which may be the mechanistic basis for cerebrovascular impairment-induced Aβ accumulation.

Possible physiological roles for Aβ: Although linked to several toxicities, Aβ is critically important to neurons as Aβ depletion in culture results in cell death (Plant et al. 2003). While one of the most commonly cited sources of insulin signaling dysfunction in AD is IR antagonism by Aβo (Zhao et al. 2008), there is strong evidence indicating that Aβ monomers positively modulate insulin pathway activation (Giuffrida et al. 2009). Aβ and insulin are structurally similar (Kurochkin 1998, Kurochkin 2001) and both Aβ and insulin are degraded by insulin degrading enzyme (IDE) (Kurochkin et al. 1994, Qiu et al. 1998, Farris et al. 2003). Therefore, while it remains unclear if the brain can synthesize insulin, the brain does appear to have an endogenously produced IR agonist in the form of Aβ.

The pro-survival properties of Aβ appear to involve a modulatory effect on excitatory neuronal signaling. NMDAR activation increases Aβ production (Lesne et al. 2005) and Aβ regulates synaptic activity (Kamenetz et al. 2003). When combined with data showing that inhibition of γ secretase increases excitatory postsynaptic current frequency and APP deficient mice are hypersensitive towards kainite induced seizures (Steinbach et al. 1998, Kamenetz et al. 2003), these findings indicate that Aβ may be a critical negative regulator of excitatory signals. Aβ may also modulate excitability by increasing the expression of inactivating potassium channel subunits (Plant et al. 2006), which are key determinants of cell survival and apoptotic processes (Yu 2003).

The physiological role of Aβ may also involve regulation of self-renewing NSC (neural stem cell) proliferation and differentiation. This was first suggested after APP was found to be critically involved in brain development (Hartmann et al. 1999, Herms et al. 2004, Guenette et al.

2006). In accordance, both sAPPα and Aβ influence NSC fate and replication rate (Lopez-Toledano et al. 2004, Diaz-Moreno et al. 2013, Itokazu et al. 2014). sAPPα (Caille et al. 2004, Baratchi et al. 2012, Demars et al. 2013) and Aβ (Diaz-Moreno et al. 2013, Itokazu et al. 2014) increase NSC proliferation and sAPPα causes NSC differentiation into astrocytes (Baratchi et al. 2012, Bailey et al. 2013) while Aβ drives NSCs towards a neuronal fate (Lopez-Toledano et al. 2004). Interestingly, because of the neurogenic effect of Aβ, age-related increases of Aβ levels may be a means of counter-acting decreases in neurogenesis (Kuhn et al. 1996, Heine et al. 2004, Morgenstern et al. 2008).

Summary: The mechanisms that underlie Aβ accumulation in sporadic AD remain unknown but may involve increased expression and activity of the APP cleaving enzyme BACE1. Low concentrations of Aβ are neuroprotective and may be an acquired adaptation to defend against a multitude of insults. However, because of the toxicity of Aβo, a reduction in cerebral amyloid remains a primary goal of AD therapeutics.

1.3: THE TAU HYPOTHESIS OF ALZHEIMER'S DISEASE

Tau is a protein that stabilizes microtubules in healthy neurons but is aberrantly phosphorylated and aggregated in neurodegenerative diseases such as frontotemporal lobe dementia (FTD), Parkinson's disease and AD. Furthermore, chronic traumatic encephalopathy is characterized by the presence of tau aggregates (Omalu et al. 2011a, Omalu et al. 2011b, McKee et al. 2013). Tau is encoded by the *MAPT* gene located on chromosome 17 and mutations to *MAPT* are a direct cause of FTD and Parkinson's (Foster et al. 1997, Hutton et al. 1998) thus demonstrating that dysregulation of tau homeostasis is sufficient to cause neurodegeneration.

Tau is normally unfolded and associates with, and stabilizes, microtubules (Kadavath et al. 2015) and is consequently concentrated in neuronal axons (Binder et al. 1985). The

conversion of soluble, monomeric tau to insoluble aggregates is hypothesized to underlie neurodegeneration (Goedert et al. 2017). The mechanistic causes of tau aggregation remain poorly understood but aberrant phosphorylation appears to play a central role. Hyperphosphorylation of tau not only promotes aggregation but also leads to tau dissociation from microtubules (Bramblett et al. 1993, Yoshida et al. 1993).

NFTs appear early in AD (Braak et al. 1991, Braak et al. 2011) and better correlate with cognitive decline than plaques (Arriagada et al. 1992, Guillozet et al. 2003). The Braak stages of Alzheimer's classify disease progression on a scale of 1-6 based on the accumulation of tau deposits. In the early Braak stages, tau aggregates are found only in the superior layers of the transentorhinal cortex with deposits spreading into the hippocampus and neocortex in later stages (Braak et al. 1991, Braak et al. 1995, Braak et al. 1997). Tau harboring a Pro301Ser mutation causes FTD in humans (Bugiani et al. 1999) and neurodegeneration in mouse models (Allen et al. 2002). When injected into normal mice, protein extracts from the brains of Pro301Ser expressing animals cause initial tau deposition at the injection site that continues to spread to adjacent regions of the brain (Clavaguera et al. 2009). This work supports the hypothesis that the toxicity of aberrant tau involves a prion disease-like pathological cascade (Woerman et al. 2016).

Tau toxicity: Dissociation of tau monomers from microtubules may contribute to neurodegeneration as microtubule loss is co-localized with tau deposits in mutated tau expressing mice (Hall et al. 2000) and tau KO mice display motor deficits, cognitive impairment and disorganization of axonal microtubules (Harada et al. 1994, Ikegami et al. 2000, Lei et al. 2012). However, mice with at least one functional *MAPT* gene have a normal phenotype (Morris et al. 2013) demonstrating that a reduction in tau function of at least 50% is well tolerated by the brain in mice.

Although AD is diagnosed by the presence of macroscopic NFTs, tau toxicity is likely mediated by intermediately sized oligomers (Bobinski et al. 1997, Wittmann et al. 2001, Berger et al. 2007, Mocanu et al. 2008, Lasagna-Reeves et al. 2011). Like Aβo, tau oligomers compromise membrane integrity and cause dysregulation of intracellular calcium and mitochondrial dysfunction (Flach et al. 2012). Despite the absence of a secretory sequence, tau peptides are released from cells in culture (Yamada et al. 2011) and are found in cerebrospinal fluid (Munroe et al. 1995). Aggregation stimulates tau release from neuronal cells (Saman et al. 2012) and allows oligomers to interact with cell surface receptors. Tau oligomers aberrantly activate NMDAR (Amadoro et al. 2006) and cholinergic receptors causing calcium influx (Gomez-Ramos et al. 2009). The localization of M1/M3 receptors in the entorhinal cortex and hippocampal CA1/CA3 fields may account for these regions selective vulnerability in early AD (Levey 1996, Gomez-Ramos et al. 2006, Gomez-Ramos et al. 2009, Padurariu et al. 2012). The extracellular release of tau aggregates may underlie the propagation of tau deposits as tau filaments from the brains of AD patients are taken up by neurons *in vitro* resulting in aggregation of endogenous tau proteins (Kfoury et al. 2012, Santa-Maria et al. 2012).

Summary: Tau hyperphosphorylation leading to aggregation may be a primary cause of neurotoxicity in AD (Stamer et al. 2002, Gomez-Ramos et al. 2006). Although tau deposits are found intracellularly, their toxic effects appear to involve extracellular interactions with cholinergic receptors, NMDARs and the plasma membrane. Furthermore, intracellular tau inclusions can be released into the extracellular space and taken up by neighboring cells, which matches data demonstrating that experimentally seeded tau deposits can spread throughout the various regions of the brain. Dysregulated tau may also cause neuronal dysfunction via destabilization of microtubules resulting in destruction of intra-neuronal transport systems

(Schneider et al. 1999, Stamer et al. 2002, Mandelkow et al. 2003). In total, because mutated tau is a direct cause for neurodegenerative brain disease and tau pathology is present before clinical symptoms in AD patients, dysregulation of tau homeostasis may play a pathogenic role in Alzheimer's and be the mechanistic connection between AD onset and risk factors, such as diabetes.

1.4: THE DIABETES HYPOTHESIS OF ALZHEIMER'S DISEASE

Not only is T2DM a risk factor for AD (Cheng et al. 2012) but AD increases risk of developing T2DM by up to 3-fold (Janson et al. 2004). Furthermore, both AD and T2DM display insulin signaling impairment (Aytug et al. 2003, Morino et al. 2005, Moloney et al. 2010). Abnormal glucose utilization (Friedland et al. 1985, Fukuyama et al. 1994, Desgranges et al. 1998, Li et al. 2016, Oh et al. 2016) and cognitive decline (Stewart et al. 1999, Logroscino et al. 2004) are common in both diseases. Insulin signaling dysfunction is present in the AD brain even in the absence of systemic diabetes (Frolich et al. 1998, Frolich et al. 1999, Talbot et al. 2012) which may be due to reduced insulin levels (Gil-Bea et al. 2010) as well as increased inhibitory phosphorylation of insulin receptor substrate (IRS) proteins. Feedback inhibition of insulin signaling involves serine phosphorylation of IRS proteins which attenuates insulin signaling by inhibiting IRS binding with IR (Paz et al. 1997, Rui et al. 2001, Aguirre et al. 2002). Aberrant IRS phosphorylation is hypothesized to contribute to insulin resistance in both diabetic and Alzheimer's disease states (Paz et al. 1997, Werner et al. 2004, Talbot et al. 2012). AD brain tissue is insensitive to ex vivo insulin (Talbot et al. 2012) which is associated with dysregulated IRS phosphorylation and blunted IR/IGF1R activation. These results support earlier work which revealed that tyrosine kinase activity of IR is decreased in AD (Frolich et al. 1998). Therefore,

insulin signaling impairment in AD is likely a combination of decreased IGF1/insulin availability, blunted receptor activity and inappropriate phosphorylation of IRS.

Overview of the insulin signaling pathway: The brain is a major metabolic organ that accounts for ~25% of the body's total glucose use (Zlokovic 2008, Zlokovic 2011). While glucose uptake in peripheral tissues requires insulin, in the brain this is considered to be an insulin-independent process. Insulin and IGF1 are required for proper brain function as they provide critical neurotrophic support for neurons. IGF1 and insulin share similar amino acid sequences/ tertiary structures (Bondy et al. 2004) and bind to and activate the others' receptor (Yamaguchi et al. 1993). Both insulin and IGF1 receptors are tyrosine kinases (Kasuga et al. 1982, Jacobs et al. 1983, Rubin et al. 1983) that phosphorylate substrate proteins such as IRS. IRS phosphorylation leads to downstream activation of phosphoinositide-3 kinase (PI3K) and AKT, a serine/threonine kinase and key mediator of the neurotrophic effects of insulin and IGF1.

Origins of cerebral insulin; insulin is peripherally derived: High concentrations of insulin are maintained in the brain ranging from 10-100 fold greater levels than that observed in the plasma (Havrankova et al. 1979, Baskin et al. 1983). While IGF1 is produced locally by neurons (Bach et al. 1991, Bondy et al. 2004) the brain's source of insulin remains unclear. Despite the presence of pre-proinsulin mRNA in neurons (Schechter et al. 1996) it's been suggested that the brain's sole source of insulin is the pancreas and arrives via the systemic circulation (Banks 2004, Burns et al. 2007, Erol 2008).

Neuronal insulin receptors: Insulin receptors are present in one of two isoforms, the IR-A isoform that lacks exon 11 that the other isoform, IR-B, expresses (Ebina et al. 1985, Ullrich et al. 1985). A major functional difference between the two isoforms is that IR-A has a higher affinity for the neurotrophic factor IGF2 (Frasca et al. 1999) and a slightly higher affinity for

insulin (Mosthaf et al. 1990). Brain specific insulin receptors are mainly the IR-A isoform and as result of differential glycosylation have a lower molecular weight than their peripheral counterparts (Park 2001).

Structurally, the insulin receptor is a homodimer composed of 2α chains and 2β chains held together with disulphide bonds (Hedo et al. 1981, Massague et al. 1981, Siegel et al. 1981). Insulin receptor binding of insulin/IGF1 results in a conformational change that activates the catalytic tyrosine kinase activity of the β subunits (Kanzaki 2006). This activation of the insulin receptor results in autophosphorylation at multiple tyrosine residues (Frattali et al. 1993, Lee et al. 1993) including tyrosine 960 (tyrosine 972 in mice) in the juxtamembrane region of the β subunit (White et al. 1988, Lee et al. 1994). Phosphorylation at this site is a vital component of the insulin signaling cascade because it provides a binding motif for the phospho-tyrosine binding domain of IRS (White et al. 1988, Lee et al. 1994). Once docked to the insulin receptor, IRS is phosphorylated on tyrosine residues (Kanzaki 2006).

IRS, PI3K and AKT: Tyrosine phosphorylation of IRS proteins creates binding sites for Src homology 2 domain containing proteins such as PI3K (White 1998). PI3K catalyzes the production of 3'phosphoinositide secondary messengers which are critical to the insulin signaling cascade. PI3K is composed of a catalytic p110 subunit and a regulatory p85 subunit that contains Src homology 2 domains that interact with activated IRS (Myers et al. 1992). Formation of the IRS/PI3K complex increases the catalytic activity of the p110 subunit (Virkamaki et al. 1999).

Phosphoinositides produced by PI3K are important signal conductors that bind to pleckstrin homology domains on proteins such as IRS (Virkamaki et al. 1999) and AKT (Mayer et al. 1993). This interaction brings IRS and AKT proteins towards the inner layer of the plasma

membrane near the juxtamembrane region of the insulin receptor (Yenush et al. 1996) and to activating kinases, respectively (Coffer et al. 1991, Burgering et al. 1995, Andjelkovic et al. 1996, Kohn et al. 1996, Bellacosa et al. 1998, Soskic et al. 1999). Furthermore, binding of 3'phosphoinositides is required for AKT to be competent for phosphorylation (Alessi et al. 1997a, Stokoe et al. 1997, Bellacosa et al. 1998, Currie et al. 1999).

There are two phosphorylation sites on AKT, Thr 308 and Ser 473, capable of inducing catalytic activity (Alessi et al. 1996). PDK1, which also depends on 3'phosphoinosites for its function, phosphorylates AKT at Thr 308 (Alessi et al. 1996, Alessi et al. 1997b). While overexpression of PDK1 has been shown to activate AKT (Alessi et al. 1997a), optimal activation of AKT requires additional phosphorylation at Ser 473 by mTORC2 (Sarbassov et al. 2005) which stabilizes the conformation state of AKT (Yang et al. 2002).

AKT mediates the neurotrophic effects of insulin/IGF1, in part, by inhibiting pro-apoptotic machinery (Cardone et al. 1998) and concomitantly activating anti-apoptotic proteins (Datta et al. 1997, del Peso et al. 1997, Blume-Jensen et al. 1998, Du et al. 1998, Wang et al. 1999) . The role of AKT in neurotrophic support also involves the regulation of survival (Du et al. 1998, Kane et al. 1999) and apoptotic (Biggs et al. 1999, Brunet et al. 1999, Kops et al. 1999) transcription factors. Moreover, AKT is involved in production of the neurotrophin brain BDNF (Du et al. 1998), activation of proteins involved in neurite outgrowth (Read et al. 2009) and regulation of the metabolic protein GSK-3 (Bhat et al. 2000).

Figure 2: The insulin signaling pathway

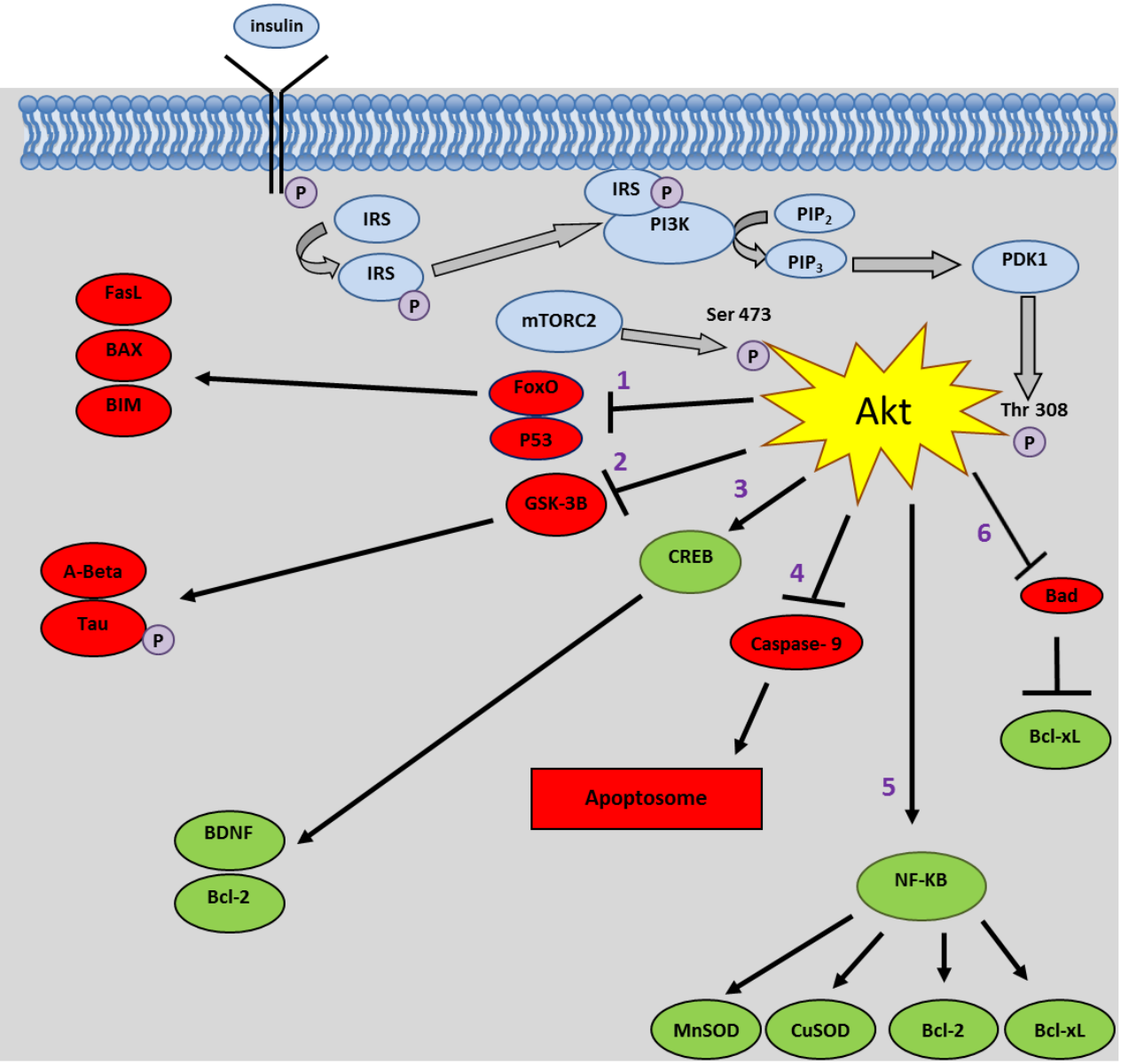


Figure 2: The insulin signaling pathway *- Insulin receptor binding of insulin triggers a complex signaling cascade (in blue) leading to activation of the serine/threonine kinase AKT. Upon binding of insulin, insulin receptors are autophosphorylated and subsequently bind IRS proteins. IRS proteins are then phosphorylated by activated insulin receptors and complex with PI3K*

resulting in PI3K activation. Activated PI3K produces phospholipid secondary messengers by catalyzing the conversion of phosphatidylinositol 4,5-bisphosphate (PIP_2) to phosphatidylinositol 3,4,5-trisphosphate (PIP_3). PIP_3 messengers activate PDK1 which phosphorylates AKT at Threonine 308. AKT is further activated by phosphorylation at Ser 473 by mammalian target of rapamycin 2 (mTORC2). Targets of activated AKT include pro-apoptotic mediators (in red) as well as pro-survival machinery (in green). Loss of insulin signaling (at sites labeled with numbers 1-6 in purple) allows FoxO and p53 transcription factors to remain active and (1) transcribe genes for pro-apoptotic proteins such as BIM, BAX and FasL. AKT inhibits the activity of GSK-3β that, when active, (2) causes increased amyloidogenic processing and hyperphosphorylation of tau. Other pro-apoptotic proteins inhibited by AKT include (4) caspase-9, which forms an apoptotic structure known as the apoptosome, and (6) Bad, which blocks activity of the ant-apoptotic protein Bcl-xL. Pro-survival modulators regulated by AKT include CREB and NFκB. Reduction of CREB transcriptional activity as a result of a loss of insulin signaling leads to (3) decreased BDNF and Bcl-2 expression while inhibition of NFκB leads to (5) reduced expression of anti-oxidants such as MnSOD and CuSOD as well as anti-apoptotic Bcl-2 family members.

<u>*Bcl-2 family*</u>: The Bcl-2 family is a structurally related group of proteins that regulate cell survival through their effects on mitochondria (Llambi et al. 2011). Mitochondrial dysfunction results in mitochondrial membrane permeability and the release of pro-apoptotic factors such as Cytochrome c, and apoptosis inducing factor (Bossy-Wetzel et al. 1999, Lemasters et al. 2009, Paradies et al. 2009). Bcl-xL is an anti-apoptotic Bcl-2 family member that maintains mitochondrial membrane integrity (Tornero et al. 2011). AKT activates Bcl-xL by

phosphorylating BAD which causes dissociation of BAD from Bcl-xL (del Peso et al. 1997, Blume-Jensen et al. 1998, Datta et al. 1999, Wang et al. 1999) allowing for mitochondrial stabilization. AKT also regulates cell survival prior to Cytochrome c release by inhibiting the proteolytic activity of Caspase 9 (Cardone et al. 1998).

AKT and the regulation of transcription factors: AKT stimulates expression of pro-survival Bcl-2 members by activating the transcription factor CREB (Zhang et al. 2013). Also under CREB transcriptional control is BDNF (Shieh et al. 1998, Tao et al. 1998) which is essential for neuronal development, differentiation, synaptic plasticity, and neuroprotection against a broad range of cellular insults (Hu et al. 2008). BDNF stimulates non-amyloidogenic APP processing pathways (Fu et al. 2002, Rohe et al. 2009) and is protective against Aβo toxicity (Arancibia et al. 2008). Insulin signaling impairment resulting in decreased BDNF production may contribute to AD development. Both AD and T2DM patients have decreased levels of BDNF (Zhen et al. 2013, Forlenza et al. 2015).

The transcription factor NFκB is also under AKT control (Kane et al. 1999). Like CREB, NFκB plays critical roles in neuron survival (Maggirwar et al. 1998, Brunet et al. 1999, Riccio et al. 1999) and is also involved in neurite outgrowth, myelin formation and axonal regeneration (Mincheva et al. 2011). Genes for antioxidant proteins such as MnSOD (Maehara et al. 2000) and Cu/ZnSOD (Rojo et al. 2004) as well as Bcl-2 and Bcl-xL are targets of NFκB (Tamatani et al. 1999).

When inactive, NFκB is bound to IκB proteins that sequester it to the cytosol (May et al. 1997, Mercurio et al. 1999, Kaltschmidt et al. 2009). NFκB is activated when IκB proteins are phosphorylated by IκB Kinase (IKK) complexes and targeted for degradation which allows NFκB to translocate to the nucleus where it binds to regulatory DNA sequences (Perkins 2007).

The IKK complex consists of catalytic IKKα and IKKβ subunits and a regulatory IKKγ subunit (Perkins 2007). AKT facilitates NFκB activation by phosphorylating IKKα at a critical regulatory site that promotes IKK activation (Ozes et al. 1999) and subsequent IκB degradation.

AKT influence over transcription factor activity extends to pro-death modulators as well (Miyashita et al. 1995, Van Der Heide et al. 2004). The forkhead box class O (FoxO) family of transcription factors contribute to apoptosis through the induction of pro-death genes such as FasL (Reif et al. 1997, Brunet et al. 1999, Stahl et al. 2002) and the Bcl-2 member BIM-1 (Dijkers et al. 2000). FasL facilitates apoptosis by activating caspases (Graham et al. 2001) while BIM-1 activates the pro-apoptotic Bcl-2 family member BAX (Yamaguchi et al. 2002). In the absence of AKT, FoxO transcription factors are transcriptionally active in the nucleus (Biggs et al. 1999, Brunet et al. 1999, Kops et al. 1999). The FoxO family of transcription factors is known to play a role in the cell's response to oxidative stress, however, their prolonged activation results in apoptosis (Reif et al. 1997, Brunet et al. 1999, Dijkers et al. 2000, Stahl et al. 2002, Barthel et al. 2005). AKT phosphorylates FoxO family members at a conserved c-terminal sequence (Van Der Heide et al. 2004) which leads to nuclear exclusion and inhibition of transcriptional activity.

Another pro-death transcription factor known to be inactivated by AKT is p53 (Yamaguchi et al. 2001) which induces the expression of BAX. Oligomers of BAX insert into the outer mitochondrial membrane and provide a passageway for Cytochrome c and other pro-apoptotic proteins (Kowaltowski et al. 2000). Increased p53 activity leading to BAX expression has been linked to neuronal deprivation of neurotrophic factors (Aloyz et al. 1998).

The effects of diabetes on the CNS, lessons from animal models: One of the earliest connections between insulin signaling dysfunction and AD-associated pathology came from

IRS2 KO mouse studies (Schubert et al. 2003). Homozygous IRS2 KO mice had impaired brain growth, reduced neuronal proliferation and hyperphosphorylated tau. Follow up studies have focused on the impact of peripheral insulin on Aβ and tau and have demonstrated that peripheral insulin deficiency exacerbates pathology in APP-overexpressing AD mice (Jolivalt et al. 2010). Furthermore, diabetic AD mice display increased learning and memory impairments compared to non-diabetic controls. Experimental T2DM, induced by a high fat diet or ablation of the leptin gene, also increases Aβ and worsens cognitive performance in AD mice (Takeda et al. 2010, Vandal et al. 2014).

Despite convincing evidence that the induction of T1DM and T2DM in AD mice exacerbates pathology and learning and memory deficits, insulin signaling impairment alone appears insufficient to replicate all features of AD. For example, mice fed a high fat diet display no histopathological or inflammatory changes associated with AD (Moroz et al. 2008). Although neuronal specific insulin receptor KO mice present with increased tau phosphorylation and increased neuronal apoptosis, no differences in memory, glucose metabolism or amyloid production/deposition have been found in this model (Schubert et al. 2004). These results seem to suggest that diabetes only exacerbates the AD condition but is not causative. However this appears to be refuted by recent work involving the induction of streptozotocin (STZ) diabetes in non-human primates. When rendered diabetic by STZ, vervet monkeys display hyperphosphorylated tau, increased Aβ deposition and signatures of insulin resistance consistent with findings in the human AD brain (Morales-Corraliza et al. 2016). Therefore, whether or not diabetes is sufficient to cause AD remains unknown and the answer appears to be restrained by the availability of clinically relevant models. Nevertheless, studies modeling diabetes using a

wide range of methods across multiple species have determined that dysfunction of the insulin signaling pathway contributes to pathological changes that are associated with AD.

Mechanisms of diabetes induced amyloidosis: Analysis of AD brain tissue suggests that disease-associated Aβ accumulation may be due to a combination of increased BACE1 expression/activity, decreased brain efflux of Aβ, and diminished expression of enzymes that degrade Aβ, all of which are influenced by insulin signaling (Carro et al. 2002, Miners et al. 2010, Zhang et al. 2011a). APP processing is location dependent (Golde et al. 1992, Vetrivel et al. 2006) and insulin increases α-secretase cleavage of APP by increasing APP trafficking towards enzymes located at the plasma membrane (Parvathy et al. 1999, Gasparini et al. 2001). Disruption of the trafficking process by the attenuation of PI3K/AKT or MAPK results in APP becoming concentrated in the TGN and in the vicinity of BACE1, which increases the likelihood of beta cleavages taking place. Exacerbation of Aβ levels in AD mice by STZ diabetes has also been linked to activation of the UPR (Wang et al. 2010, Devi et al. 2012).

Mechanisms of diabetes induced tau pathology: Both STZ induced T1DM and experimental T2DM alone or in conjunction with APP overexpression induces hyperphosphorylation of tau at multiple sites (Planel et al. 2007b, Vandal et al. 2014, El Khoury et al. 2016, Gratuze et al. 2017a). Trophic factors such as insulin, IGF1, and BDNF (Hong et al. 1997, Elliott et al. 2005) prevent aberrant tau phosphorylation therefore dysregulation of insulin signaling in AD leading to tau aggregation is a potential mechanistic link between AD and diabetes. Early *in vitro* studies analyzing insulin regulation of tau phosphorylation suggested that diabetes associated tau phosphorylation was the result of overactivation of the tau kinase GSK3 (Hong et al. 1997), which is directly phosphorylated and inhibited by AKT (Bhat et al. 2000). However, *in vivo* studies demonstrate that inactivating phosphorylation of GSK3 is increased in

the insulin depleted brain (Clodfelder-Miller et al. 2006) and the failures of clinical trials involving GSK3 inhibitors (Hampel et al. 2009) call into question the role of GSK3 in disease progression.

Over activity of other tau kinases such as JNK and PKA have been suggested to underlie tau hyperphosphorylation in the STZ diabetic brain (van der Harg et al. 2017); however this doesn't seem to be the case for T2DM models (El Khoury et al. 2016, Gratuze et al. 2017a). MAPK is another tau kinase that may contribute to diabetes-induced phosphorylation of tau as MAPK is aberrantly active in both AD (Perry et al. 1999) and in the brains of STZ diabetic monkeys (Morales-Corraliza et al. 2016). In addition to increased kinase activity, diabetes may cause hyperphosphorylation of tau by inactivating tau phosphatases (Liu et al. 2005), particularly PP2A (Gratuze et al. 2017b). The mechanisms that underlie dysregulation of PP2A in both T1DM and T2DM remain unknown, but disease-induced hypothermia may be involved (Planel et al. 2007b, El Khoury et al. 2016). Similarly, AD patients are hypothermic and have suppressed PP2A activity (Sontag et al. 2014, Carrettiero et al. 2015). Interestingly, hypothalamic insulin regulates thermogenesis (Sanchez-Alavez et al. 2010) suggesting that insulin-depletion *in vivo* causes aberrant tau phosphorylation via down regulation of hypothalamic mediated thermogenesis resulting in suppression of tau phosphatases.

Insulin therapy in Alzheimer's Disease: Experimental approaches in AD patients have focused on increasing cerebral insulin by treatment with insulin sensitizers or intranasal insulin. While these strategies have produced promising results in animal models, results in human in patients have been mixed.

Insulin sensitizers: Insulin-sensitizing PPARγ agonists are currently used alone or as part of combinational therapy for the management of T2DM (Mayerson et al. 2002). In animal

studies, oral administration of rosiglitazone was successful at improving memory and reducing amyloid concentrations in APP mice (Escribano et al. 2010). However, despite success in early trials (Watson et al. 2005, Risner et al. 2006), a phase III trial revealed that rosiglitazone (2 or 8mg once daily) was ineffective at improving global function or ADAS-Cog scores in patients with mild to moderate AD after 24 weeks of treatments (Gold et al. 2010).

Intranasal insulin Direct administration of insulin via an intranasal spray that by-passes the blood brain barrier is effective at reversing cognitive deficits in patients with mild to moderate probable AD. Daily intranasal insulin was found to improve memory (assessed by story recall capability), ADAS-Cog scores, and functional ability in AD patients (Craft et al. 2012). Interestingly, PET imaging revealed that intranasal insulin increased glucose uptake in parietotemporal and frontal brain regions. Intranasal insulin also modulates APP processing (Reger et al. 2008) resulting in increased plasma Aβ40/Aβ42 ratios (Dhamoon et al. 2009). In total, these results provide evidence that targeting insulin signaling via direct stimulation of IR is a therapeutically viable strategy in the treatment of AD and warrants the pursuit of further studies.

Summary - Diabetes is a risk factor for AD and even in the absence of systemic diabetes, the AD brain displays insulin signaling impairment in the form of aberrant IRS phosphorylation, decreased insulin levels and unresponsive IR. Activation of the insulin signaling pathway shifts the balance of pro/anti-apoptotic signals in favor of survival through promotion of Bcl-2 members that maintain mitochondrial integrity (Hockenbery et al. 1990, Yang et al. 1997, Pugazhenthi et al. 2000) and by activation of survival transcription factors such as NFκB (Kane et al. 1999) and CREB (Du et al. 1998). In diabetic rodents, insulin is a key regulator of amyloid production and tau phosphorylation, which are the two pathological hallmarks of AD. Taken

together, these findings suggest that restoration of insulin signaling is a compelling approach toward developing effective AD therapeutics.

1.5: sAPPα AS A THERAPEUTIC FOR ALZHEIMER'S DISEASE

Evidence that sAPPα activates insulin signaling: Despite extensive research, a putative sAPPα receptor remains elusive. sAPPα treatment of hippocampal neurons results in both AKT and GSK3 phosphorylation (Cheng et al. 2002) and sAPPα-mediated activation of AKT can be blocked with the PI3K inhibitor wortmannin. PI3K is activated by a multitude of cellular messengers including JAK/STAT, IRS, G-proteins, and others (Cantley 2002) however IRS is required for sAPPα-mediated neurite outgrowth (Wallace et al. 1997). These results support data demonstrating that sAPPα activates IRs in neuronal cell lines (Jimenez et al. 2011). Furthermore, the effects of sAPPα on GSK3 can be blocked *in vitro* by the IR/IGF1R antagonist AG1024 and *in vivo* by infusions of Aβo. While these results indicate that sAPPα signaling involves activation of IR, this seems to be contradicted by recent studies which suggest that full length APP and G-protein signaling are required for sAPPα-mediated neuroprotection, AKT phosphorylation and inhibitory phosphorylation of GSK3 (Milosch et al. 2014), although these results do not exclude a role for IR in sAPPα signaling. Nevertheless, sAPPα may be used to restore neuroprotective activation of AKT/PI3K that is dysfunctional in AD (Griffin et al. 2005).

sAPPα and cellular protection: In general, sAPPα has neurotrophic effects and the reduction of sAPPα in AD may contribute to neurodegeneration (Moechars et al. 1996). At the cellular level, sAPPα is protective against a wide range of insults hypothesized to underlie neuropathology in AD.

Excitotoxicity: Excitotoxic cell death may cause neuronal loss in AD (Hynd et al. 2004) which involves excessive stimulation of glutamate receptors resulting in dysregulated calcium

influx. Elevated intracellular calcium levels initiates apoptotic signaling via the activation of proteases such as caspases and calpains and by opening the mitochondrial transition pore that causes impaired bioenergetics and reactive oxidative species (ROS) release (Orrenius et al. 2003, Celsi et al. 2009). sAPPα protects neurons from glutamate-induced excitotoxicity *in vitro* which may be due to a combination of sAPPα-mediated activation of NFκB (Barger et al. 1996) and modulation of glutamatergic-NMDARs (Furukawa et al. 1998). Dysregulated calcium flux though NMDA channels contributes to cell death in AD (Bezprozvanny et al. 2008). The NMDAR is a therapeutically viable target as the NMDAR antagonist Memantine is currently being used to alleviate symptoms in late stage AD patients (Tariot et al. 2004). sAPPα decreases current through NDMARs in hippocampal neurons which may contribute to the protective effect of sAPPα against excitotoxicity (Furukawa et al. 1998). Because of the involvement of NMDAR in long term potentiation (LTP) (Muller et al. 1988, Lu et al. 2001) these results indicate that sAPPα would be negatively involved in memory formation. However, sAPPα increases LTP and enhances spatial memory (Taylor et al. 2008); therefore, further investigation into sAPPα modulation of NMDARs is required.

Effects of sAPPα on learning and memory: sAPPα may be able to reverse memory impairment in AD as increasing sAPPα production via ADAM10 overexpression prevents behavioral and LTP deficits in APP mice (Postina et al. 2004). Furthermore, infusions of sAPPα have been found to enhance memory performance in mice, rats, and chicks (Roch et al. 1994, Bour et al. 2004) and rescue behavioral abnormalities in APP-KO mice (Ring et al. 2007). sAPPα may also play a role in learning and memory in the healthy brain as genetic ablation of APP in mice results in cognitive deficits and reduced locomotor activity (Senechal et al. 2008). Moreover, mutations to ADAM 10 cause neuropathological changes such as necrosis, apoptosis

and astrogliosis with the hippocampus of APP mice (Taylor et al. 2008) and sAPPα targeting antibodies cause deficits in passive and active avoidance tasks (Doyle et al. 1990, Huber et al. 1993, Mileusnic et al. 2000).

AD is characterized by hippocampal dependent memory impairment (Mormino et al. 2009), and neuronal loss within the CA1 and CA3 hippocampal fields (Simic et al. 1997, Bobinski et al. 1998) both of which correlate with reduced cerebrospinal concentrations of sAPPα (Almkvist et al. 1997). The cause of AD-associated hippocampal neuronal loss remains unclear but reductions in NSC activity may be involved. Neurogenesis within the dentate gyrus of the hippocampus and the subventricular zone occurs in adulthood and may contribute to learning and memory (Eriksson et al. 1998, Gould et al. 1999, Shors et al. 2001, Magavi et al. 2002). Decreased neurogenesis occurs in the brains of AD-mice (Haughey et al. 2002). This supports data from human studies demonstrating reduced numbers of viable NSCs in the AD brain (Lovell et al. 2006). Treatment of NSCs harvested from old SAMP8 mice with sAPPα increases NSC proliferative rates (Demars et al. 2013) and infusions of sAPPα increases NSC proliferation within the subventricular zone of healthy adult mice (Caille et al. 2004). Therefore, the effects of sAPPα on learning and memory may be mediated by sAPPα-mediated modulation of NSC activity and neurogenic capacity.

sAPPα and Aβ: The therapeutic potential of sAPPα as a treatment for AD was recently demonstrated using viral mediated gene transfer to increase sAPPα levels and ameliorate pathology in the brains of APP/PS1 mice (Fol et al. 2016). Not only does sAPPα reduce Aβ production *in vitro* and *in vivo* (Obregon et al. 2012), but sAPPα is also protective against the toxic effects of Aβ. In neuronal cultures treated with Aβ, sAPPα blocks oxidative stress and enhances cell survival (Goodman et al. 1994). The effects of sAPPα on Aβ formation appear to

multifaceted as sAPPα negatively influences pathways involved in BACE1 expression and interacts with, and inhibits, BACE1 (Obregon et al. 2012).

CDK5: CDK5 is a serine/threonine kinase that stimulates BACE1 expression (Cruz et al. 2006, Wen et al. 2008) and tau phosphorylation (Baumann et al. 1993). Interestingly, sAPPα appears to be a negative regulator of CDK5 as genetic ablation of APP causes increased CDK5 expression *in vivo* and *in vitro* (Han et al. 2005). sAPPα also blocks up-regulation of CDK5 expression and inhibits CDK5 activity (Hartl et al. 2013). The mechanisms by which sAPPα regulates CDK5 activity remain unclear; however sAPPα-mediated inhibition of calpain may be involved. For example, the excitoprotective effects of statins, which are protective against human AD, have been attributed to statin-mediated sAPPα release and subsequent PI3K activation resulting in repressed calpain activity (Ma et al. 2009). Interestingly, calpains cleave and release sAPPα from APP (Nguyen et al. 2012) suggesting that calpain, CDK5 and sAPPα form a regulatory loop that is dysfunctional in AD.

APP dimers: Under non-pathological conditions, BACE1 has a shorter half-life than that observed in stressed tissue (Lefort et al. 2012) which appears to be regulated by trafficking of BACE1 to lysosomes, mediated in part by the cellular sorting protein GGA3 (Kang et al. 2010). In support, genetic depletion of GGA3 results in BACE1 accumulation and Aβ production (Walker et al. 2012). During insult, cellular GGA3 is degraded by activate caspase-3 enzymes resulting in BACE1 accumulation and enhanced levels of Aβ (Vassar 2007). Caspase-3 activity is elevated in the AD brain (Su et al. 2001). Antibody-mediated multimerization of APP is sufficient to increase caspase-3 levels and BACE1 stability in hippocampal neurons (Lefort et al. 2012) and sAPPα binds APP and disrupts APP dimerization resulting in decreased caspase-3 activity and increased GGA3 expression (Thornton et al. 2006, Gralle et al. 2009).

BACE1: Overproduction of Aβ in AD appears to be a result of not only increased BACE1 expression but also increased BACE1 enzymatic activity (Zetterberg et al. 2008). Recent evidence indicates that the proteolytic activity of BACE1 is modulated by interactions between BACE1 and various cellular messengers. Regulators that both bind and inhibit BACE1 include the reticulon family of proteins (He et al. 2004) and the lipid messenger sphingosine-1-phosphate (Takasugi et al. 2011). Reticulon binds BACE1which prevents BACE1 from interacting with APP. Interestingly sAPPα also binds BACE1 and overexpression of human sAPPα in the 5xFAD mouse model decreases Aβ production (Obregon et al. 2012), although it remains unclear whether this decrease is attributable to an interaction between sAPPα and BACE1

Transthyretin: The anti-amyloid effects of sAPPα do not appear to be limited to sAPPα-mediated suppression of BACE1 expression or direct interference of BACE1 activity, rather it appears that sAPPα may also indirectly suppress amyloid toxicity in the brain by stimulating expression transthyretin (Li et al. 2010). Transthyretin is an Aβ binding protein that protects against amyloid toxicity in the APP mouse brain and in Aβ treated neuronal cultures (Schwarzman et al. 1994, Tsuzuki et al. 2000, Costa et al. 2008). The expression of transthyretin is concomitantly decreased with sAPPα in AD (Sousa et al. 2007) and levels of transthyretin levels are elevated along with sAPPα in APP mice (Stein et al. 2002). Furthermore, infusion of anti-transthyretin antibodies into the brain of APP mice accelerates Aβ deposition and increases cell death (Stein et al. 2004). Similarly, *in vitro* studies demonstrate that sAPPα-mediated protection against Aβ can be blocked by anti-transthyretin antibodies (Stein et al. 2004).

Summary - Therapies geared at restoring insulin function have produced promising results in human AD patients. sAPPα not only activates insulin signaling but also antagonizes Aβ

production and enhances learning and memory. Therefore, sAPPα is a potential therapeutic for AD.

1.6: THERAPEUTIC POTENTIAL OF sAPPα IN THE PERIPHERAL NERVOUS SYSTEM

Sensory neuropathy is characterized by degeneration, demyelination, and loss of fibers within the peripheral nerve (Kennedy et al. 1996, Christianson et al. 2003) and is a common complication associated with diabetes. Previous studies demonstrate that a variety of pathological changes associated with diabetic neuropathy can be reversed with insulin treatment (Saleh et al. 2013). As we have reported previously, the underlying mechanism of insulin-mediated reversal of pathological markers within diabetic DRG neurons appears to involve enhancement of NFκB transcription factor activity in neurons (Saleh et al. 2013). NFκB is vital for neuronal survival (Maggirwar et al. 1998, Fernyhough et al. 2005, Mattson et al. 2006) and deficits in NFκB activity lead to oxidative stress, neuronal degeneration (Mattson et al. 2006) and cell death (Fernyhough et al. 2005). NFκB is activated by growth factors such as insulin and IGF1 (Heck et al. 1999) as well as increased ROS (Mattson et al. 1997).

The NFκB family of transcription factors consists of five subunits; p65, RelB, cRel, p50 and p52, that form homo/hetero dimers and transcribe stress response genes including the antioxidant MnSOD (Bernard et al. 2001). Decreased MnSOD may underlie diabetes-induced sensory neuropathy and AD-associated neurodegeneration by increasing oxidative stress (Marcus et al. 2006, Saleh et al. 2013).

MnSOD is a 26kDa protein localized in the mitochondria that catalyzes the dismutation of superoxide free radicals to hydrogen peroxide. Genetic ablation of MnSOD results in perinatal lethality in mice (Li et al. 1995, Lebovitz et al. 1996) and *in vitro* MnSOD depletion causes cell

death in multiple tissue types (Mattson et al. 1993, Van Remmen et al. 2001, Justilien et al. 2007). MnSOD has been demonstrated to protect peripheral neurons from STZ diabetic-associated injury (Vincent et al. 2007) and improve learning and memory of AD mice (Massaad et al. 2009). In our previous study (Saleh et al. 2013), we discovered that decreases in MnSOD expression in diabetic rat DRG are associated with decreased activity of cRel-p50 NFκB heterodimers. Because insulin stimulates NFκB activity (Saleh et al. 2013), reduced p50/cRel activation may be the mechanistic connection between insulin dysfunction and decreased MnSOD levels in the diabetic nervous system.

Besides insulin, activators of NFκB include inflammatory cytokines (Duh et al. 1989, Hohmann et al. 1990) and sAPPα (Barger et al. 1996). Despite evidence that APP is expressed in DRG neurons (Scott et al. 1991, Sisodia et al. 1993) where it appears to play an important anti-apoptotic role (Nishimura et al. 2003), studies exploring the role of APP and its derivatives in peripheral neurons are limited. Furthermore, experiments involving APP overexpressing cell lines suggest that sAPPα increases MnSOD expression (Kogel et al. 2005) . In total, these data indicate that sAPPα is a potential therapeutic for the treatment of diabetes-induced peripheral neuropathy.

1.7: HYPOTHESIS AND RATIONALE

sAPPα can prevent amyloid and tau pathology in AD mice (Deng et al. 2015, Fol et al. 2016) and is protective against trophic depletion in DRG neuronal cultures (Nishimura et al. 2003), however, the effects of sAPPα on the diabetic nervous system are unknown. Because sAPPα activates the insulin signaling pathway (Jimenez et al. 2011) *we hypothesized that sAPPα could inhibit pathology in the diabetic nervous system*. This hypothesis was tested in the following aims:

Aim 1: To determine if overexpression of sAPPα can reduce Alzheimer's-like pathology in the diabetic brain.

Aim 2: To evaluate the effects of sAPPα on peripheral neuropathy in diabetic mice.

In our final aim (**Aim 3**), we examined the effects of sAPPα-overexpressing NSCs implanted into the hippocampi on MWM performance of healthy mice.

Figure 3: Scope of this thesis

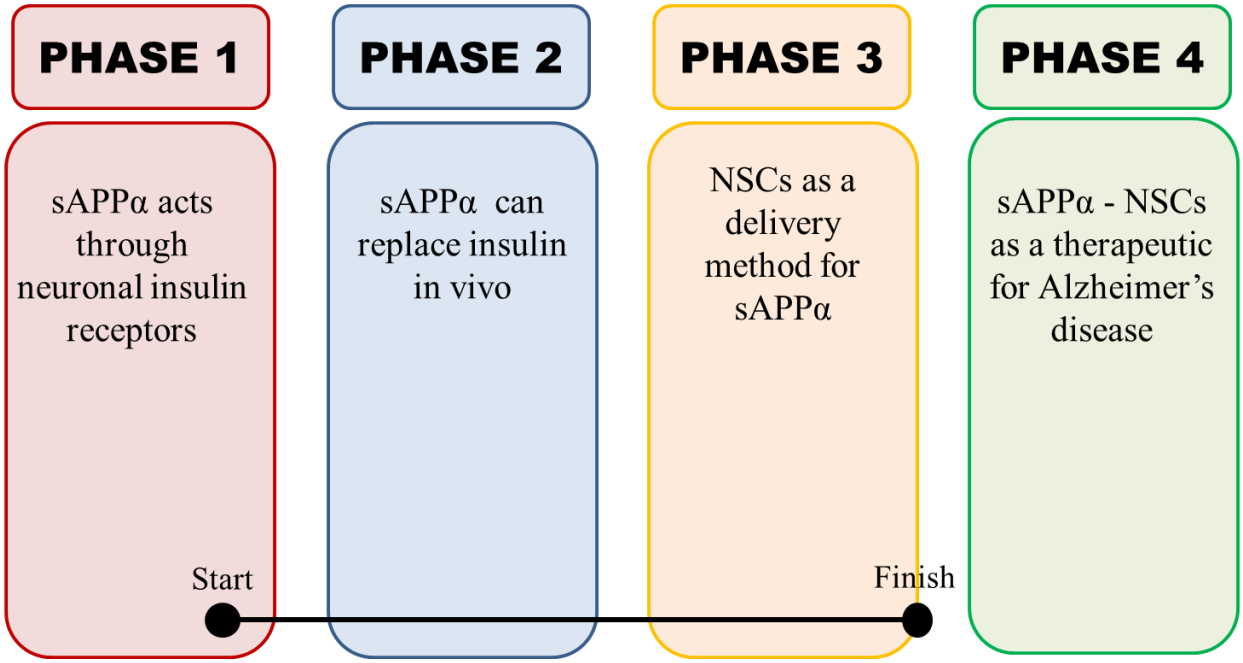


Figure 3: Scope of this thesis *- The scope of this thesis begins with data demonstrating that sAPPα binds and activates neuronal insulin receptors. We then evaluate the effects of sAPPα in the diabetic CNS and PNS. Finally, we test the effects of sAPPα-overexpressing NSCs on MWM of healthy mice.*

Summary: Diabetes and AD are linked both etiologically and biochemically. Furthermore, stimulation of the insulin signaling pathway is a promising therapeutic strategy in AD. Although it remains unclear whether or not sAPPα acts directly on IR or IGF1R (Jimenez et al. 2011), sAPPα nevertheless activates the insulin signaling pathway, reduces tau phosphorylation, improves learning and memory, protects against excitotoxicity and unlike IGF1 and insulin, directly blocks Aβ production by inhibiting Aβ generating enzymes. sAPPα also has

potent cognitive effects, which may be due to a combination of insulin signaling activation as well as modulation of NSC activity. In total, sAPPα appears to be an ideal therapeutic for AD and diabetes-induced pathology in the nervous system. Therefore, this work tested the hypothesis that sAPPα can replace the effects of insulin *in vivo*. The results described here may provide a basis for the development and implementation of sAPPα-based therapeutics.

CHAPTER 2: DETAILED MATERIALS AND METHODS

Immunoblotting/immunoprecipitation: Cortical tissue from experimental mice were isolated, frozen on dry ice and stored at -80ºC until processed. Protein was extracted by homogenization in 4% SDS buffer (125mM Tris-HCl, pH 6.8, 4% SDS). Protein concentration was determined and western blot samples made. Either cell culture or cortical tissue samples (10-20 ug) were separated by SDS-PAGE, transferred to PVDF membrane, and blocked with either 5% milk or 5% bovine serum albumin (BSA) in 0.1% tris buffered saline- tween (TBST). The same solution was used for incubation with primary antibodies (incubation in primary antibodies was carried out overnight at 4 degrees Celsius, see table 3 for the antibodies used). The following day, membranes were incubated with an appropriate species-specific secondary antibody, washed in TBST, and imaged using a FluorSMax (Biorad) or chemidoc (Biorad) imaging system. ECL prime (General Electric) detection kit was used to detect blots in figures 4 and 6 while Clarity Western ECL (Biorad) was used for all other blots. Quantity one software was used to quantify bands in figures 4 and 6 while all other blots were quantified with Image lab (Biorad) software.

For immunoprecipitation, either cortical neuronal cultures or brain tissue were homogenized in non-denaturing lysis buffer (50 mM Tris pH 7.4, 150 mM NaCl, 1 % Triton, 1 mM EDTA, 10 mM NaF, 2 mM sodium orthovanadate and protease inhibitors [Roche]). Homogenates were incubated at 4ºC for 20 minutes to assist cell lysis, followed by a centrifugation at 14,000xg for 10 minutes to remove cellular debris. Following protein quantification, 500 µg of protein was incubated with 2 µg antibodies to insulin receptor (Santa Cruz Biotechnology) or APP (6E10, signet) overnight at 4 degrees Celsius. Protein A/G sepharose beads (Pierce) were added and samples were mixed vigorously for 4 hours. Mixtures

were washed twice in lysis buffer and once in ice-cold PBS before eluting with 4% SDS for immunoblotting.

Table 1: Antibodies used for study

Antibodies used for western blot analysis				
Antibody	**Species**	**Dilution**	**Vendor**	**Catalog #**
phospho-AKT (S473)	Rabbit	1/1000	Cell Signaling	9271
AKT	Rabbit	1/1000	Cell Signaling	9272S
phospho-AMPK (Thr172)	Rabbit	1/1000	Santa Cruz	sc-33524
APP (6E10)	Mouse	1/1000	Signet	SIG-39320
APP (A4)	Mouse	1/1000	Millipore	MAB348
β-actin	Mouse	1/1000	Santa Cruz	sc-47778
phospho-eIF2α (Ser51)	Rabbit	1/1000	Cell Signaling	3597S
eIF2α	Rabbit	1/1000	Santa Cruz	sc-133132
phospho-GSK3α (Ser21)	Rabbit	1/1000	Cell Signaling	9327S
GSK3α	Rabbit	1/1000	Cell Signaling	9338S
phospho-IR (Y972)	Rabbit	1/1000	Millipore	07-838
IR	Rabbit	1/1000	Santa Cruz	sc-713
phospho-MAPK (T202/Y204)	Rabbit	1/500	Cell Signaling	9106S
MAPK	Rabbit	1/1000	Cell Signaling	9102
phospho-PERK (Thr980)	Rabbit	1/500	Cell Signaling	3179
PERK	Rabbit	1/1000	Santa Cruz	sc-377400
PP2A-C	Rabbit	1/1000	Millipore	05-421
phospho-Tau (Thr231)	Rabbit	1/2000	Millipore	ab151559
Tau	Rabbit	1/2000	Millipore	ab39524
Antibodies used for immunoprecipitation				
Antibody	**Species**	**Amount**	**Vendor**	**Catalog #**
IR	Rabbit	2µg	Santa Cruz	sc-713
Antibodies used for immunohistochemistry				
Antibody	**Species**	**Dilution**	**Vendor**	**Catalog #**
GFAP	Rabbit	1/500	Thermofisher	PA3-16727

Data Analysis: Where appropriate, data were subjected to 2-way repeated measures ANOVA 1-way ANOVA with Tukey's or Dunnet's post-hoc tests or an unpaired, two-sided, student's t-test using Graphpad Prism software (La Jolla, CA).

Induction of type 1 diabetes in experimental animals by STZ: STZ is selectively taken up by GLUT2 receptors at the cell surface of insulin producing beta cells in the pancreas. Once taken up, STZ destroys beta cells via a genotoxic mechanism resulting in insulin-depletion in the whole animal. To render male Sprague Dawley rats (300g) diabetic, we used a single intraperitoneal injection STZ (85 mg/kg; Sigma). For Wt and sAPPα overexpressing mice, 90mg/kg of STZ was given via intraperitoneal injection on 2 consecutive days. Rats were maintained in parallel with age-matched controls for 3-5 months while mice were maintained in parallel for 10 or 16 weeks. Tail blood glucose was assayed three days after injection to confirm diabetes. Only animals with blood glucose values higher than 18mmol/L were considered diabetic. For CRND8 mice, a dose of 90 mg/kg was used on three successive days. Blood glucose was determined prior to a fourth injection; if levels were higher than 18.0mmol/L, the animal was considered diabetic and not reinjected. Otherwise, animals were re-injected once every three days until diabetic. CRND8 mice were housed for 6 weeks prior to euthanasia for tissue collection. Only animals diabetic for the same amount of time and same age were used for assaying purposes. Furthermore only male animals were used.

Euthanasia: Since the pathways we were interested in (AKT/GSK3/Tau signaling, PP2A activation) are modified by common forms of anesthesia (Planel et al. 2009), all mice were

euthanized by conscious cervical dislocation. Rats used for DRG cultures were euthanized by conscious decapitation.

Ethics statement: All animal studies were in accordance with the University of Manitoba Animal Care Committee rules using Canadian Council of Animal Care guidelines. Animal studies were completed under University of Manitoba protocol number 14-029.

Embryonic cortical neuron culture: Cortices from E16 embryonic CD1 mice were dissected out and triturated to release neurons. For the first 24 hours, neurons were plated in Neurobasal media supplemented with 10% FBS and B27 and then in Neurobasal + B27 for the next 6 days. Three days after plating, media containing 500nM cytosine arabinoside was used to inhibit glial cell growth.

sAPPα dose response experiment: 7 days after plating, cortical neurons were plated in Neurobasal media in the absence of B27 for 2.5 hours. After this starvation period, cells were treated with 10, 30, or 100 nM sAPPα for 15 minutes then scraped in denaturing lysis buffer and processed for western blot.

CRND8 mice: CRND8 mice overexpress APP695 harboring 3 familial AD mutations (Swedish, K670N & M671L; Indiana, V717F) which cause increased Aβ production. In these mice, expression of mutant APP is driven by the Syrian hamster prion protein promoter on a C57Bl/6 background (Chishti et al. 2001).

Prestoblue assay of viability: Cultured cortical neurons were in treatment for 16 hours prior to the addition of Prestoblue (thermo fisher). Prestoblue was added to cell culture media according to the manufacturer's instructions. Two hours after the addition of Prestoblue, mean absorbance of reduced resorufin (a marker of cell viability) was standardized to absorbance of non-metabolized resazurin to generate data (expressed as viability).

sAPPα overexpressing mice: Breeding pairs of Tg- sAPP overexpressing mice (Bailey et al. 2012) were kindly supplied by Dr. Jun Tan, University of South Florida. In brief, transgenic mice were created by pronuclear injection of human sAPPα expression constructs into C57Bl/6 embryos. Hemizygous Tg mice were confirmed by PCR and used for experiments while non-transgenic littermates served as controls. In these mice, sAPPα overexpression is driven by the mouse prion promoter. The concentration of sAPPα in whole brain homogenates from hemizygous Tg mice is ~20ng/mg (Bailey et al. 2012) and WB analysis has been used previously to confirm the overexpression of sAPPα in hemizygous cortices and hippocampi (Bailey et al. 2013).

HbA1c analysis: Blood samples were collected from diabetic sAPPα-overexpressing mice, plus controls, immediately post-mortem and HbA1c levels determined using available kits (PTS diagnostics).

PP2A-C activity: Cortical tissue was homogenized in phosphatase buffer (20nM imidazole, 2mM EDTA, 2mM EGTA, 1mM benzadine, 10ug/ml pepstatin + 1 protease inhibitor cocktail tablet/10ml (Roche)) and immunoprecipitation performed on whole cortices (1 cortex/sample, N

= 3-4 animals/group) using anti-PP2A-C antibodies. Once precipitated, activity of PP2A-C proteins was detected using available kits (Millipore cat #17-313). Some of the immunoprecipitated PP2A-C sample from each animal was set aside for a protein assay. Briefly, precipitated PP2A-C proteins were incubated in threonine phosphopeptides at 30^0C in a shaking incubator for 10 minutes. After incubation, samples were centrifuged (2500RPM for 10min) and supernatant collected for analysis of free phosphate. Malachite Green Phosphate Detection Solution, which changes color in the presence of free phosphate, was added to each sample of supernatant and color allowed to develop for 10-15 minutes at room temperature. Sample absorbance was read at 650nM and optical density (O.D.) values recorded (higher O.D. values corresponded with higher levels of free phosphate and therefore increased PP2A-C activity). O.D. values were then standardized to values obtained from the protein assay to give the amount of PP2A-C activity/amount of protein. Mean values of this calculation for each group were determined and used for data in figure 12.

DRG sensory neuron cultures and treatments: DRG were harvested from adult normal or diabetic Sprague Dawley rats (see Table 3) and single cells dissociated with trypsin treatment followed by trituration. Cells were grown in Hams F12 media (Sigma) supplemented with N2 (0.1mg/ml transferrin, 20nM progesterone, 0.01 mM cytosine arabinoside, 100μM putrescine, 30nM sodium selenite, 1mg/ml BSA) (Huang et al. 2005). Control neurons were cultured in the presence of 10mM D-glucose while diabetic neurons were exposed to 25mM D-glucose to mimic hyperglycemic conditions.

For experiments, cells were grown in F12 + N2 supplement without growth factors/insulin for 24 hours. sAPPα used for experiments was purchased from sigma (catalogue #

S9564). See supplementary materials section for an example data sheet and example of Sigma's quality control. Thirty nM sAPPα (Sigma) was determined to be the minimum dose required to induce a significant increase in DRG neuronal outgrowth. Subsequent experiments used treatments of 100nM sAPPα as this concentration was found to stimulate robust neurite outgrowth in cultured DRG cells. Some wells were incubated with 20uM AG1024 (Santa Cruz), an antagonist of IR/IGF1R, for 60min prior to insulin or sAPPα treatment. This concentration of AG1024 was found to completely block insulin-induced neurite outgrowth in our cultures. Insulin used for experiments was purchased from Sigma.

Immunocytochemistry staining of cultured DRG neurons for NFκB subunits and β-tubulin class III: After appropriate treatment, cultured DRG neurons were fixed with 2% paraformaldehyde in phosphate buffered saline (PBS, pH 7.4) for 15min at room temperature then permeabilized with 0.3% TritonX-100 in PBS for 5min. Cells were then incubated in blocking buffer (Roche, Indianapolis, IN) diluted with FBS and 1mM PBS (1:1:3) for 1hr then rinsed three times with PBS. Primary antibodies against β-tubulin isotype III (1:500) (neuron specific; Sigma Aldrich, Oakville, ON) or the NFκB subunits p50, p65, and c-Rel (Santa Cruz Biotechnology, Santa Cruz, CA,) were added to wells and plates were incubated at 4°C overnight. The following day, cells were rinsed 3X with PBS and incubated with AlexaFluor 488-conjugated secondary antibodies (1:500) (Invitrogen) or Cy3-conjugated secondary antibodies (1:500) (Jackson Laboratories) for 1hr at room temperature. Cells were then washed 3X with PBS. Coverslips were mounted on glass slides with DAPI-supplemented mounting medium. Cells were imaged using a Carl Zeiss Axioscope-2 fluorescence microscope (20X

objective) equipped with an AxioCam camera. Images were captured using AxioVision3 software.

Quantification of neurite outgrowth: To determine neurite outgrowth, fluorescent images of cultured cells stained with β-tubulin class III/Cy3 conjugated antibodies were captured. In order to quantify neurite outgrowth, the mean fluorescent pixel area of neurites was determined by removing the fluorescence of the cell body using ImageJ (NIH) software leaving only neurite fluorescence remaining in the image. Total pixel area of the remaining neurites was then divided by the total cell number, to give mean neurite outgrowth area per cell. Data is reported as the average outgrowth/cell ratio between the three coverslips imaged for each group.

Analysis of nuclear/cytoplasmic ratios for NFκB subunits: To determine nuclear: cytoplasmic ratios of NFκB subunits, the nucleus of neurons stained for the NFκB subunits p50, p65, or cRel was traced and mean fluorescence intensity measured using ImageJ software. This value was then divided by the mean fluorescence value obtained from the cytoplasm to obtain the nuclear/cytoplasmic fluorescent ratio (Saleh et al. 2013)

RT-PCR: Whole DRGs were isolated from adult wild type and Tg sAPPα over-expressing mice. DRGs were homogenized in Trizol (Thermo Fisher) and RNA was extracted as per the manufacturer's instructions. The cDNA template was prepared using the iScript cDNA Synthesis Kit (BIO-RAD) as per the manufacturer's instructions. Finally, amplification of sAPPα was performed using the REDExtract-N-Amp™ Tissue PCR Kit (Sigma Aldrich) as per the manufacturer's instructions. The primers used for amplification of sAPPα (457 bp) and β

actin (100 bp) are as follows (5' to 3'): β actin forward: GATGTATGAAGGCTTTGGTC; β actin reverse: TGTGCACTTTTATTGGTCTC; sAPPα alpha forward: GCCTGGACGATCTCCAGC; sAPPα reverse: TGGCCCGGTGTTAGCACTGGC. Annealing temperatures used for PCR amplification of sAPPα and β actin are 69°C (40 cycles) and 60°C (30 cycles), respectively. PCR products were run on a 3% agarose gel in 1x TAE at 100V for 35 minutes. Images were obtained on a ChemiDoc MP (BIO-RAD).

Quantification of thermal sensitivity (Hargreaves test): Hind paw thermal response latencies were measured using the Hargreaves thermal sensitivity machine. In short, rodents were placed in plexiglass cubicles on top of the modified Hargreaves device (UCSD, La Jolla, CA, USA) and the heat source was placed directly below the middle of one of the hind paws and the time until paw withdrawal measured. Response latency of each paw was measured 4 times at 5 min intervals and the median value of trials 2-4 used to represent the response latency for that paw. Median values were then averaged with between the two hind paws for each animal to generate data points (Jolivalt et al. 2012).

Mice: Wt, SAMR1 mice that were injected with NSCs and subjected to MWM testing were obtained from Harlan Laboratories and maintained in conventional housing until 6 months of age. 6-month old mice then had NSCs implanted and were housed 2 more months in conventional housing before MWM testing. After testing, mice were sacrificed by cervical dislocation and brain tissue processed for analysis by immunohistochemistry (IHC). *eGFP mice*: Homozygous eGFP mice expressing mice were purchased from Jackson Laboratories (Stock No. 003291). In these mice, expression of enhanced green fluorescent protein (eGFP) is under the control of a chicken beta-actin promoter and cytomegalovirus enhancer.

Generation NSCs: NSCs used for implantation were generated by breeding homozygous, eGFP expressing, C57/Bl6 dams (driven by the beta-actin promoter) with C57/Bl6 wild-type sires, to generate Wt-NSCs, or homozygous sAPPα-overexpressing sires, to generate sAPPα-NSCs. NSCs were harvested from the cortices of the resulting (E14) embryos. Tg, sAPPα-overexpressing NSCs have been demonstrated to secrete >10fold more sAPPα into culture media compared to Wt-NSCs (Bailey et al. 2013).

NSC culture: Cortices were dissected out, triturated and cells passed through a 40uM mesh. Cells were maintained in media containing basic fibroblast growth factor (bFGF) (20ng/ml) and human epidermal growth factor (hEGF) (20ng/ml), in order to maintain NSC characteristics, until implantation. Cultured Wt-NSCs and sAPPα-NSCs were analyzed for nestin expression by WB to confirm NSC characteristics (Supplemental Figure 1).

NSC implantation: Six-month old SAMR1 mice were anesthetized with isoflurane and incisions made along the midline of the scalp. 3ul of NSCs (100,000 cells/ul) suspended in ACSF were injected into each hippocampi, according to previous studies (Blurton-Jones et al. 2009) using the following coordinates (relative to the bregma): anteroposterior -2mm, mediolateral +/- 1.75mm, and dorsoventral -1.75mm After surgery, scalp incisions were closed with sutures and mice returned to their home cages after the effects of anesthesia had worn off. Carprofen was given up to 24 hours post-operatively for pain control. A total of 29 mice were injected for this study (10 were injected with ACSF, 11 with sAPPα-NSCs and 8 with Wt-NSCs). 2 mice injected with Wt-NSCs had to be euthanized due to health reasons before the end of the study.

MWM: Eight weeks after the implantation of NSCs, cognitive ability was assessed in our mice using the MWM. The maze consisted of a circle pool filled with water that was made opaque by the addition of non-toxic, black water-based paint. A circular platform was hidden 2cm below the surface of the water. Visual cues were placed at cardinal points around the pool. Mice were placed into the pool and allowed to swim freely for 1 minute or until the hidden platform was found. If the platform was not found after 1 min, mice were placed onto the platform and remained there for 20 seconds before being removed from the pool. Each day of training consisted of 4 trials (up to 1 minute or when mice found the platform) in which mice were placed under each visual cue. A total of 5 days of training was performed. After the training sessions on day 5, the hidden platform was removed and mice were placed back into the pool to swim for 60 seconds. Time in the quadrant where the hidden platform had been previously, as well as crosses over the previous location of the platform, were recorded and quantified.

IHC analysis of hippocampal slices: Hippocampi were removed immediately after sacrifice and fixed in 4% paraformaldehyde (PFA) for 1 hour. After fixation, tissue was placed into 30% sucrose overnight then placed into molds containing OCT. 20um slices were prepared from frozen blocks of OCT containing tissue then analyzed by IHC. Briefly, slices were incubated in anti-GFAP antibodies (Thermofisher; 1:500) diluted in blocking buffer (Roche) overnight at 4 degrees Celsius. The next day, slices were washed with PBS and incubated in cy-3 conjugated anti-rabbit antibodies 1:500) for 1 hour at room temperature. Slices were then washed with PBS and sealed to glass coverslips using DAPI-containing hard set mounting medium.

[2]CHAPTER 3: sAPPα ACTIVATES NEURONAL IR

INTRODUCTION:

Despite evidence that sAPPα activates the PI3K/AKT axis, stimulates MAPK signaling, and requires IRS to stimulate neuronal outgrowth, the putative receptor sAPPα receptor remains unknown. Previous studies demonstrate that the protective effects of sAPPα can be blocked by inhibitors of IR/IGF1R and by Aβ oligomers which antagonize IR. While these studies provide a clear link between sAPPα and the insulin signaling pathway, several unknowns remain. For instance, it is unclear if the effects of sAPPα are mediated through activation of IR or the structurally similar IGF1R. Furthermore, a direct interaction between sAPPα and IR has yet to be established and it's unknown whether or not sAPPα would bind the same ligand binding sites on IR as insulin. Therefore, the goals of this work were to determine if sAPPα activates IR specifically, and if so, is that activation mediated by direct binding or by an indirect method such as inhibition of IR phosphatases.

RESULTS:

sAPPα increases insulin receptor phosphorylation in neuronal cultures – To determine if sAPPα would activate IR *in vitro,* neuronal cultures were insulin depleted for 2.5 hours in order to remove endogenous insulin signaling. After this washout period, sAPPα (10, 30

[2] Sections of text from chapter 3 and 4, and figures 4, 6, 8, 9, and 10, are published in Experimental Neurology, 2018 Brent D. Aulston, Jason Shapansky, YaWen Huang, Gary L.Odero, Gordon W. Glazner ," Secreted amyloid precursor protein alpha activates neuronal insulin receptors and prevents diabetes-induced encephalopathy". -see permissions section

and 100 nM) was added for 15 minutes after which cells were lysed and lysates prepared for western blot. To assess insulin receptor activation, we analyzed phosphorylation of neuronal IR at Tyr 972 as this site in particular is required for activation of the insulin signaling pathway by providing a docking site for IRS (White et al. 1988). As demonstrated in Figure 4A, sAPPα increased IR phosphorylation in a dose-dependent manner. Because antibodies targeted to phosphorylation sites on IR can also cross-react with phosphorylation sites on IGF1R, we performed immunoprecipitation on sAPPα-treated lysates using antibodies specific for the insulin receptor. Western blot analysis of these precipitated lysates revealed that sAPPα specifically increased IR phosphorylation (Fig. 4B).

Figure 4: sAPPα increases insulin receptor (IR) phosphorylation in cultured mouse cortical neurons

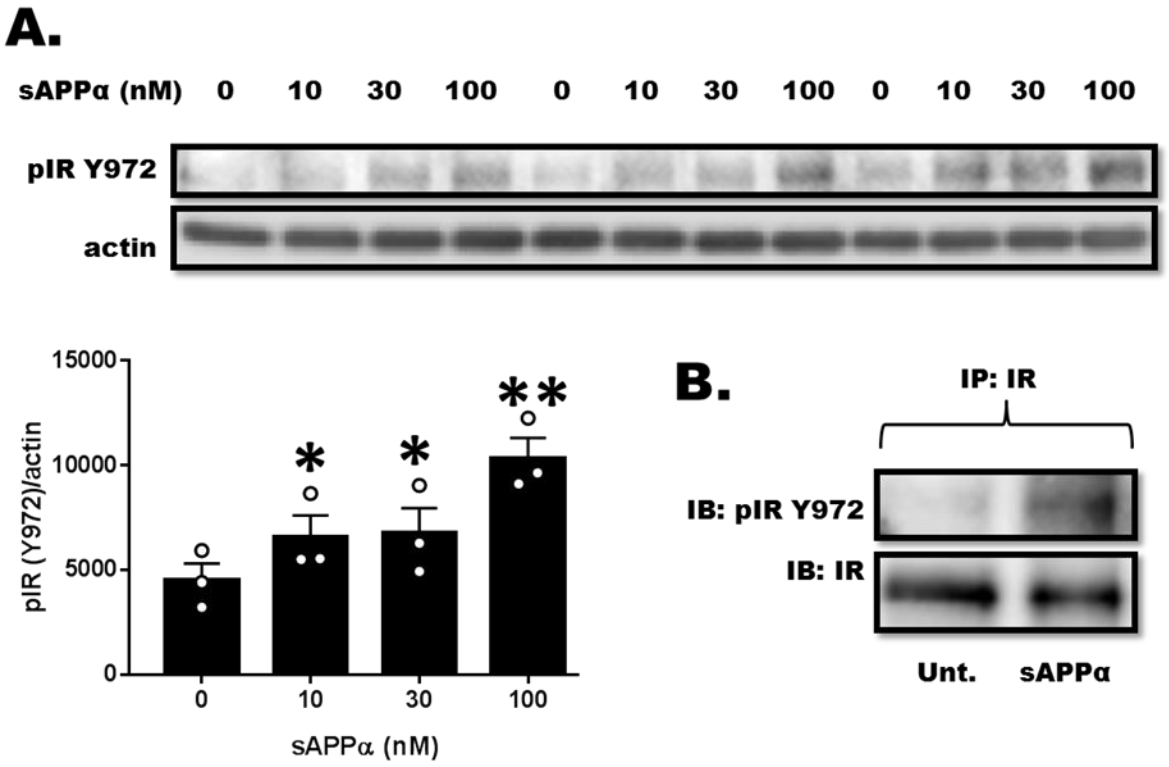


Figure 4: sAPPα increases insulin receptor (IR) phosphorylation in cultured mouse cortical neurons *- (A) Mouse embryonic cortical neurons were cultured for 7 days, then insulin depleted for 2.5 hours prior to treatment with sAPPα (10 nM, 30 nM, or 100 nM). 15 minutes after the addition of treatments, cells were lysed and lysates prepped for western blot. Immunoblotting with phosphorylated insulin receptor (pIR, Y972) antibody revealed a dose-related increase relative to actin versus controls. Data is mean pIR/actin ratio +/- SEM. Graph units are arbitrary. Data was analyzed with a one-way ANOVA, Tukey post-hoc, *p<0.05, **p<0.01 vs 0nM sAPPα. N= 3 wells. (B) Mouse embryonic cortical neurons were cultured for 7 days, and then insulin depleted for 2.5 hours prior to treatment with sAPPα (100nM). Cell lysates were collected 15 minutes after the addition of sAPPα. IR were immunoprecipitated (IP:IR) from untreated (Untr.) and sAPPα treated (sAPPα) lysates and probed by immunoblot for phospho-IR (IB: pIR Y972) and total IR (IB: IR). This figure is the work of Jason Schapansky.*

sAPPα is protective against insulin depletion – Since sAPPα activated neuronal IRs *in vitro*, we next analyzed if sAPPα is protective against insulin depletion. To test this, cortical neurons were cultured in the absence of insulin for 16 hours and then cell viability evaluated (Fig 5B, for cell viability assay details, see Presto Blue assay in the Materials and Methods). In order to determine the protective effects of endogenous sAPPα production on cell survival, some cultures were treated with GI254023X, an inhibitor of α-secretase. Only insulin-depleted cells cultured in GI254023X showed a difference in cell viability compared to cultures with both insulin and endogenous sAPPα production. These results suggest that sAPPα is protective

against insulin depletion in neurons which is supported by the finding that APP/sAPPα binds IR in brain tissue from healthy rats (Fig 5A).

Figure 5: APP/sAPPα binds IR *in vivo* and is protective against insulin depletion *in vitro*

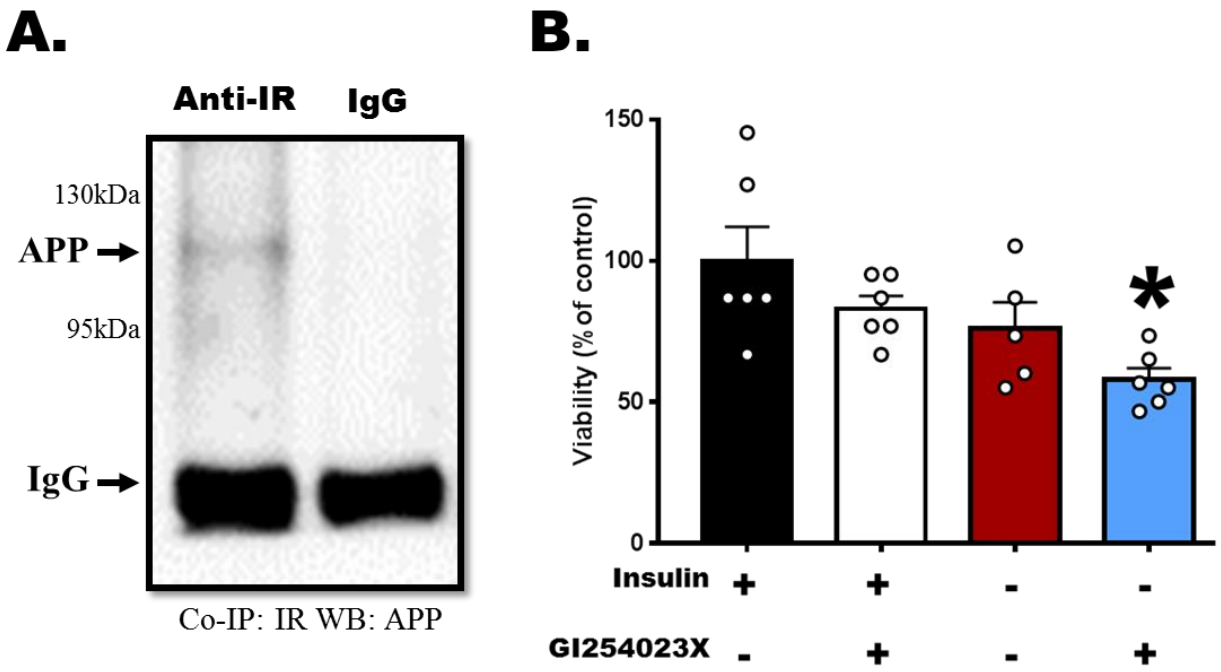


Figure 5: APP/sAPPα binds IR in vivo and is protective against insulin depletion in vitro *– A. Cortical tissue from a 3month old wild-type rat was homogenized in RIPA buffer and immuno-precipitation performed using antibodies against the beta subunit of insulin receptor (IR) or non-specific IgG antibodies as a control. Following IP, precipitates were Western blotted and probed with anti-APP antibodies (raised against the N-terminus of APP). B. Cortical neurons were plated in B27 media +/- insulin, and +/- 20uM of the alpha secretase inhibitor GI254023X for 16 hrs. Cells were then incubated in Presto Blue for 2 hrs and cell viability was assessed by analyzing absorbance at 570nm (corresponding to absorbance by reduced rezofurin) standardized to absorbance at 600nm (corresponding to absorbance by oxidized resazurin). Data*

*represents mean absorbance +/-SEM (expressed as viability). N = 5-6 wells/treatment. Data was analyzed by 1-way ANOVA with Tukey's post-hoc test. * indicates p<0.05.*

APP/sAPPα physically interacts with IR and competes with insulin for IR binding sites *in vivo* - Activating phosphorylation of neuronal IR in culture can occur directly by receptor binding ligands, such as insulin and IGF1, or indirectly by treatments, such as hydrogen peroxide (Hayes et al. 1987, Mahadev et al. 2001), that inhibit phosphatases that act on IR. Therefore, a potential physical interaction between APP/sAPPα and IR was assessed *in vivo* using co-immunoprecipitation (Co-IP). Co-IP of insulin receptors in CRND8 and CRND8-STZ brain homogenates was followed by Western blot analysis of Co-IP samples with a specific sAPPα antibody. As demonstrated in Table 2, Wt and CRND8 mice were successfully rendered type 1 diabetic, and therefore insulin depleted. sAPPα/APP was detected in the both CRND8 and CRND8-STZ pulldown samples (Fig. 6A), but CRND8-STZ animals had significantly higher APP/sAPPα levels than saline injected CRND8 animals. To rule out the possibility that the increased interaction between APP and IR we observed in CRND8-STZ homogenates was a result of differential expression of APP or IR, we analyzed cortical APP (using antibodies raised against the N-terminus of APP) and IR levels in our animals and found no differences in expression between CRND8 and CRND8-STZ groups (Fig. 6B).

Table 2: Blood glucose of Wt and Tg (CRND8) mice

Group	B.G (mmol/L)
Wt	13.1 $\pm$ 0.54
Tg-CRND8	12.3 $\pm$ 0.67

Wt –STZ	31.3 ± 0.77***
Tg-CRND8 STZ	30.8 ± 1.24***

Table 2: Blood glucose and brain weight Wt and Tg (CRND8) mice *- 8-week old wild-type (Wt) and transgenic CRND8 mice were rendered diabetic with intraperitoneal injections of streptozotocin (STZ) and maintained for 6 weeks. Data represents mean blood glucose (B.G.) measurements at the time of death +/- SEM. Data was analyzed using 1-way ANOVA with Tukey's post-hoc test. *** indicates $p<0.001$ compared to Wt/Tg-CRND8 groups. N = 5-11/group.*

Figure 6: Physical interaction between APP/sAPPα and insulin receptor is greater in insulin-depleted animals

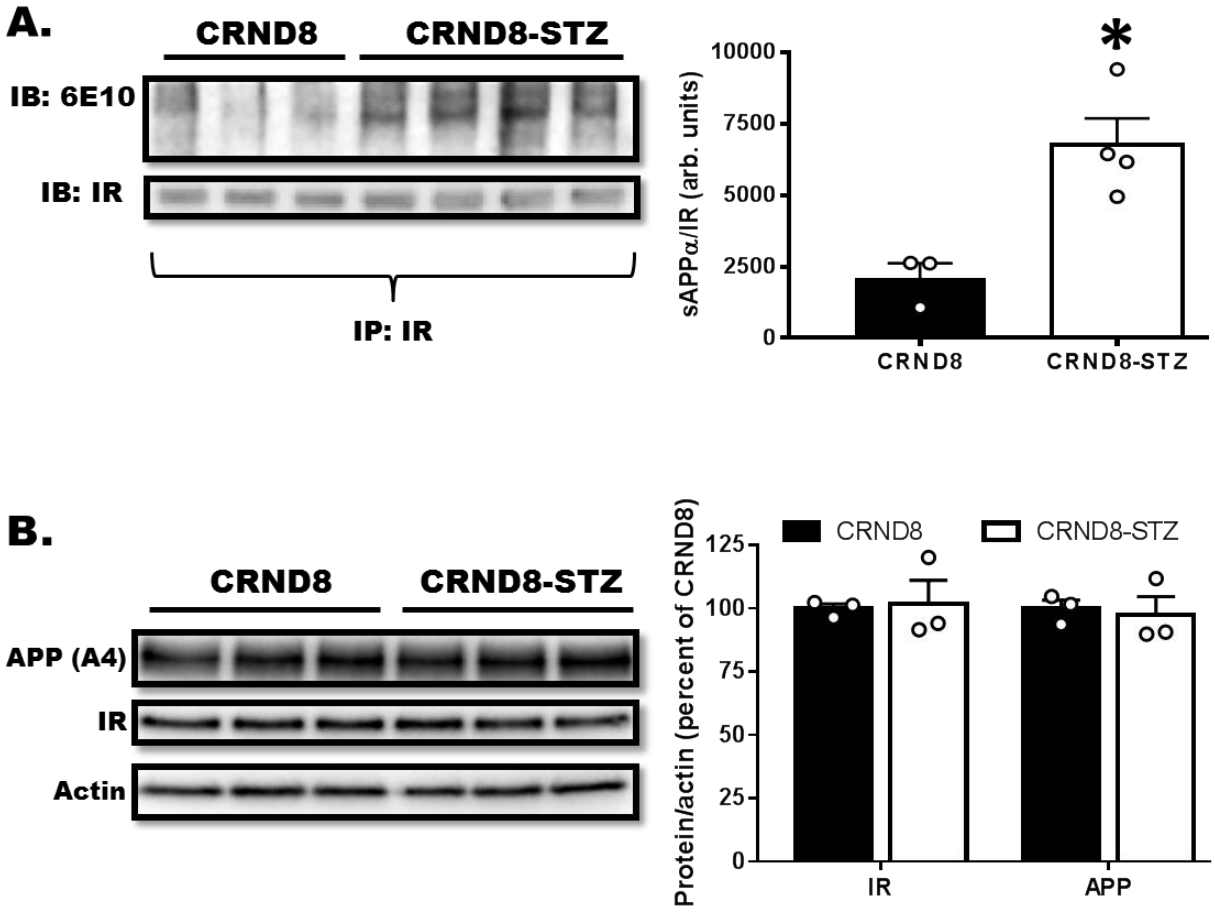


Figure 6: Physical interaction between sAPPα and insulin receptor is greater in insulin-depleted animals - *A. Co-immunoprecipitation (co-IP) of cortical lysates from CRND8 and CRND8 STZ mice with a specific IR antibody (IP: IR) followed by immunoblot with 6E10 antibody (IB: 6E10) and IR antibodies (IB: IR) revealed that the amount of sAPPα bound to IR was significantly higher in CRND8-STZ mice than non-diabetic controls (mean sAPPα/IR ratio +/- SEM, t-test, N = 3/4/group, *p<0.05). B. CRND8 and CRND8-STZ cortical lysates were probed by western blot using antibodies raised against IR and the N-terminus of full length APP (APP A4). Data represents mean protein levels standardized to actin loading controls +/- SEM.*

N =3 animals/group. Data was analyzed with student's t-test. No differences (p>0.05) in APP/IR expression were detected between groups. Part A of this figure is the work of Jason Schapansky. Part B was completed by Brent Aulston.

SUMMARY:

With this work we demonstrate that sAPPα activates IR in the absence of insulin, and sAPPα/APP physically interacts with IR and competes with insulin for ligand binding sites. The antibodies used in these experiments detect the N-terminus of APP (Fig. 5) and an epitope between amino acids within the Aβ region (antibody 6E10, Fig. 6). Because these antibodies detect both APP and sAPPα, it is unclear which form interacts with IR. Nevertheless, these results suggest that sAPPα is an activating ligand for IR.

CHAPTER 4: SECRETED AMYLOID PRECURSOR PROTEIN ALPHA PREVENTS DIABETES-INDUCED ENCEPHALOPATHY

INTRODUCTION:

Hyperphosphorylation of the microtubule associated protein tau leading to tau aggregation is hypothesized to contribute to cognitive dysfunction and cell death in AD, Parkinson's disease and diabetic encephalopathy. Previous studies demonstrate that neuronal insulin signaling directly regulates tau phosphorylation *in vitro* and *in vivo*. Despite evidence that sAPPα activates AKT and prevents tau phosphorylation in the brains of AD mice, the effects of sAPPα on diabetes-induced tau pathology remain unexplored. Therefore, we tested the hypothesis that sAPPα could prevent the development of Alzheimer's –associated tau pathology in the insulin-depleted rodent brain.

RESULTS:

Generation of diabetic sAPPα-overexpressing mice – Built off the results obtained in chapter 3, the central hypothesis tested in this thesis was that sAPPα inhibit the development of pathology in the diabetic nervous system. We tested this by rendering sAPPα-overexpressing mice insulin depleted with STZ injections. It was important to determine if STZ-injected sAPPα mice were as hyperglycemic as their Wt counterparts as any discrepancies in glycemic profiles could confound our results. As shown in Figure 7, no differences in fasting blood glucose or HbA1c levels were found between diabetic Wt and diabetic sAPPα (Tg) mice. This demonstrates that sAPPα overexpression did not affect the animals' response to STZ nor did sAPPα increase glucose uptake.

Figure 7: Characteristics of diabetic sAPPα mice

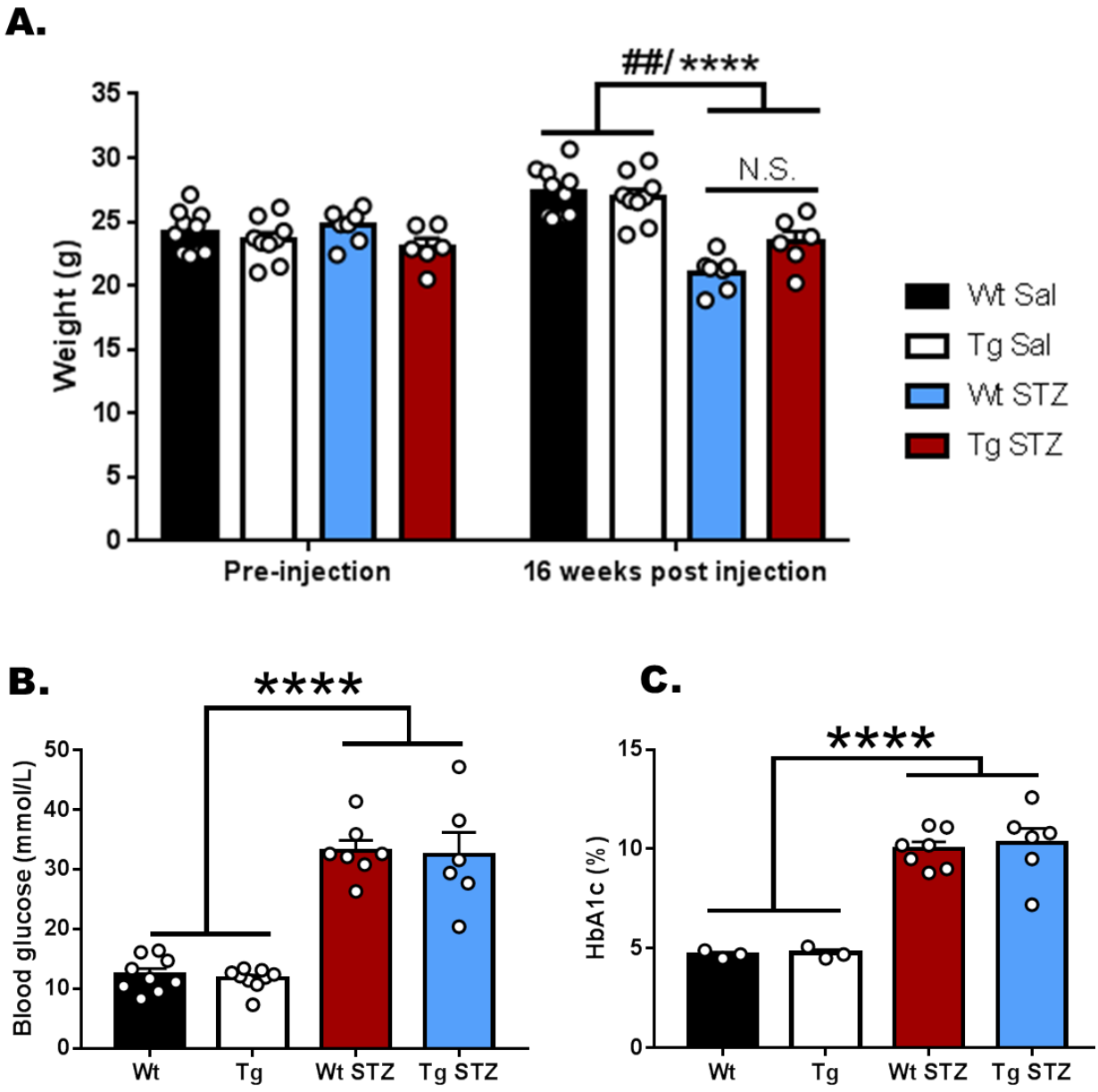


Figure 7: Characteristics of diabetic sAPPα mice *- A. Pre-injection body weight and body weight 16 weeks post-injection was determined in diabetic (STZ) wild-type (Wt) and sAPPα (Tg) overexpressing cohorts plus saline(Sal) injected controls. Data was analyzed by a 2-way, repeated measures ANOVA. ## indicates p<0.01 between saline groups and Tg STZ group. ****

*indicates p<0.0001 between saline groups and Wt STZ group. B. Fasting (3 hours) blood glucose levels were measured 2 days prior to death. Data represents mean blood glucose +/- SEM. Data was analyzed by a 1-way ANOVA and Tukey's post hoc test. **** indicates p<0.0001 between STZ injected groups and saline injected groups. No differences were detected between Tg STZ and WT STZ mice or between Tg saline and Wt saline mice. C. HbA1c percentages were determined with blood samples collected immediately post mortem. Data represents mean HbA1c % +/- SEM. Data was analyzed by a 1-way ANOVA and Tukey's post hoc test. **** indicates p<0.0001 between STZ injected groups and saline injected groups. No differences were detected between Tg STZ and WT STZ mice or between Tg saline and Wt saline mice. For A and B, N = 6-10 animals per group. For C, N = 3-7 animals/group.*

Overexpression of sAPPα prevents hyper-phosphorylation of tau in the diabetic brain – Elevated levels of sAPPα have been suggested as a mechanism for the absence of neurodegeneration in young CRND8 mice despite having supraphysiological concentrations of Aβ (Jimenez et al. 2011). To test the hypothesis that sAPPα specifically could prevent pathology in the insulin depleted brain, we examined phosphorylated tau levels in the cortices of our diabetic sAPPα mice.

Aberrant tau phosphorylation leading to tau aggregation is hypothesized to underlie neurodegeneration in AD (Braak et al. 1991, Braak et al. 1997, Delacourte et al. 1999, Price et al. 1999). Because sAPPα decreases tau phosphorylation in human cell lines as well as transgenic AD mice (Deng et al. 2015), we hypothesized that sAPPα overexpression would prevent tau hyperphosphorylation in the STZ-diabetic brain. As demonstrated in Figure 8, analysis of cortical lysates from diabetic-sAPPα mice revealed a decreased phospho-tau (Thr 231)/total tau

ratio compared to diabetic-Wt controls ($p<0.01$). No differences between non-diabetic controls and diabetic sAPPα-overexpressing mice were detected.

Figure 8: Overexpression of sAPPα prevents hyperphosphorylation of tau in diabetic cortices

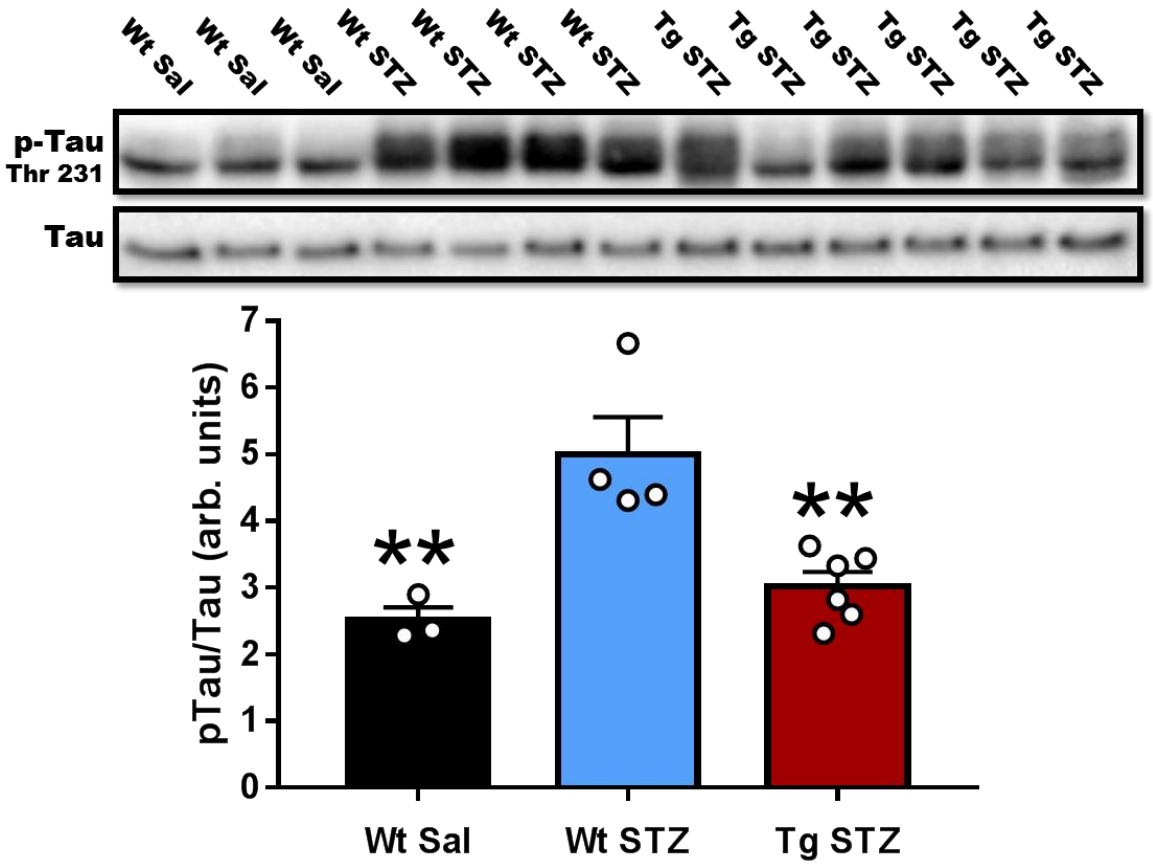


Figure 8: Overexpression of sAPPα prevents hyperphosphorylation of tau in diabetic cortices *– Cortical tissue lysates from 16 week diabetic (STZ) or non-diabetic, saline injected (Sal) wild-type (Wt) and sAPPα-overexpressing mice (Tg) were analyzed by WB for phosphorylated Tau (T231). Data represents mean phospho-protein/loading control ratio +/- SEM. Data was*

*analyzed by a 1-way ANOVA and Tukey's post-hoc test. N= 3 animals for Wt group, 4 animals for Wt-STZ group and 6 animals for Tg-STZ group. Graph units are arbitrary. ** indicates p < 0.01 compared to Wt-STZ group.*

sAPPα suppresses diabetes-induced UPR activation - To test whether or not sAPPα negatively regulated stress signaling in the diabetic brain, we next analyzed activation of the UPR in the cortices of our animals. The UPR is a highly conserved cell survival pathway that reduces protein translation in response to insult (Verkhratsky et al. 2002, Kogel et al. 2003, Brewster et al. 2006). Activating phosphorylation of the ER kinase PERK (T980) results in PERK-mediated phosphorylation of eIF2α (Ser51) and inhibition of translational complex formation. In addition to being an indicator of cell stress, diabetes induced activation of the UPR has been linked mechanistically to the exacerbation of amyloid pathology in AD mice via increased BACE1 expression (Devi et al. 2012). In our animals, we found that sAPPα prevented PERK activation in diabetic cortices (Fig. 9, $p<0.05$ compared to Wt-STZ) although no differences in eIF2α phosphorylation were detected between groups.

Figure 9: sAPPα suppresses diabetes-induced UPR activation

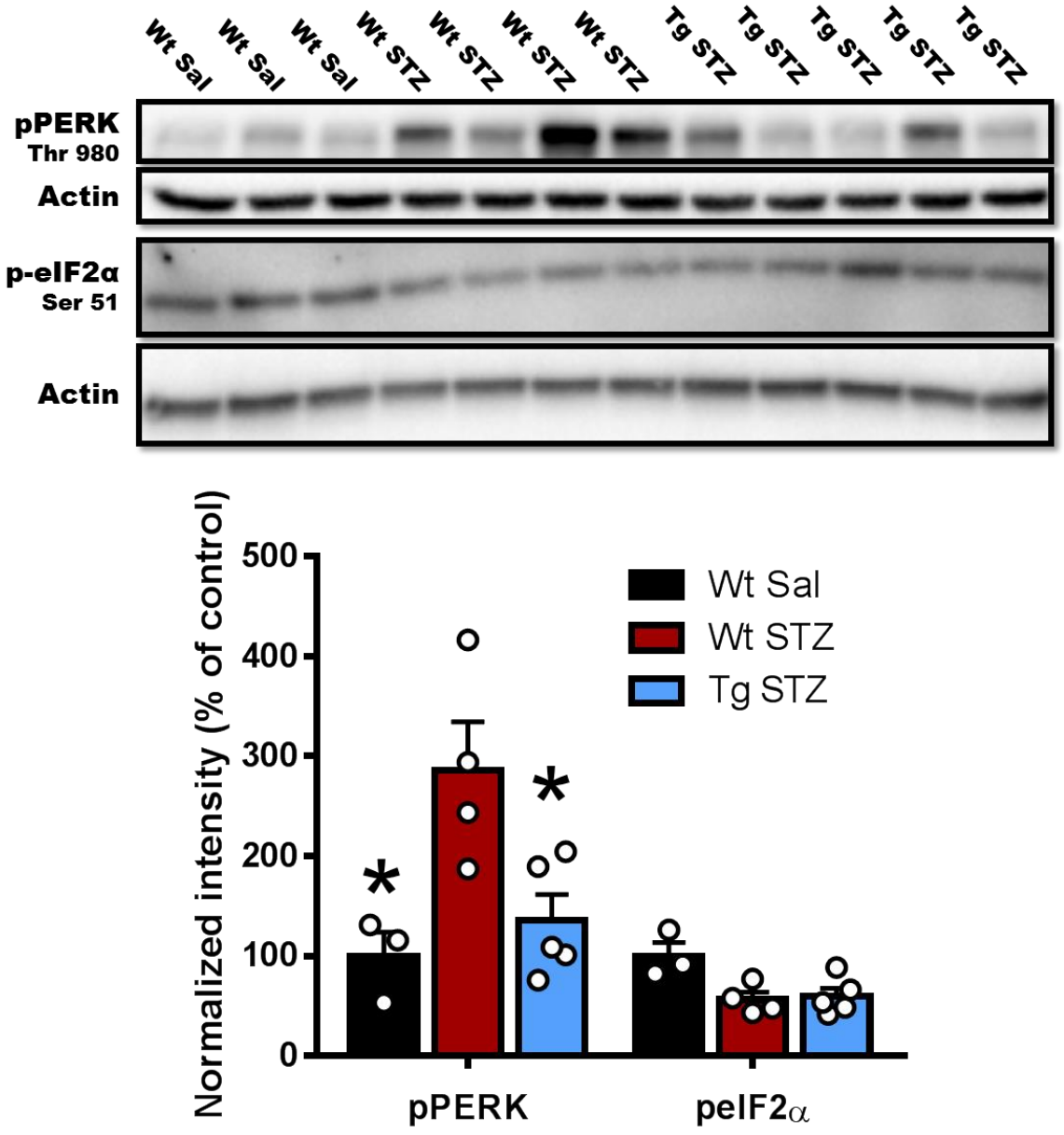


Figure 9: sAPPα suppresses diabetes-induced UPR activation *– Cortical tissue lysates from 16 week diabetic (STZ) or non-diabetic, saline injected (Sal) wild-type (Wt) and sAPPα-overexpressing mice (Tg) were analyzed by WB for UPR activation by determining*

*phosphorylation of PERK (Thr 980) and eIF2α (Ser51). Data represents mean protein/loading control ratio in whole cortical lysates +/- SEM. Data was analyzed by a 1-way ANOVA and Tukey's post-hoc test. * indicates p<0.05 compared to Wt STZ group. N= 3 animals for Wt group, 4 animals for Wt-STZ group and 5 animals for Tg-STZ group.*

sAPPα blocks aberrant AKT/GSK3 phosphorylation - Abnormal activation of the tau kinase GSK3 is hypothesized to contribute to tau pathology in AD (Hooper et al. 2008) and pharmacological inhibition of GSK3 reduces tau phosphorylation in rats (Georgievska et al. 2013). GSK3 activity is suppressed by phosphorylation of serine (Ser 9 on the GSK3β isoform and Ser 21 on the GSK3α isoform) residues by the survival kinase AKT (Cross et al. 1995). Cell culture studies indicate that the protective effects of sAPPα are mediated by activation of AKT (Wallace et al. 1997, Cheng et al. 2002) that may underlie sAPPα-mediated inhibition of GSK3 both *in vitro* and the brains of AD mice *in vivo* (Deng et al. 2015). In contrast to cell culture studies, *in vivo* insulin depletion increases phosphorylation of GSK3 and AKT in brain tissue (Clodfelder-Miller et al. 2006) suggesting an activation of stress response pathways in order to counteract the loss of trophic support. Therefore, we analyzed the effects of sAPPα on AKT and GSK3 phosphorylation the STZ diabetic brain. Furthermore, because sAPPα appears to be an activating ligand for neuronal IR and competes with insulin for binding sites on IR (Figures 5 and 6) we analyzed the effects of sAPPα on AKT/GS3 in the non-diabetic Tg mice. Because sAPPα is a competitive agonist for IR, we hypothesized that sAPPα overexpression in the non-diabetic brain may impact pathway activation downstream of IR, however, no differences in AKT or GSK3 phosphorylation were detected between Wt and Tg mice. Moreover, we found that diabetes-induced phosphorylation of both AKT and GSK3α was prevented by sAPPα-

overexpression (Fig. 10). WB analysis of IR expression and IR phosphoprylation revealed no differences between groups (Supplemental Figure 2).

Figure 10: sAPPα blocks aberrant AKT/GSK3 phosphorylation

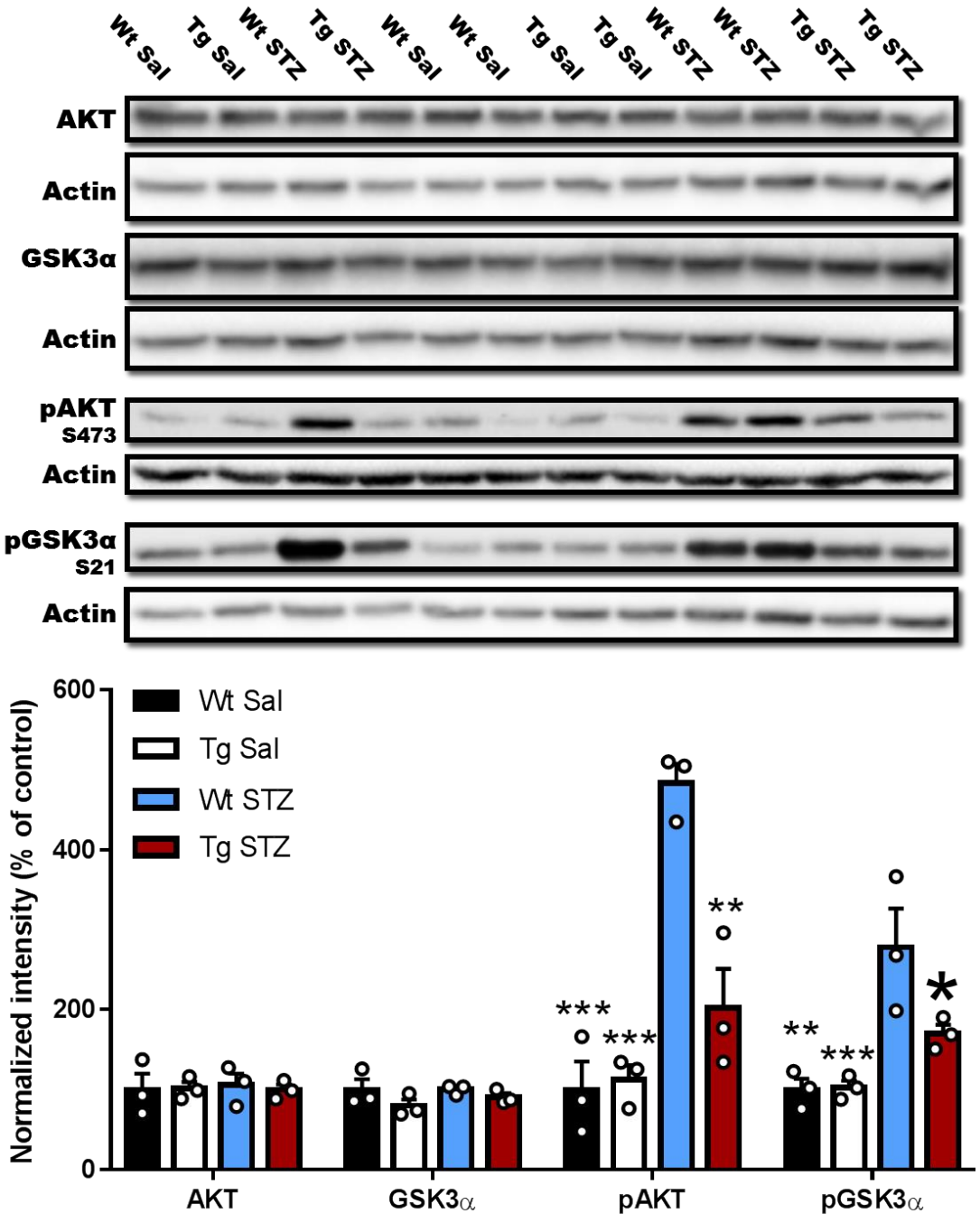


Figure 10: sAPPα blocks aberrant AKT/GSK3 phosphorylation *– Cortical tissue lysates from 16 week diabetic (STZ) or non-diabetic, saline injected (Sal) wild-type (Wt) and sAPPα-overexpressing mice (Tg) were analyzed by WB for phosphorylated AKT (S473) and phosphorylated GSK3α (ser 21). Data represents mean protein/loading control ratio +/- SEM. Data was analyzed by a 1-way ANOVA and Tukey's post-hoc test. N= 3 animals/group. Graph units are arbitrary. * indicates p < 0.05 compared to Wt STZ group. ** indicates p < 0.01 compared to Wt STZ group. *** indicates p < 0.001 compared to Wt STZ group.*

The effects of sAPPα on PP2A-C activity and MAPK/AMPK/PKA signaling in the diabetic brain – The protective effects of sAPPα in culture have been attributed to both activation of the IRS/AKT cascade and the MAPK pathway. The results in Figure 10 ruled out the possibility that sAPPα reduced tau phosphorylation by inhibiting GSK3 activity, therefore we next examined the effects of sAPPα on MAPK signaling as the MAPK pathway is neuroprotective and activated by sAPPα but is also a positive modulator of tau phosphorylation (Guise et al. 2001). However, WB analysis of cortical lysates detected no differences between non-diabetic and diabetic Wt/Tg mice (Fig. 11). In addition to the MAPK pathway, we analyzed activation of AMPK, a tau kinase that's abnormally active in AD (Vingtdeux et al. 2011) but no differences in AMPK activity were observed in our animals.

Recent work has identified PKA as a positive regulator of tau phosphorylation that is up-regulated in the diabetic brain (van der Harg et al. 2017). However, contrary to this report, activity of PKA, determined by phosphorylation of PKA at Thr 197, was significantly decreased in the brains of our Wt-STZ mice compared to non-diabetic controls ($p<0.05$). We observed a

trend towards decreased phospho-PKA in the Tg-STZ group compared to Wt controls but no significant differences were detected.

The lack of differences in tau kinase activation we observed suggested that differences in tau phosphorylation between diabetic Wt and Tg mice could be the result of altered tau phosphatase activity. The phosphatase PP2A accounts for approximately 70% of the tau phosphatase activity in the brain (Liu et al. 2005) and PP2A activity has been reported to be decreased by diabetes (Planel et al. 2007b). In our animals, expression of the catalytic subunit of PP2A (PP2A-C) was unchanged between groups (Fig. 12A) however activity of immunoprecipitated PP2A-C proteins was decreased in WT-STZ cortical lysates which was patially normalized in the sAPPα diabetic group (12B) (See supplementary Figure 1 for WB analysis of IP samples). These results suggest that the effects of sAPPα on tau phosphorylation are mediated by maintenance of PP2A-C activity.

Figure 11: The effects of sAPPα on tau phosphorylation are not mediated by AMPK, MAPK or PKA

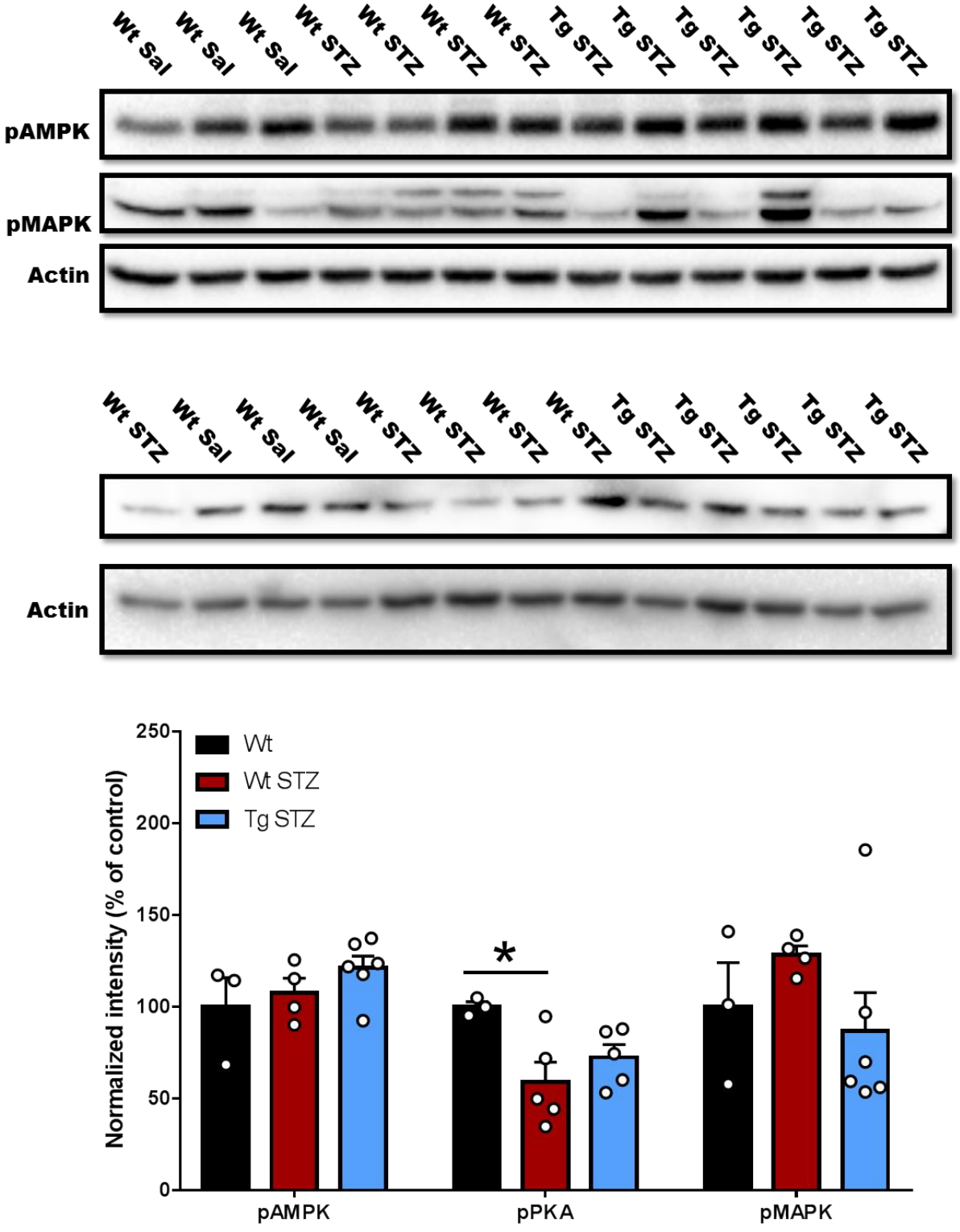


Figure 11: The effects of sAPPα on tau phosphorylation are not mediated by AMPK, MAPK or PKA *– Cortical tissue lysates from 16 week diabetic (STZ) or non-diabetic, saline injected (Sal) wild-type (Wt) and sAPPα-overexpressing mice (Tg) were analyzed by WB for activating phosphorylation of AMPK (Tyr 172), MAPK (Thr 202/Tyr 204) and PKA (Thr 197). Data are mean protein/loading control ratio in whole cortical lysates +/- SEM. Data was analyzed with a 1-way ANOVA and Tukey's post-hoc test. * indicates p<0.05 vs Wt group. N= 3 animals for Wt group, 4 animals for Wt-STZ group and 6 animals for Tg-STZ group.*

Figure 12: Overexpression of sAPPα maintains PP2A phosphatase in the diabetic brain

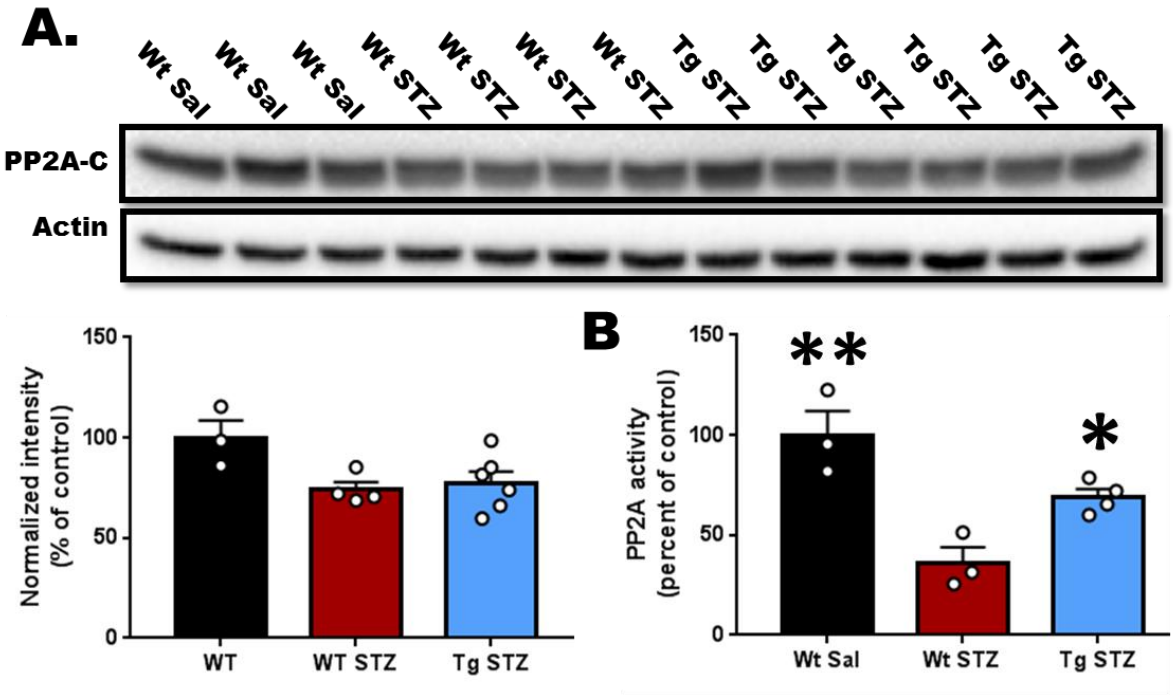


Figure 12: Overexpression of sAPPα maintains PP2A phosphatase in the diabetic brain *– A. Cortical lysates from diabetic sAPPα (Tg STZ) and diabetic Wt mice (Wt STZ) plus saline injected wild-type controls (Wt sal) were probed for PP2A-C expression. Data represents mean protein/actin ratio +/- SEM. 1-way ANOVA with Tukey's post-hoc test revealed no differences in*

*PP2A-C expression between groups. B. Cortical tissue was homogenized in phosphatase buffer (see Materials and Methods) and IP performed on lysates using anti-PP2A-C antibodies. Activity of precipitated PP2A-C proteins was assessed using available kits (see Materials and Methods). Data represents mean activity level standardized to protein level +/- SEM. Data is expressed as a percentage of the control (Wt Sal) group. Data was analyzed with 1-way ANOVA with Tukey's post-hoc test. ** indicates p<0.01 compared to Wt STZ group. * indicates p<0.05 compared to Wt STZ group. For A, N= 3 animals for Wt group, 4 animals for Wt-STZ group and 6 animals for Tg-STZ group. For B, N = 3-4 animals/group.*

SUMMARY:

With this work, we studied the effects of sAPPα on the diabetic brain by rendering Tg, sAPPα-overexpressing mice STZ diabetic. As expected, overexpression of sAPPα blocked diabetes-induced tau phosphorylation (T231) in cortical tissue after 16 weeks of diabetes which was associated with decreased activation of the UPR suggesting an overall protective effect of sAPPα in the STZ diabetic brain. However, despite *in vitro* studies demonstrating that sAPPα modulates tau phosphorylation by modulating AKT/GSK3, we found elevated phosphorylation of AKT and GSK3 in the cortices of Wt-STZ mice which was reduced by sAPPα overexpression. In order to identify alternative mechanisms that may mediate the effect of sAPPα on tau phosphorylation, we next analyzed activity of PP2A, a phosphatase that acts on tau, GSK3 and AKT. Here, we show that the catalytic subunit of PP2A has reduced activity in diabetic brain tissue which was reversed by sAPPα overexpression. These data demonstrate that sAPPα prevents aberrant phosphorylation of tau, AKT and GSK3 in the diabetic brain which may be related to sAPPα-mediated activation of PP2A phosphatases.

CHAPTER 5: SECRETED AMYLOID PRECURSOR PROTEIN ALPHA REVERSES NFKB DEFICITS IN INSULIN-DEPLETED DRG NEURONS

INTRODUCTION:

The development of a type 1 diabetic, sAPPα overexpressing model enabled us to evaluate the protective effects of sAPPα in both the diabetic CNS and the PNS. APP is expressed in DRG neurons and is protective against cell death in trophic deprived DRG cultures, yet the role of APP/sAPPα in the diabetic PNS has yet to be evaluated. Previously, we found that diminished activity of the neuroprotective transcription factor NFκB may underlie the development of sensory neuropathy via decreased expression of the antioxidant MnSOD. Both NFκB activity and MnSOD expression are influenced by the insulin signaling pathway and sAPPα. Therefore, we hypothesized that sAPPα would prevent sensory neuropathy in diabetic mice and reverse NFκB activity/MnSOD expression deficiencies in insulin depleted DRG neurons.

RESULTS:

Table 3: Blood glucose measurements of rats used in study

Group	B.G. (mmol/L)
Control	6.9 ± 2.5
Diabetic	25.6 ± 4.8***

Table 3: Blood glucose measurements of rats used in study *- 3-5 month STZ-diabetic and age matched control rats were maintained in parallel until the end of the study. Diabetes was induced by a single intraperitoneal injection of STZ (85mg/kg) while control animals were injected with saline. STZ induced diabetes was confirmed three days after injection by tail blood glucose assay. Values reflect mean blood glucose measurements at the time of sacrifice, +/- SEM. Data was analyzed by students t-test. N = 8 animals/group. ******* indicates p<0.001.*

Expression of NFκB gene targets in diabetic DRG – STZ-diabetic rats display peripheral neuropathy characterized by the dying back of axons and reduced intraepidermal nerve fiber density (Kennedy et al. 1996, Christianson et al. 2003, Mizisin et al. 2007, Quattrini et al. 2007). We and others have reported decreased transcriptional activity of the neuroprotective transcription factor NFκB in the DRG of STZ-diabetic rats (Berti-Mattera et al. 2011). In order to determine if the expression of neuroprotective proteins reported to be, at least partially, under the transcriptional control of NFκB is altered in the DRGs of STZ-diabetic rats, Western blotting of whole DRG homogenates from control and diabetic animals was performed with antibodies against MnSOD (SOD2), Cu/ZnSOD (SOD1) and calbindin. As expected, we observed a significant decrease in MnSOD expression in STZ-diabetic DRGs compared to age matched controls however no difference in calbindin expression was detected (Fig. 13). Interestingly, a significant increase in Cu/ZnSOD expression was observed in the STZ-diabetic DRGs (Fig. 13). These results suggest that diabetes decreases specific NFκB targets while leaving others unaffected or upregulated via NFκB-independent mechanisms.

Figure 13: Expression of NFκB gene targets in diabetic DRG

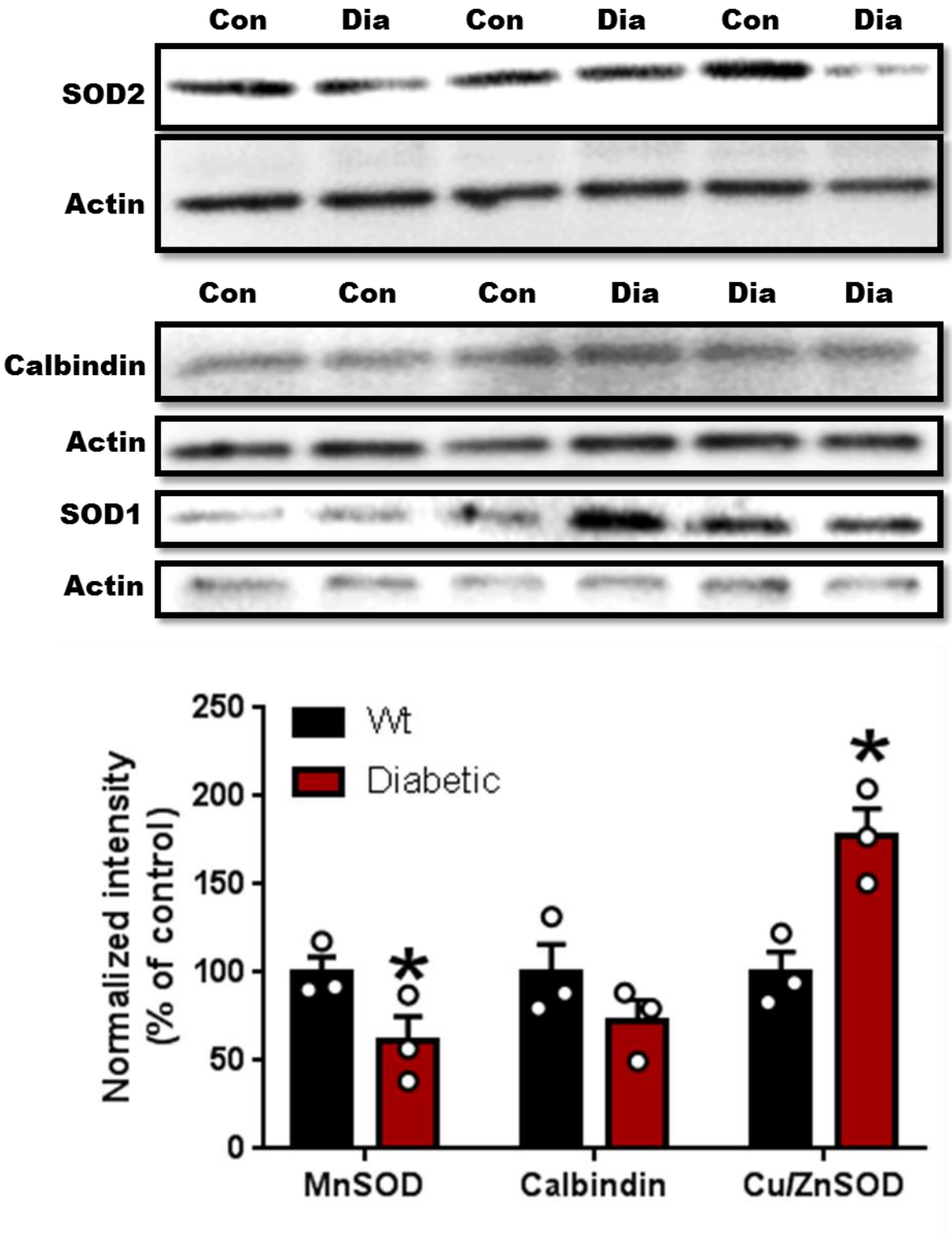


Figure 13: Expression of NFκB gene targets in diabetic DRG *- Intact DRGS were removed from STZ-diabetic rats (Dia) or controls (Con), homogenized and analyzed by Western blot. MnSOD, Cu/ZnSOD and Calbindin bands were standardized to actin loading controls. Data represent mean protein/actin ratio expressed as a percentage of the Wt group +/- SEM. Student's t-test was used to evaluate differences between control and diabetic groups. N = 3 animals/ group. * indicates p < 0.05.*

sAPPα slows the development of diabetes-induced neuropathy *in vivo* – Loss of sensation in the hands and feet is indicative of peripheral neuropathy in diabetic patients (Pittenger et al. 2004). Thermal hypoalgesia, an indicator of sensory neuropathy, appears in diabetic C57Bl/6 mice ~8 weeks following STZ injection (O'Brien et al. 2014). Therefore, to determine if sAPPα overexpression in the peripheral nervous system could delay the onset of diabetes-induced sensory neuropathy, a second cohort of Wt/sAPPα were rendered STZ diabetic (Fig 14) and thermal sensitivity determined using Hargreaves method (see Materials and Methods) (Fig 14D). Compared to Wt-STZ animals, Tg-STZ mice displayed decreased latency paw withdrawal ($p<0.05$) after 10 of weeks diabetes while no differences were detected between Tg-STZ and non-diabetic controls indicating a preservation of sensation to thermal stimuli in diabetic sAPPα mice.

Figure 14: Overexpression of sAPPα delays the development of thermal hypoalgesia in diabetic mice

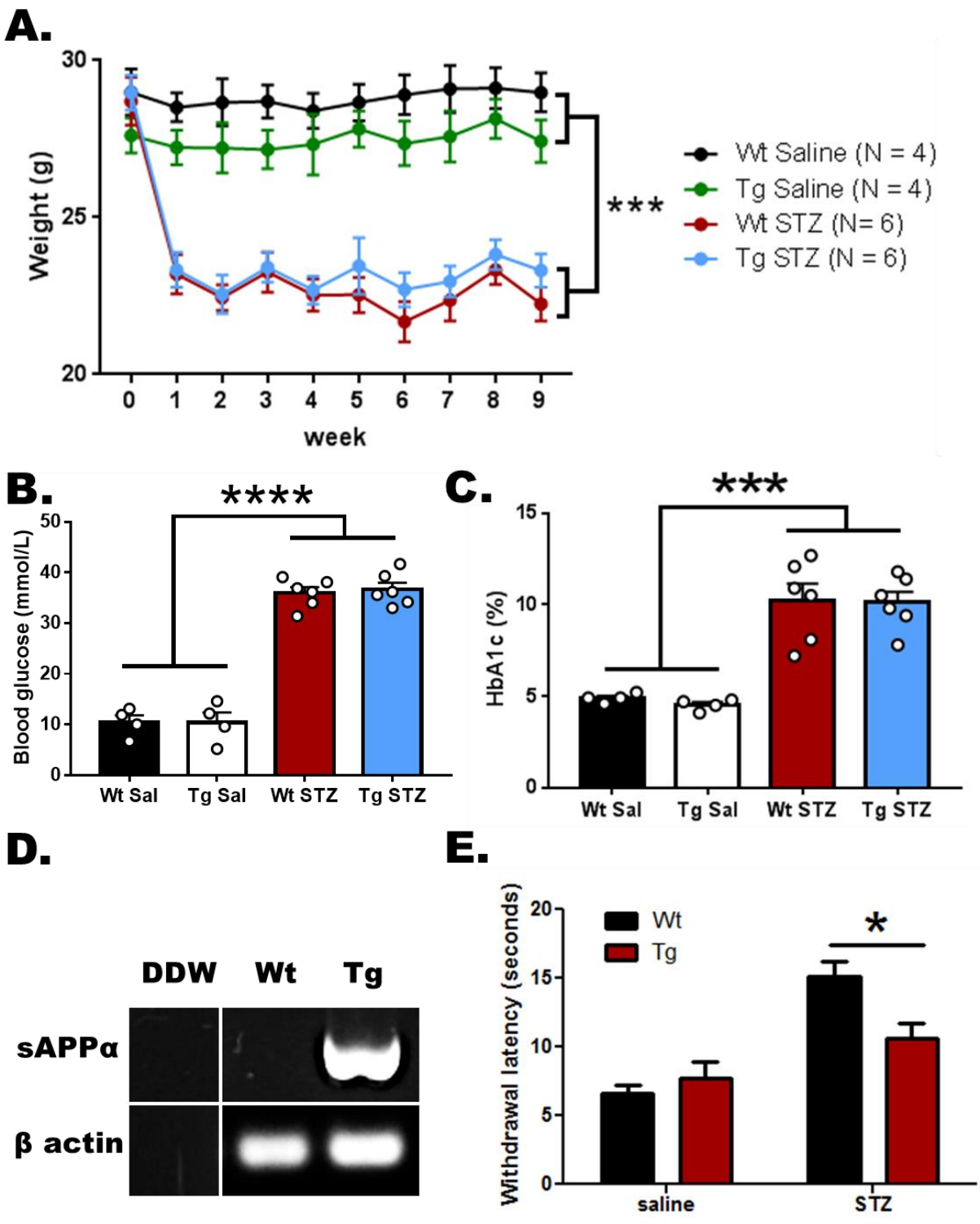


Figure 14: Overexpression of sAPPα delays the development of thermal hypoalgesia in diabetic mice *– A. Body weight of diabetic (STZ) wild-type (Wt) and sAPPα (Tg) overexpressing mice and saline (sal) injected controls was determined prior to STZ injection (week 0) and weekly until the duration of the study. Data represents mean body weight +/- SEM. Data was analyzed by a 2-way ANOVA. *** indicates p<0.001 between saline and STZ injected groups. .No differences were detected between Tg STZ and WT STZ mice or between Tg saline and Wt saline mice. B. Fasting (3 hours) blood glucose levels were measured at the time of death. Data represents mean blood glucose +/- SEM. Data was analyzed by a 1-way ANOVA and Tukey's post hoc test. **** indicates p<0.0001 between STZ injected groups and saline injected groups. No differences were detected between Tg STZ and WT STZ mice or between Tg saline and Wt saline mice. C. HbA1c percentages were determined with blood samples collected immediately post mortem. Data represents mean HbA1c % +/- SEM. Data was analyzed by a 1-way ANOVA and Tukey's post hoc test. **** indicates p<0.0001 between STZ injected groups and saline injected groups. No differences were detected between Tg STZ and WT STZ mice or between Tg saline and Wt saline mice. D. Dorsal root ganglia were isolated from wild type (Wt) and sAPPα over-expressing (Tg) mice. Trizol was used to extract RNA and RT-PCR was used to prepare the cDNA template for subsequent PCR amplification of sAPPα. β actin and samples with no template (double de-ionized water: DDW) were used as internal PCR controls. E. Thermal sensitivity of the lower extremities (palmer surface of hind paws) in control and STZ-diabetic Wt/Tg sAPPα mice was determined using Heargraves method (see Materials and Methods). Data are withdrawal latency (in seconds) +/- SEM. Data was analyzed by 2-way ANOVA followed by Bonferonni post-hoc test *p < 0.05 vs Tg STZ and Wt STZ. No significant differences were detected between Tg STZ mice and non-diabetic controls. Gary Odero completed part D of this*

figure under the direction of Brent Aulston. Darrell Smith completed part E of this figure under the direction of Brent Aulston.

T**he neurotrophic effects of sAPPα are mediated by neuronal insulin receptors** - In order to elucidate the mechanisms underlying the neurotrophic effects of sAPPα we next quantified neurite outgrowth of DRG neurons cultured co-treated with sAPPα +/- AG1024, an antagonist of IR/IGF1R ligand binding sites . As demonstrated in Figure 15, sAPPα treatment increased neurite outgrowth of DRG neurons cultured from Wt and diabetic rats. Furthermore, we determined that the effects of sAPPα on neurite outgrowth were completely inhibited by the application of AG1024 (Fig. 16). In the absence of other treatments, AG1024 had no effect on neurite outgrowth in our cultures. Additional experiments confirmed the specificity of AG1024 inhibition of IR/IGF1R as the dose of AG1024 used was found not to affect NGF-induced neurite outgrowth (Supplemental Figure 6). These data indicate that ligand binding sites on IR/IGF1R are necessary for the neurotrophic effects of sAPPα on DRG neurons.

Figure 15: sAPPα enhances neurite outgrowth of DRG neurons cultured from healthy and diabetic rats

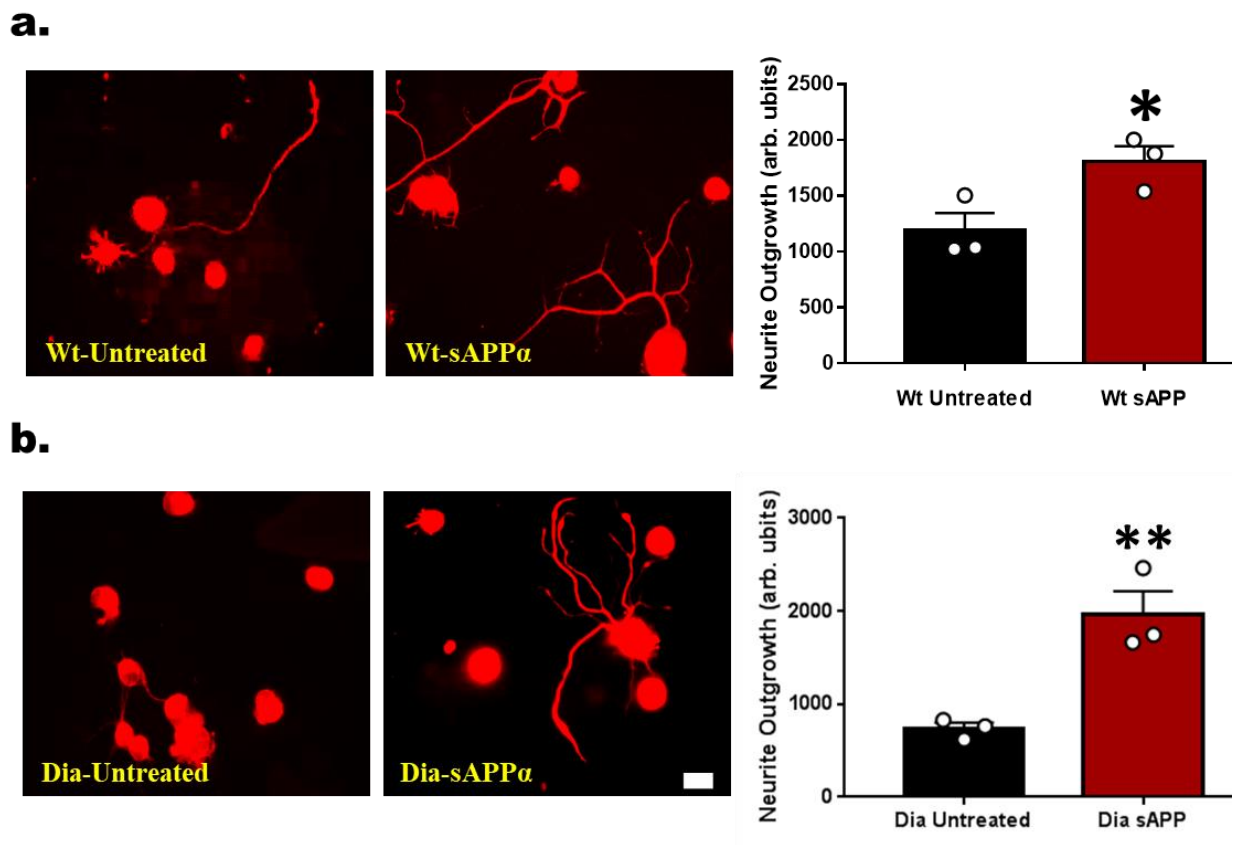
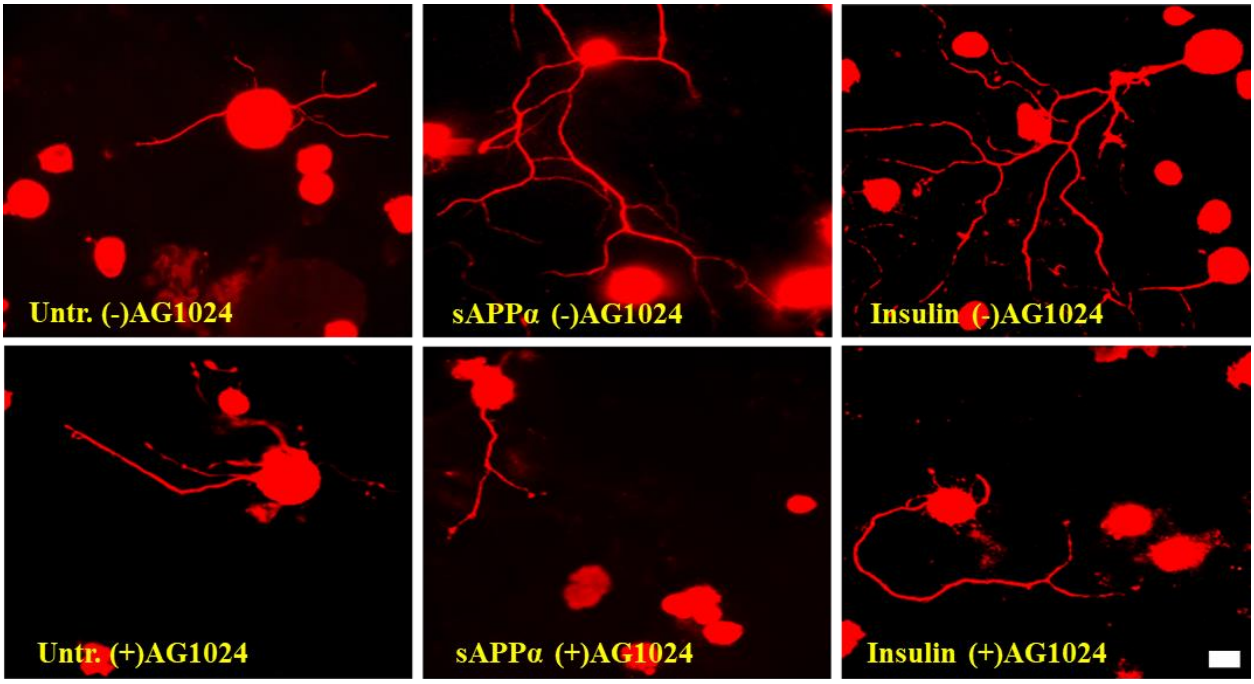


Figure 15: sAPPα enhances neurite e outgrowth of DRG neurons cultured from healthy and diabetic rats *- DRG neurons were cultured from non-diabetic (Wt) and 2-month diabetic (Dia) rats as described in Materials and Methods. Wells were treated with 30nM sAPPα or left untreated for 24 hours before fixation in PFA. Fixed neurons were then stained with antibodies against β-tubulin class III (RED) and imaged using a fluorescent microscope. Area of neurite outgrowth/cell body was assessed as described in the Materials and Methods section. Data represent mean neurite outgrowth/well +/- SEM. N = 3 wells Data were analyzed by student's t-test. Graph units are arbitrary. * indicates p<0.05. ** indicates p<0.01 vs untreated. Scale bar is 20μm.*

Figure 16: sAPPα-mediated neurite outgrowth of cultured DRG neurons requires IR/IGF1R ligand binding sites



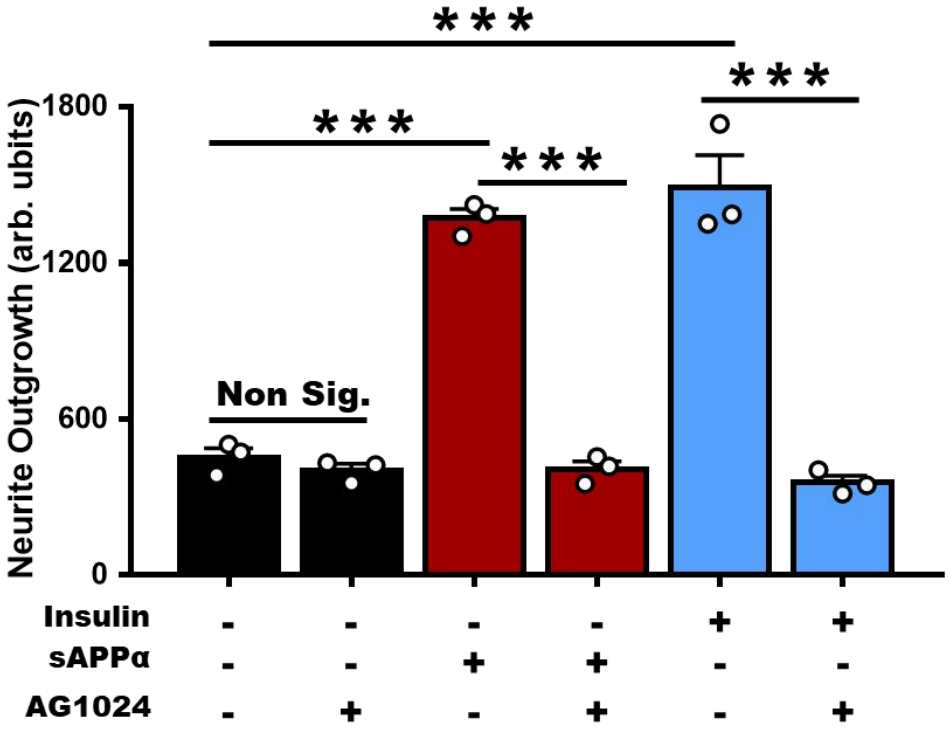
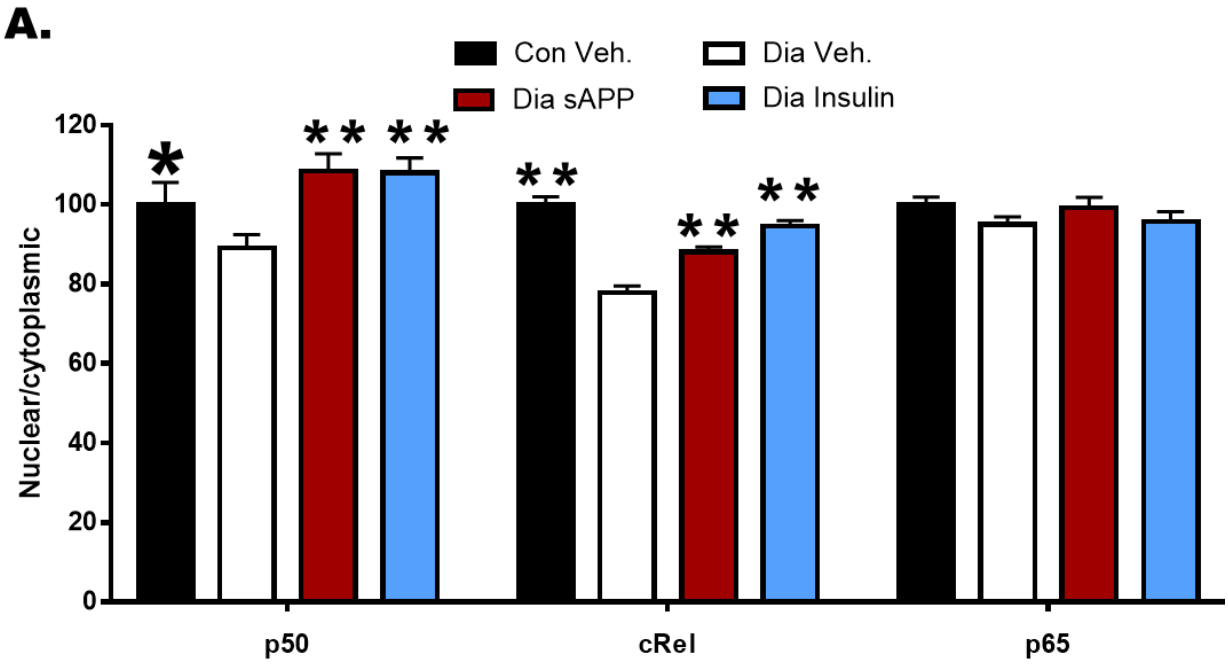


Figure 16: sAPPα-mediated neurite outgrowth of cultured DRG neurons requires IR/IGF1R ligand binding sites *- Control DRG neurons were cultured as described in Materials and Methods. Wells were treated with AG1024 (20μM), an antagonist of IR/IGF1R, or vehicle for 60 minutes prior to 100nM sAPPα or 10nM insulin treatment and maintained in treatment for 24 hours before fixation. Neurons were then stained with antibodies against β-tubulin class III and imaged using a fluorescent microscope. Area of neurite outgrowth/cell body was assessed as described in the Materials and Methods section. Data represent mean neurite outgrowth/well +/- SEM. N = 3 wells. Data were analyzed with 1-way ANOVA and Tukey's post-hoc test. Graph units are arbitrary. * indicates p<0.001 vs indicated sample. Scale bar is 20μm*

sAPPα activates NFκB and increases MnSOD expression in cultured DRG neurons - Decreased NFκB activity is associated with diabetes-induced neuropathy that may contribute to degeneration of peripheral neurons by decreasing expression of the antioxidant MnSOD. To determine the effects of sAPPα on NFκB activity in our cultures, DRG neurons from control and diabetic animals were cultured and treated with 100nM sAPPα, 10nM insulin or otherwise left untreated for 24hrs then fixed and immuno-stained for the NFκB subunits p50, cRel, and p65 (Fig. 17). Our data show that nuclear localization for both p50 ($p<0.05$) and cRel ($p<0.01$) was decreased in untreated diabetic DRG neurons compared to untreated control neurons while no difference in the nuclear/cytoplasmic ratio of p65 was observed. Both sAPPα and insulin treatment of diabetic DRG neurons significantly increased the nuclear/cytoplasmic ratios of p50 (insulin: $p<0.01$, sAPPα: $p<0.01$) and cRel (insulin: $p<0.01$, sAPPα: $p<0.01$) subunits compared to untreated diabetic neurons. Consistent with our previous work demonstrating that p65 activity is unaltered in diabetic DRG (Saleh et al. 2013), neither insulin nor sAPPα treatment of diabetic

DRG neurons produced a difference in the p65 nuclear/cytoplasmic ratio compared to untreated diabetic cells.

Figure 17: sAPPα treatment of diabetic DRG neurons increases the nuclear/cytoplasmic ratio of the NFκB subunits cRel and p50



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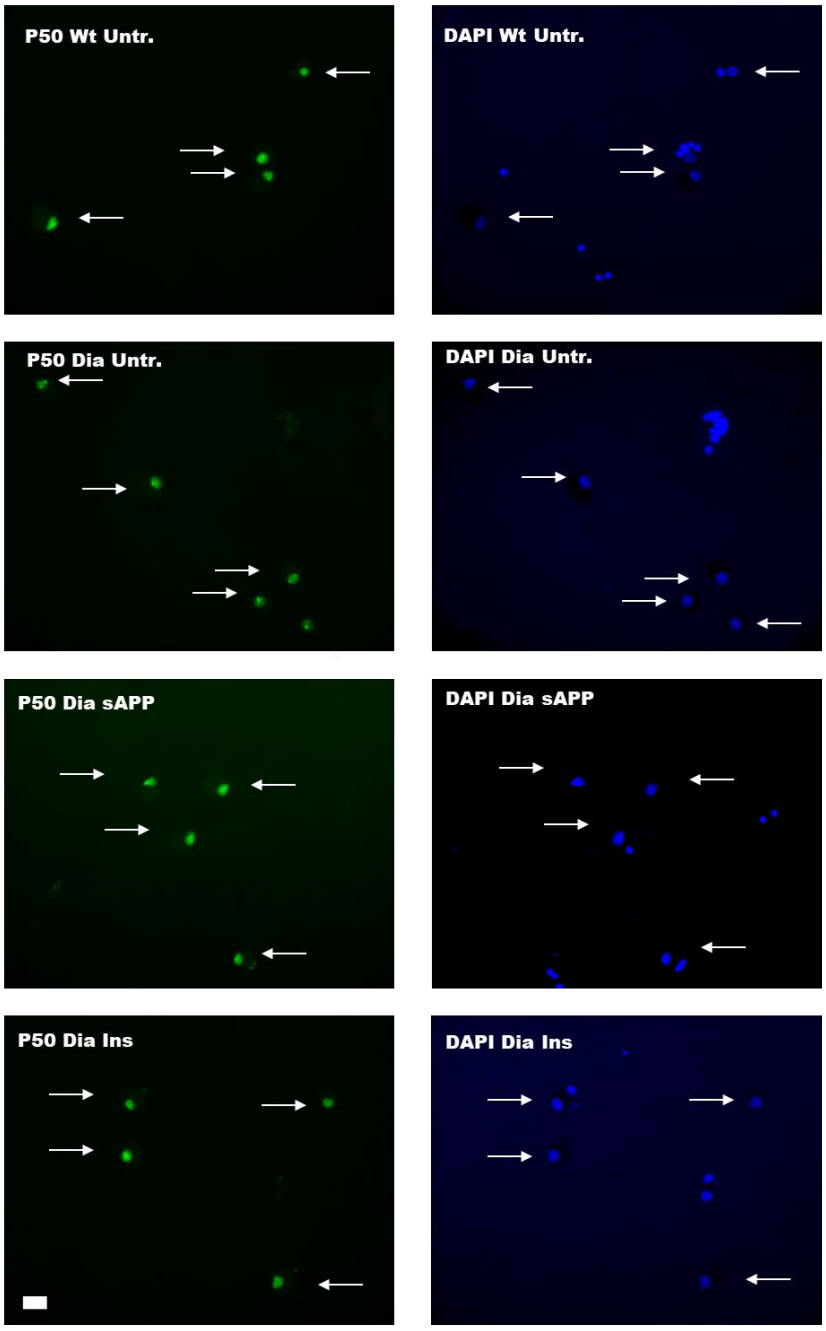
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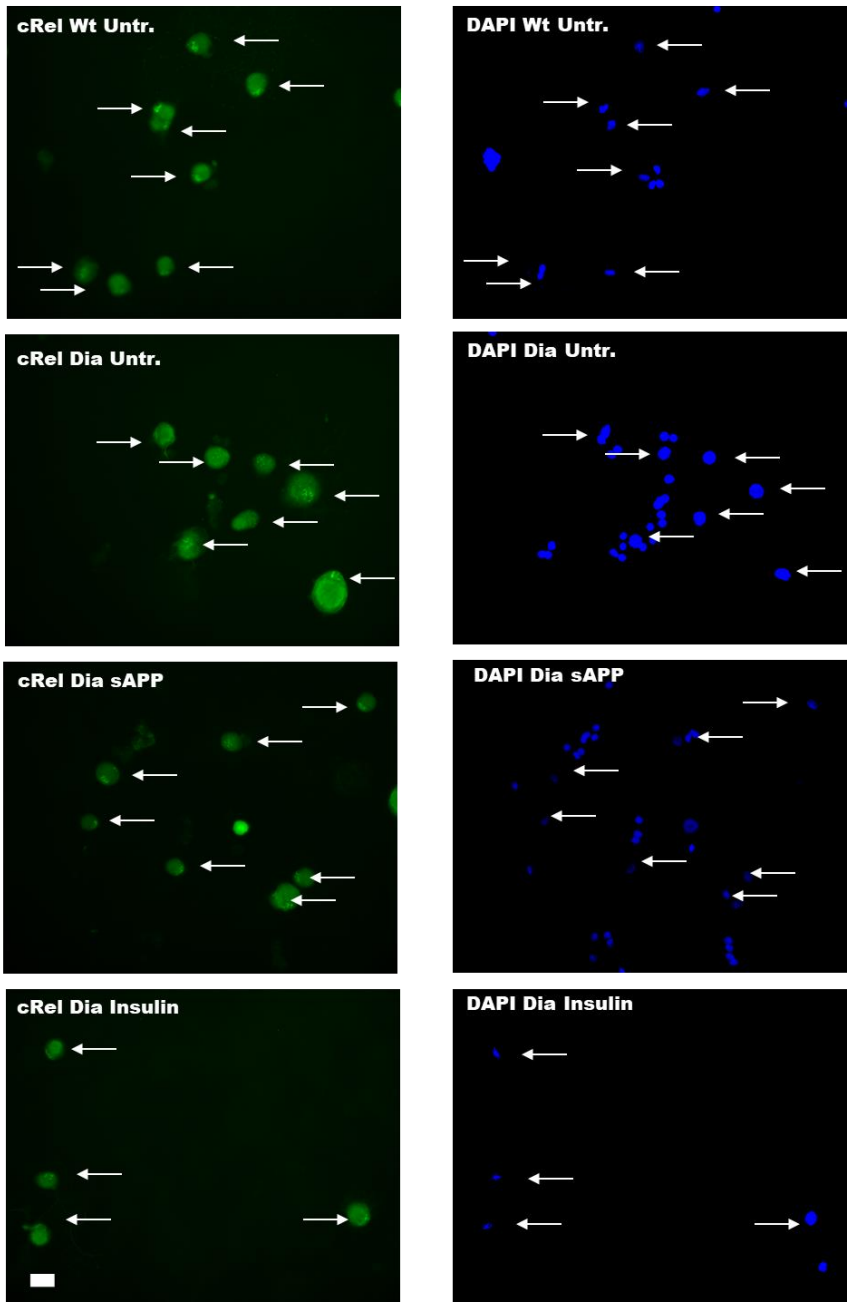
C.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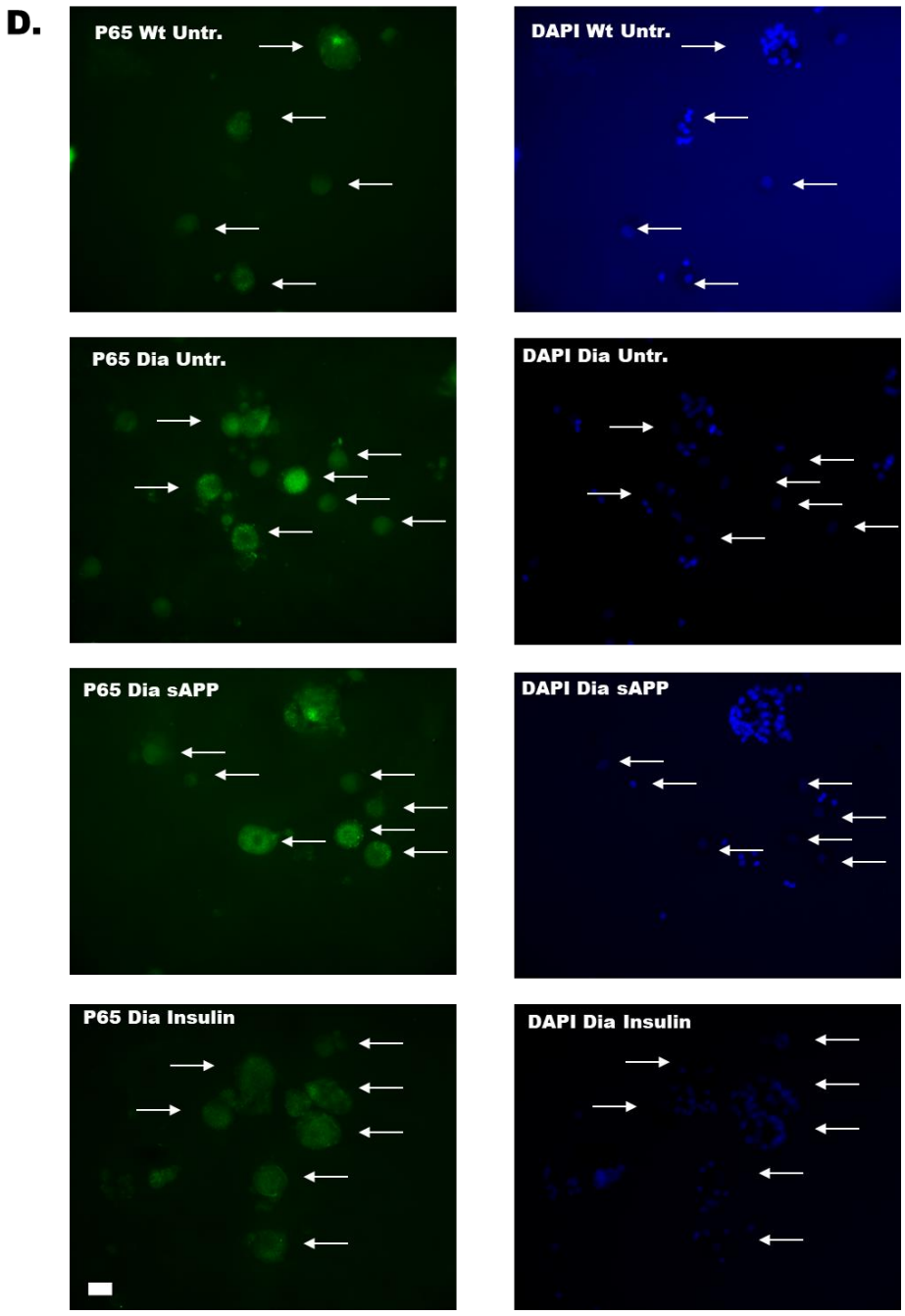


Figure 17: sAPPα treatment of diabetic DRG neurons increases the nuclear/cytoplasmic ratio of the NFκB subunits cRel and p50 - *Untreated non-diabetic control DRG neurons (Con Unt.) or diabetic DRG neurons that were untreated (Dia Unt.) or treated with 100nM sAPPα (sAPP) or treated with 10nM insulin (Ins) were cultured for 24 hours and then immunostained with*

*antibodies against the NFκB subunits p50, cRel, or p65. Nuclei (marked with arrows) were stained with DAPI. Nuclear/cytoplasmic ratios were determined as described in the Materials and Methods. A. Data represents mean nuclear/cytoplasmic ratio expressed as a percentage of the Con Unt. group, +/- SEM. N= 80-120 cells/treatment group. Data were analyzed with 1-way ANOVA and Dunnett's post-hoc test. * indicates p<0.05 compared to untreated diabetic group. ** indicates p<0.01 compared to untreated diabetic group. *** indicates p<0.001 compared to untreated diabetic group. Representative images of neurons stained for P50 (B) cRel (C) and p65 (D). Scale bars are in the lower left corners of B C and D and are 20μm.*

We next cultured DRG neurons in starvation media (F12 absent of insulin/growth factors) +/-100nM sAPPα for 24 hours (Fig. 18) to determine if exogenous sAPPα would increase expression of MnSOD in our cultures. Western blot analysis of cell lysates revealed a significant increase ($p<0.05$) in MnSOD expression in the sAPPα treated group compared to untreated cells.

Figure 18: sAPPα increases MnSOD expression in insulin-deprived DRG neurons

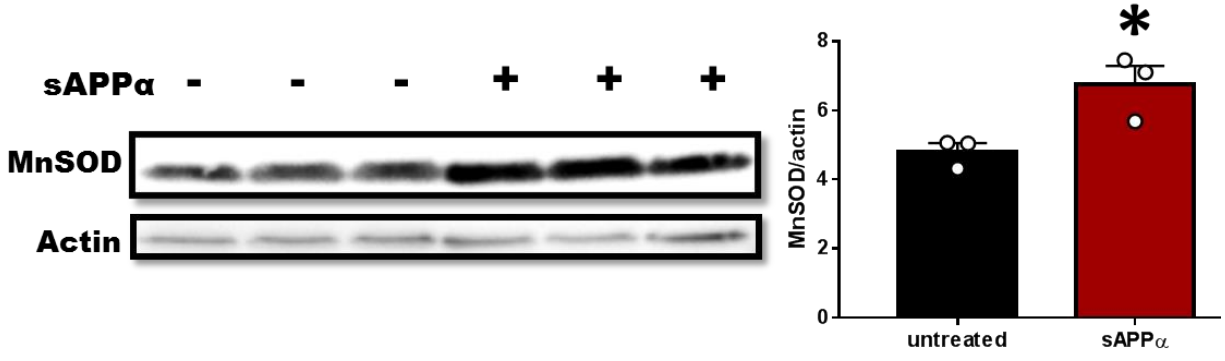


Figure 18: sAPPα increases MnSOD expression in insulin-deprived DRG neurons *– Adult Wt rat DRG neurons were cultured as described in the Materials and Methods. After plating, cells*

*were incubated in starvation media (F12+N2 supplement without growth factors) for 24 hours +/- 100nM sAPPα. After treatment, cells were lysed and lysates prepped for WB. Data represents mean MnSOD/actin ratio +/- SEM. Student's t-test was used to evaluate differences between untreated and sAPPα groups. N= 3 wells. Graph units are arbitrary. * indicates p < 0.05.*

SUMMARY:

Sensory neuropathy is characterized by peripheral nerve degeneration and is a common complication in patients with diabetes. We have found previously that diabetes may contribute to the development of peripheral neuropathy by reducing the activity of the transcription factor NFκB, and in turn, decreasing the expression of neuroprotective genes such as MnSOD, in DRG neurons. With this work, we found that sAPPα slowed loss of sensation in the lower extremities of diabetic mice, and, in culture, increased MnSOD expression and reversed diabetes-induced deficits in NFκB. Moreover, we determined that ligand binding sites on IR/IGF1R are required for the neurotrophic effects of sAPPα on DRG neurons. In total, these data indicate that the neurotrophic effects of sAPPα in the PNS involve activation of an insulin signaling/NFκB axis and provide preliminary evidence that sAPPα is a potential treatment for diabetes-associated peripheral nerve disease.

CHAPTER 6: THE EFFECTS OF SECRETED APP ALPHA OVEREXPRESSING NEURAL STEM CELLS ON MORRIS WATER PERFORMANCE OF HEALTHY MICE

INTRODUCTION:

NSCs are currently being evaluated for the treatment of many neurodegenerative diseases. In AD mice, NSCs have been shown to reverse cognitive impairment but not aberrant tau phosphorylation or amyloid deposition (Blurton-Jones et al. 2009). However, given the toxicity of Aβ oligomers and aggregated tau in culture and *in vivo*, NSC-based therapies could be improved upon by targeting AD-associated pathologies. For example, NSCs genetically modified to overexpress neprilysin, a protease that degrades Aβ, have been found to improve cognition and decreased amyloid levels compared to Wt-NSCs in AD mice (Blurton-Jones et al. 2014). Because sAPPα reduces tau and amyloid pathology, increases learning and memory and provides neurotrophic support by activating the insulin signaling pathway, sAPPα is an ideal candidate for NSC-based therapeutics in the treatment for AD. With this work, we examined the effects of sAPPα-NSCs on MWM performance in healthy mice.

RESULTS:

Both Wt and sAPPα – NSCs differentiate primarily into astrocytes after implantation – Wt and sAPPα NSCs were generated as described in the Materials and Methods section. In brief, homozygous sAPPα mice (or Wt controls) were bred to mice homozygous for eGFP. Resulting embryos where hemizygous for sAPPα (sAPPα-NSCs) (or nullizygous for sAPPα (Wt-NSCs)) and hemizygous for eGFP. NSCs were harvested from E14 embryos and maintained in culture before implantation.

Previous work demonstrates that embryonic NSCs cultured from the same line of Tg, sAPPα overexpressing mice used in our studies secrete >10 fold more sAPPα into culture media than Wt-NSCs (Bailey et al. 2013). Furthermore, in preliminary studies, we found that starvation media (no growth factors or supplements) conditioned by sAPPα-NSCs increased the survival of cultured neurons compared to media conditioned by Wt-NSCs (Supplementary Figure 2).

NSCs have the potential to differentiate into oligodendrocytes, neurons, and astrocytes and cell culture studies demonstrate that sAPPα steers NSCs towards an astrocytic fate (Baratchi et al. 2012). Therefore we anticipated a disparity in differentiation characteristics between Wt and sAPPα -NSC grafts. However, IHC analysis of hippocampal slices taken from our NSC injected animals with antibodies raised against the astrocytic marker GFAP demonstrate that the vast majority of both NSC types differentiated into astrocytes (Fig. 19).

Figure 19: Differentiation of NSC grafts

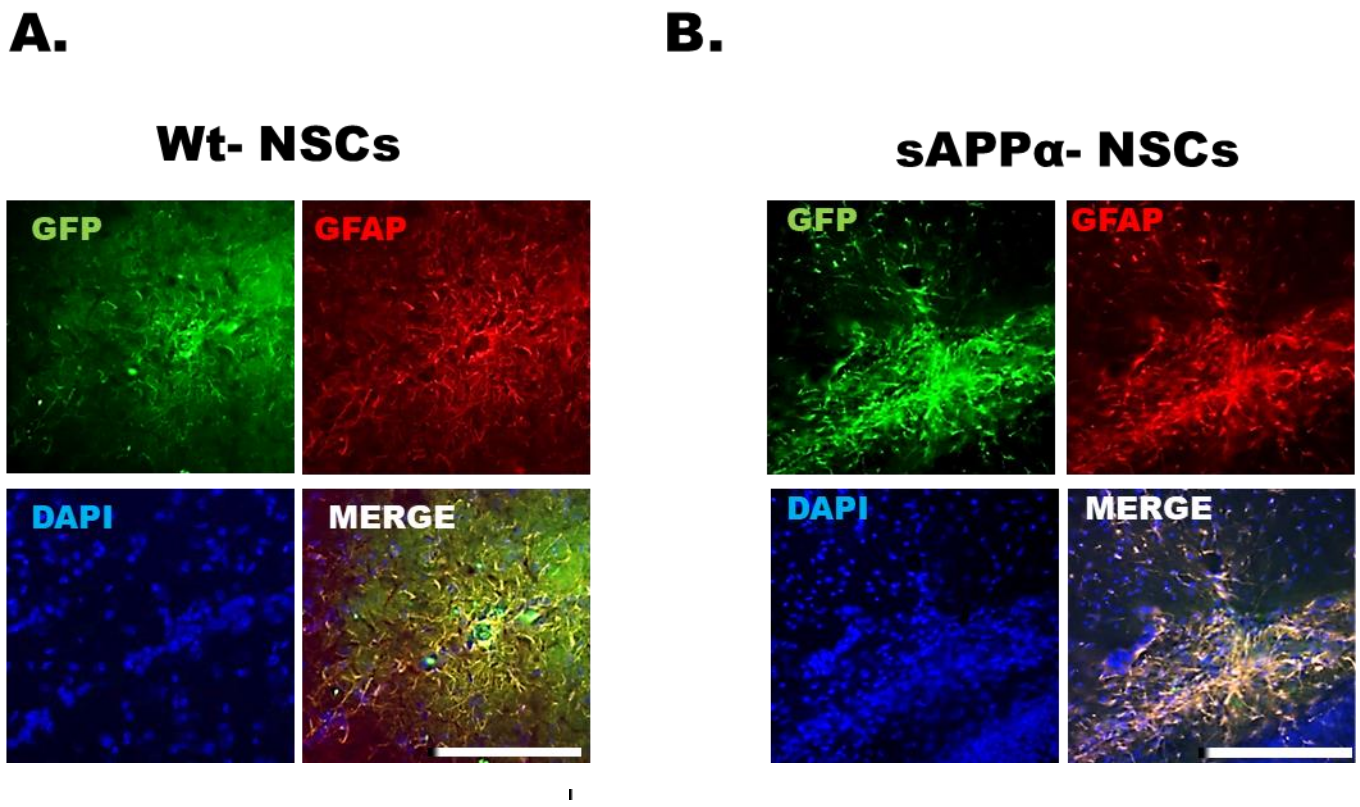
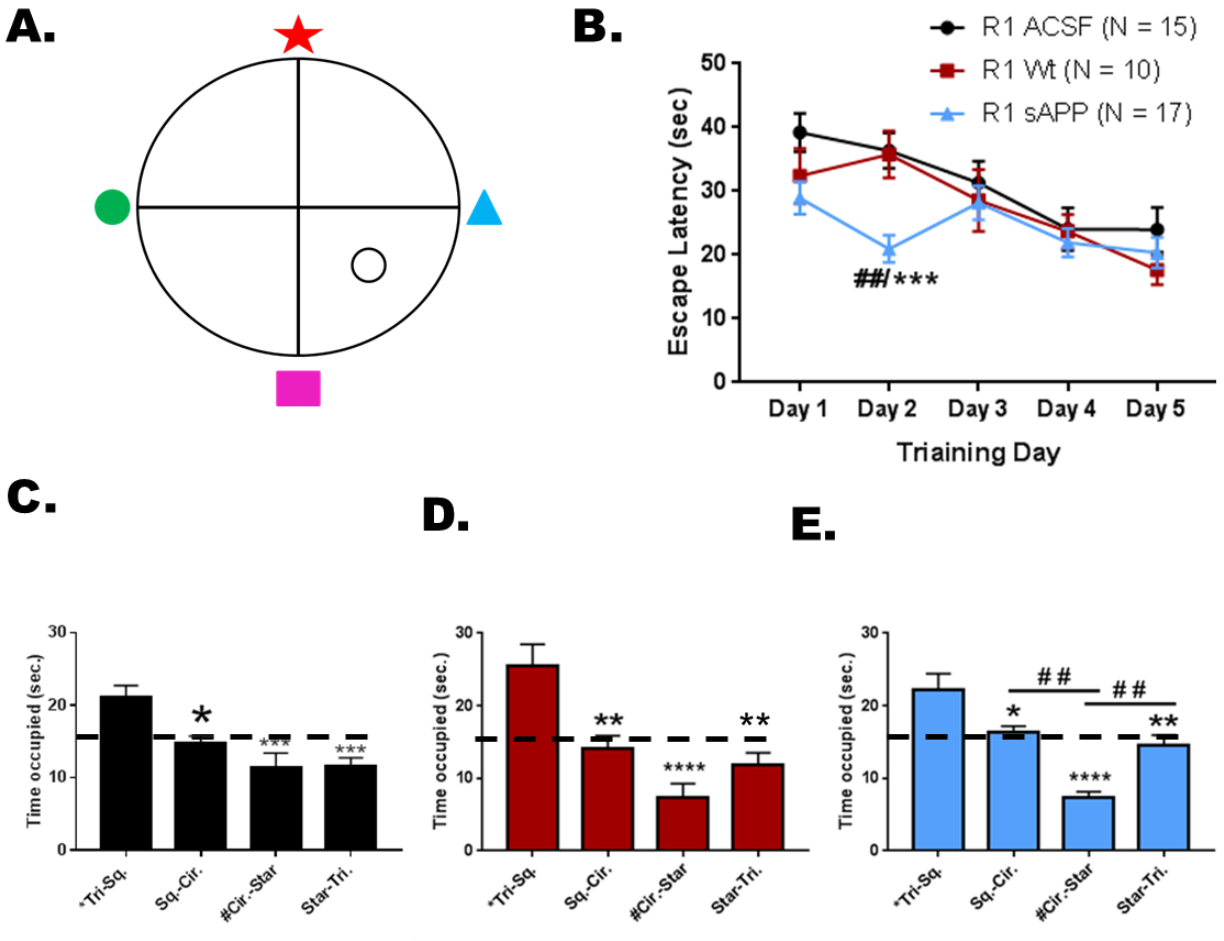


Figure 19: Differentiation of NSC grafts *- To determine differentiation characteristics of NSC grafts, brains were removed 2 months after NSC injection (after MWM testing) and hippocampal slices were probed with antibodies raised against the astrocytic marker GFAP (RED) followed by the addition of cy-3 conjugated secondary antibodies and the nuclear stain DAPI (BLUE). Overlap of green eGFP/red GFAP signals (YELLOW) confirmed astrocytic differentiation of Wt-NSCs (A.) and sAPPα-NSCs (B.). Scale bar is 0.5mm.*

sAPPα-NSCs alter MWM performance of healthy mice – During the training phase of the MWM, all groups learned the maze (Figure 20 C-E), however sAPPα-NSC injected mice had significantly faster escape times on day 2 compared to both ACSF and Wt -NSC injected groups (Figure 20B). Furthermore, sAPPα-NSC injected mice had significantly more platform crosses

during the probe trial compared to Wt-NSC and ACSF treated controls (Figure 20G) despite not spending an increased amount of time in the target quadrant (Figure 20F). These discordant results suggest a difference in search strategies by sAPPα-NSC treated mice, although additional studies are needed.

Figure 20: sAPPα-NSCs alter MWM performance of healthy SAMR1 mice



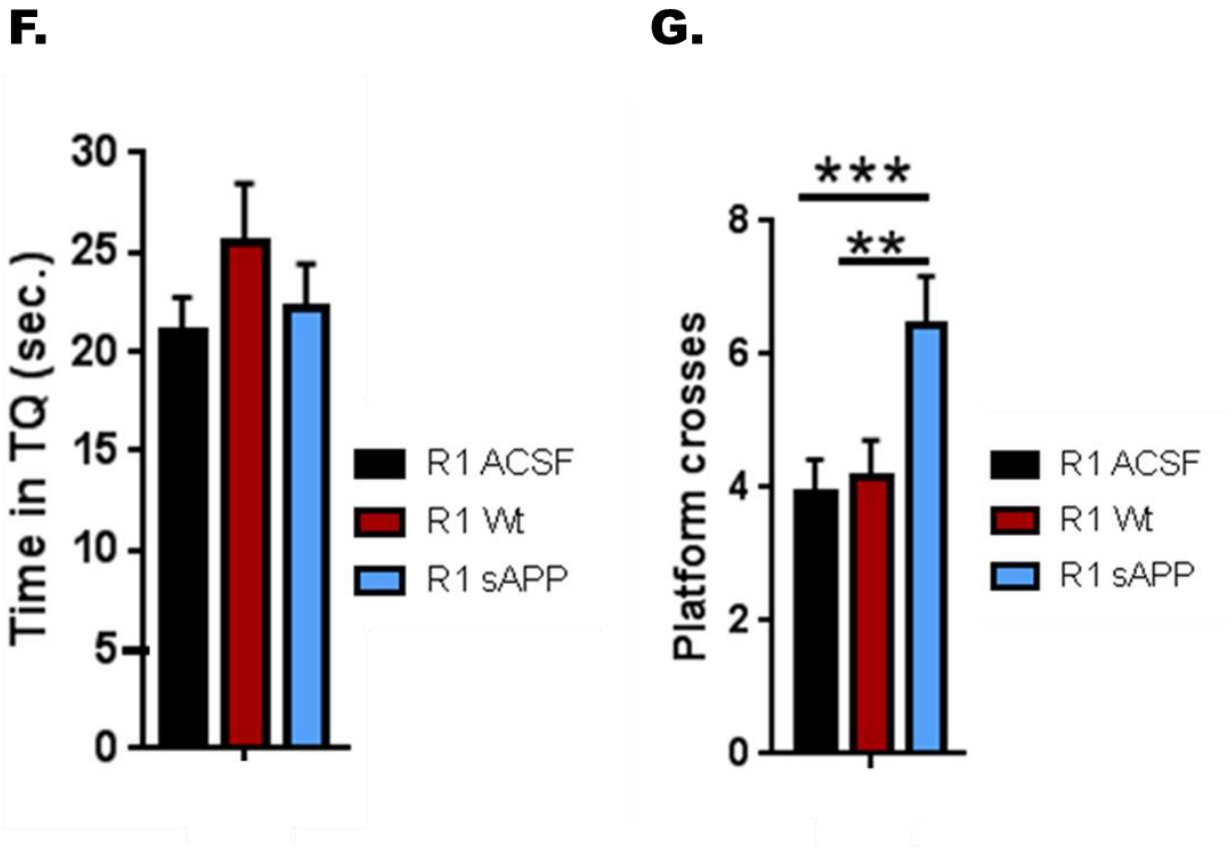


Figure 20: **sAPPα-NSCs alter MWM performance of healthy SAMR1 mice** *– A. Design of MWM used for testing. Colored symbols were placed around the outside of the pool. The circle within the triangle-square quadrant represents the target platform. B. Data represents average escape latency over 4 trials for each group during the 5 days of training +/- SEM. ## indicates p <0.01 compared to Wt-NSC treated group, *** indicates p <0.001 compared to ACSF treated group. C.-E, Date represents mean time spent in each quadrant by ACSF (C.) Wt-NSC (D.) and sAPPα-NSC (E.) treated mice +/- SEM. * indicates p<0.05 compared to time spent in the target quadrant. ** indicates p<0.01 compared to time spent in the target quadrant. *** indicates p<0.001 compared to time spent in the target quadrant. ## indicates p<0.01 between indicated groups. Data was analyzed by a 1-way ANOVA and Tukey's post-hoc test. Dashed line indicates threshold for time being due to random chance. F. Data represents mean time spent in the target*

*quadrant during the probe trial +/- SEM. Data was analyzed by a 1-way ANOVA and Tukey's post-hoc test. No differences in mean time spent in the target quadrant were detected amongst groups. G. Data is mean platform crosses during the probe trial. ** indicates p<0,01. *** indicates p<0.001. Data was analyzed by a 1-way ANOVA and Tukey's post-hoc test.*

SUMMARY:

The use of genetically modified NSCs for the delivery of therapeutics has been tested for spinal cord injury, Parkinson's disease and stroke. Given the apparent effects of sAPPα on AD pathology, sAPPα-NSCs are an intriguing treatment for AD and diabetic encephalopathy. With this work, we found that both Wt and sAPPα - NSCs survive implantation into the hippocampus long term (8 weeks) and that both types of cells differentiate primarily into astrocytes, as determined by labeling with GFAP antibodies. Furthermore, we found that sAPPα-NSCs altered MWM performance of healthy mice. Although further studies are needed to determine if sAPPα-NSCs can increase cognition in healthy mice, these results provide preliminary evidence that sAPPα-NSCs are a potential therapeutic for the nervous system.

CHAPTER 7: DISCUSSION AND CONCLUSIONS

With this work we tested the hypothesis that sAPPα can replace insulin in the diabetic nervous system. We found that overexpression of sAPPα in neural tissue prevented Alzheimer's-like pathology in the brain and prevented peripheral, sensory neuropathy. We also found that grafts of sAPPα-overexpressing NSCs implanted into the brain increased cognition in healthy mice. In total, these findings demonstrate that sAPPα is an activating ligand for IR that can replace the effects of insulin *in vivo*.

7.1: sAPPα BINDS AND ACTIVATES NEURONAL INSULIN RECEPTORS

Insulin and IGF ligands engage in cross-over activation with the others' receptor (Belfiore et al. 2009). Interestingly, Aβ and insulin share a sequence homology (Yip 1992, Kurochkin 1998) suggesting that sAPPα, like Aβ, may be an activating ligand for IR. As our data demonstrate, sAPPα was able to activate neuronal IR in the absence of insulin (Fig 4.) Furthermore, not only does sAPPα bind IR directly, it also competes with insulin for binding sites on IR as a greater interaction between IR and sAPPα was discovered in the insulin-depleted rodent brain (Fig 5.) suggesting that sAPPα is insulin mimetic.

The antibody used for the IP experiment in Figure 6A detects amino acids 3-8 within the Aβ sequence .The arginine to glycine amino substitution in mouse Aβ, which lies within the epitope recognized by the 6E10 antibody, likely confirms the human specificity of this antibody. An antibody raised against the N-terminal of APP was used to quantify overall APP levels (mouse and human) in CRND8 homogenates. Given the size of the bands detected (~100kDa), we can conclude that we observed an interaction between either APP or sAPPα, of human origin,

and IR in the brain homogenates of CRND8 mice. This interaction may explain, in part, why CRND8 are protected from supra-physiological levels of Aβ early in life (Jimenez et al. 2011).

While the results obtained in Figure 6A confirm that human APP, when overexpressed, binds to mouse IR, this observation does not address whether or not APP/sAPPα, normally binds IR in the Wt rodent brain. However, as demonstrated in Figure 5A, antibodies raised against the N-terminus of APP/sAPPα (which therefore detect mouse APP) were able to detect APP/sAPPα in IR-IP samples from Wt rat cortices. This interaction suggests that APP/sAPPα normally bind IR in the healthy brain. To test the neuroprotective effects of endogenously produced sAPPα, we examined the effects of an α-secretase inhibitor on survival of insulin deprived, Wt neurons. Neither insulin depletion nor administration alone affected survival of cultured neurons which may be due the use of B27 and B27 (-insulin) supplements (Fig 5B). According to our data, it is likely that these supplements are able to protect against insulin deprivation or inhibition of sAPPα production. However, exposure to both insulin deprivation and the α-secretase inhibitor significantly decreased neuronal viability. These results, along with the previous findings that APP and α-secretase KO mice display neural degeneration, suggest that endogenously produced sAPPα is normally neuroprotective in the brain.

Both insulin and IGF1 are being evaluated for treatment of AD-associated insulin dysfunction, therefore, it's important to clarify why sAPPα based therapeutics are of interest even though more potent ligands (insulin/IGF1) are already available for use in the clinic. First, as outlined in the introduction, sAPPα directly binds and inhibits BACE1 thus lowering Aβ levels. Secondly, analysis of the amino acid sequence of sAPPα reveals that there are fewer IDE cleavage sites on sAPPα than on insulin/IGF1 meaning that sAPPα treatments could potentially stimulate insulin signaling pathways while leaving more IDE available to digest Aβ compared to

IGF1/insulin therapies. Lastly, according to our data (Figs 7 and 16), sAPPα-based therapies are unlikely to affect peripheral insulin regulation. When taken into consideration, these findings suggest that sAPPα is an ideal therapeutic for the treatment of diabetic encephalopathy, AD and other neurodegenerative diseases.

Characterization of diabetic sAPPα-mice - After our *in vitro* studies revealed that sAPPα acts as an activating agonist for neuronal insulin receptors, we next rendered sAPPα-mice type 1 diabetic in order to determine the effects of sAPPα in the absence of insulin *in vivo*. Importantly, no differences in glycemic control between diabetic sAPPα mice and diabetic Wt mice were detected (Figs 7 and 16) thus ruling out variation in glucose utilization as a confounding factor in our studies. These results also demonstrate that sAPPα expression in the nervous system does not affect glucose regulation.

Studies done in the same line of Tg, sAPPα mice that we used for our work demonstrate that despite the use of a neural tissue specific promoter, levels of sAPPα in the plasma of Tg hemizygous mice reach concentrations of 18ng/mg (~180nM) (Bailey et al. 2012). As Figure 4 demonstrates, this concentration of sAPPα is more than sufficient to activate neuronal IRs, therefore, it is unclear why circulating sAPPα did not modulate glucose levels in our mice. The inability of plasma sAPPα to affect glucose uptake is indirect evidence that concentration of sAPPα that activate neuronal IR *in vitro* do not activate peripheral IRs *in vivo*. This is in direct contradiction to *in vitro* studies demonstrating that sAPPα activates AKT and increases glucose uptake in cultured myotubes at concentrations of ~3nM (Hamilton et al. 2014). Although beyond the scope of this thesis, future studies addressing the discordant results between the effects of sAPPα on peripheral IR *in vitro* and peripheral *in vivo* IR are needed.

Typically AD development is associated with T2DM rather than T1DM although recent epidemiological studies have solidified T1DM as an AD risk factor (Whitmer et al. 2015). Nevertheless, because of the association between AD and T2DM, it begs the question of whether or not the STZ, insulin-depletion model is appropriate for use in assessing potential AD therapeutics when T2DM is a disease of hyperinsulinemia. However, obese humans and high fat diet animal models both display decreased cerebral insulin levels (Kaiyala et al. 2000, Benedict et al. 2004) which may be a result of insulin resistance at the blood brain barrier resulting in decreased insulin transport (Baura et al. 1993, Urayama et al. 2008). Furthermore, intranasal insulin increases memory and cognition in obese humans (Novak et al. 2014) suggesting, counterintuitively, that cerebral insulin dysfunction in T2DM is a result of hypoinsulinemia. These findings, coupled with the multiple studies demonstrating decreased insulin levels in the AD brain, indicate that the STZ diabetic mouse is a reasonable model for evaluating therapeutics aimed at restoring insulin signaling in order to treat AD.

7.2: sAPPα PREVENTS DIABETES INDUCED CHANGES IN THE BRAIN

Because sAPPα both activates insulin signaling (Figs. 4-6) and is protective in brain tissue of young APP mice (Jimenez et al. 2011), we hypothesized that sAPPα could prevent the development of Alzheimer's-like pathology in the diabetic brain. We found that overexpression of sAPPα prevented diabetes-induced hyperphosphorylation of tau (Fig. 8), GSK3α and AKT (Fig. 10) and also blocked activation of the UPR (Fig. 9). Development of diabetes-induced pathology in the brain is time-dependent and worsens with duration of the disease (Planel et al. 2007b, Jolivalt et al. 2008, El Khoury et al. 2016, Gratuze et al. 2017a), however, our data show that even after prolonged diabetes (16 weeks), tau phosphorylation in diabetic sAPPα-overexpressing brain tissue was similar to non-diabetic controls.

sAPPα blocks aberrant tau phosphorylation: rationale for the use of sAPPα in AD - AD and diabetes are epidemiologically linked (Ott et al. 1996) and share overlapping pathology. The presence of NFTs composed of abnormally phosphorylated tau aggregates is a pathological hallmark of AD (Iqbal et al. 2009) and aberrantly phosphorylated tau is found in the brains of type-1 and type-2 diabetic animal models (Clodfelder-Miller et al. 2006, Kim et al. 2009). The present work demonstrates that sAPPα can prevent hyperphosphorylation of tau in insulin-depleted cortices (Fig.8). Although tau is aberrantly phosphorylated at multiple sites in the STZ-diabetic brain (Clodfelder-Miller et al. 2006, Planel et al. 2007b), we analyzed phosphorylation at Thr 231 as hyperphosphorylation of this site in particular is associated with tau aggregation and neurodegeneration early in human AD (Cho et al. 2004, Luna-Munoz et al. 2005, Luna-Munoz et al. 2007, Alonso et al. 2010, Driver et al. 2014). Therefore, based on these data we hypothesize that sAPPα has the potential to prevent AD pathogenesis by blocking abnormal tau phosphorylation.

sAPPα reduces diabetes-induced stress in the brain - Activation of the UPR in the brain is both a sign of cell stress and potentially involved in AD-associated amyloid accumulation (Devi et al. 2012). In addition to protein misfolding in the ER, the UPR can be activated by oxidative stress and excitotoxicity, both of which are common to diabetes and AD (Markesbery 1997, Hynd et al. 2004, Mastrocola et al. 2005, Anu et al. 2010, Jayanarayanan et al. 2013). We found decreased activating phosphorylation of the UPR kinase PERK in diabetic sAPPα-overexpressing brain tissue compared to Wt diabetic controls which suggests that diabetic sAPPα mice maintained overall brain health even in the absence of insulin. This data also demonstrates that diabetes-induced stress in brain tissue is associated with aberrantly phosphorylated tau and not increased amyloid aggregation. Interestingly, despite being a

downstream substrate for PERK, we observed no differences in phosphorylation of eIF2α between our experimental groups. To date, the only known eIF2α PP1 (O'Loghlen et al. 2003) which is not up-regulated in the STZ diabetic brain (Gratuze et al. 2017b). Therefore, future studies will need to analyze the balance of activity of the eIF2α kinases PERK, PKR and GNC2 (Lourenco et al. 2015) in the diabetic brain in order to address the disparity in PERK and eIF2α phosphorylation.

Effects of sAPPα on AKT on GSK3 – In culture, the trophic effects of insulin/sAPPα are mediated by PI3K-AKT activation (Cheng et al. 2002, Deng et al. 2015) resulting in increased, inhibitory phosphorylation of GSK3α/β (Cross et al. 1995, Milosch et al. 2014). However, insulin-depletion *in vivo* results in increased AKT/GSK3 phosphorylation (Clodfelder-Miller et al. 2005, Clodfelder-Miller et al. 2006). The up-regulation of phospho-AKT (~5 fold) we observed in Wt-STZ cortices is likely a protective, compensatory mechanism enacted during the course of diabetes as an increase in AKT activity would provide increased neuroprotection to counteract the effects of insulin depletion. Importantly, our results demonstrate that aberrant tau phosphorylation, at least during the late stages of diabetes, is not due to diabetes induced inactivation of AKT and consequent over-activation of GSK, which is contrary to studies that demonstrate sAPPα-mediated reductions in phosphorylated tau are associated with inhibitory phosphorylation of GSK3 in AD mice (Deng et al. 2015). Alternatively, our data indicates that the effects of sAPPα on tau phosphorylation in the diabetic brain are independent of GSK3 activity.

Activity of PKA is decreased in late stage diabetes - PKA is a tau kinase (Jicha et al. 1999) recently discovered to be upregulated in the STZ diabetic brain (van der Harg et al. 2017) and therefore hypothesized to underlie diabetes-induced tau phosphorylation. However, we

found that PKA activity was decreased in the brains of our Wt STZ mice (Fig. 11). This discrepancy may be explained by the difference in the duration of diabetes of the two studies (20 days vs 16 weeks). Together, these results suggest that aberrant activation of tau kinases may mediate tau-hyperphosphorylation in early, but not late, stage diabetes which is supported by our findings that none of the tau kinases we evaluated (GSK3, MAPK, PKA and AMPK) were up-regulated in the STZ diabetic brain.

sAPPα and PP2A-C – PP2A is a trimeric phosphatase composed of regulatory PP2A-A and PP2A-B subunits plus a catalytic PP2A-C subunit. Activity of PP2A-C is decreased in the diabetic brain and is hypothesized to underlie aberrant tau hyperphosphorylation (Planel et al. 2007b). AKT is also a substrate for PP2A (Padmanabhan et al. 2009), therefore our data indicates that diabetes results in a drop in PP2A activity that consequently causes aberrant tau phosphorylation and overactive AKT. In turn, this increase in AKT activity may account for the increase of GSK3 phosphorylation we observed in Wt-STZ cortices. Our data show that sAPPα normalized AKT/GSK3 phosphorylation in diabetic brain tissue indicating that PP2A activity was preserved in sAPPα STZ mice, which was confirmed by analysis of PP2A activity in cortical lysates (Fig. 12).

Diabetes and AD, a unified hypothesis: Numerous studies demonstrate that the insulin signaling pathway is dysfunctional in the AD brain and supporting animal studies have determined that insulin has a direct impact on AD-like pathology. Yet AD research is complicated by the apparent disconnect between the effects of insulin depletion *in vitro* and *in vivo*. For example, insulin depletion in culture decreases AKT activity, increases activity of the tau kinase GSK3 and increases tau hyperphosphorylation. However, despite the presence of hyperphosphorylated tau in the diabetic and AD brain, AKT is over-active in both diseases (Fig.

10) (Talbot et al. 2012). Given these findings, we postulate that the mechanistic connection between diabetes and AD involves an initial impairment of the brain's insulin signaling pathway and subsequent inhibition of PP2A. Although PP2A inhibition may initially delay catastrophic collapse of brain function via activation of AKT, the concurrent hyperphosphorylation of tau is a plausible cause of neurodegeneration (Figure 21). Furthermore, feedback inhibition of insulin signaling by overactive AKT may underlie insulin resistance via inappropriate inhibitory phosphorylation of IRS, which is present in diabetic primate models and AD patients (Talbot et al. 2012, Morales-Corraliza et al. 2016). Although increasing PP2A activity appears to be an attractive therapeutic target for the treatment of diabetes and AD, the effects of PP2A inhibition on AKT are likely critical for survival of the diseased brain. An alternative approach is to restore baseline insulin signaling via trophic factor administration thus increasing PP2A activity, decreasing tau phosphorylation and maintaining AKT signaling at an appropriate level. In accordance with this hypothesis, we speculate that in our studies, sAPPα was able to restore baseline insulin signaling in our diabetic animals thereby normalizing PP2A function and phosphorylation of tau, GSK3 and AKT. Interestingly, hypothermia can reduce PP2A activity (Planel et al. 2007a) and STZ-diabetic mice are hypothermic (Planel et al. 2007b) which may be the result of decreased insulin/IGF1 in the thermogenic centers of the hypothalamus (Sanchez-Alavez et al. 2011). According to these results, and in conjunction with our data demonstrating the sAPPα acts as an insulin mimetic, it's possible that sAPPα may have thermogenic activity in the hypothalamus, although further studies are needed to determine the effects of brain sAPPα on body temperature in the whole animal.

Figure 21: Dysregulation of PP2A in diabetes and AD

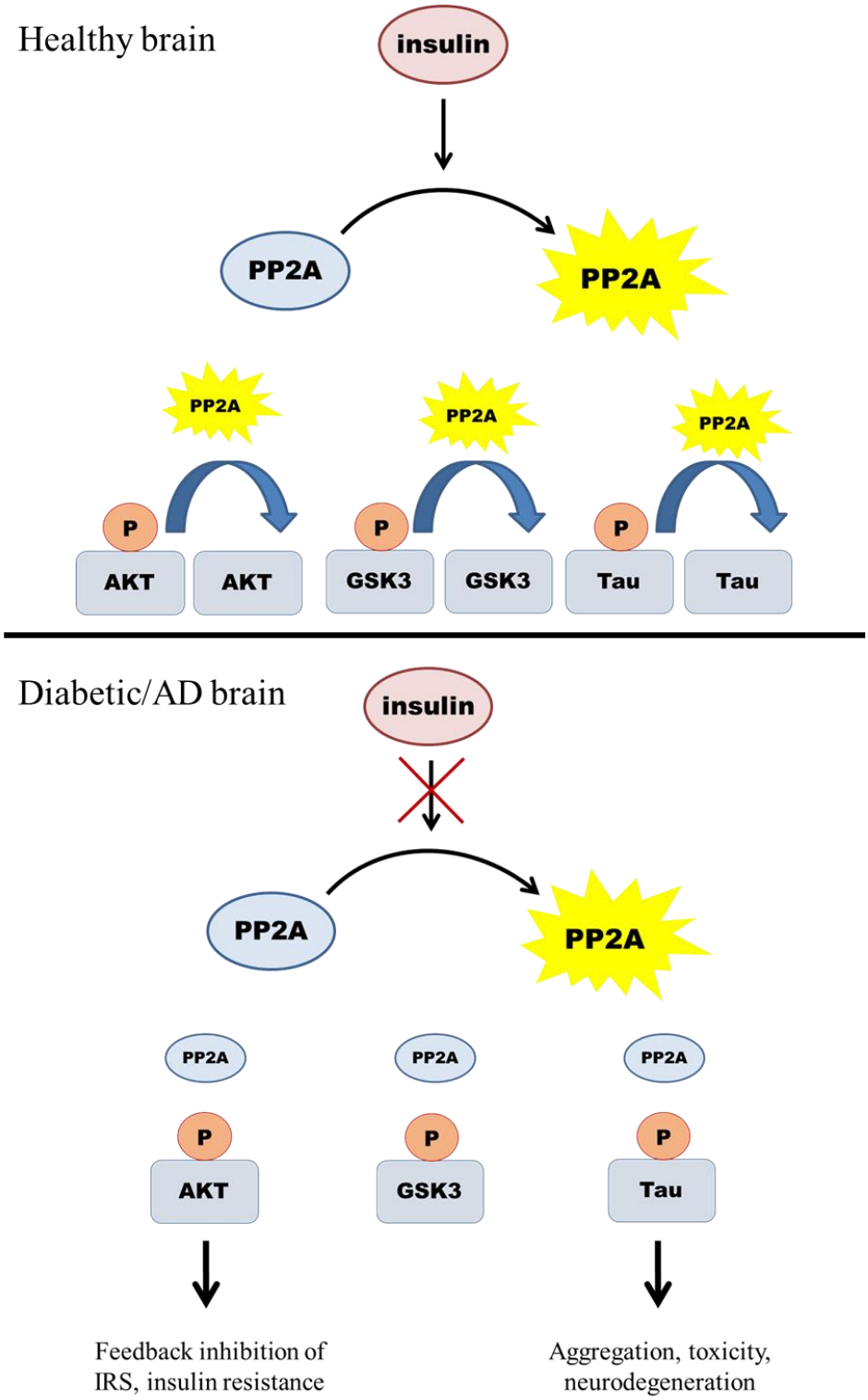


Figure 21: Dysregulation of PP2A in diabetes and AD *- Insulin signaling regulates activity of PP2A, a phosphatase that acts on AKT, GSK3 and Tau. In the healthy brain, when the insulin signaling pathway is intact, insulin stimulates PP2A activity which reduces phosphorylation of AKT, GSK3 and tau. However, decreased PP2A activity in AD and diabetes may lead to over-active AKT, feedback inhibition of IRS, insulin resistance, and hyperphosphorylation of tau leading to tau aggregates, tau toxicity and eventual neurodegeneration. Furthermore, the data presented here suggests that inactivation of PP2A, and not over-active GSK3, mediates hyperphosphorylation of tau in the diabetic brain.*

7.3: sAPPα IS PROTECTIVE IN THE SENSORY PNS

We found that sAPPα-overexpressing mice were protected from the development of diabetic, peripheral neuropathy induced by insulin-depletion despite having elevated blood glucose and HbA1c levels. Furthermore, we found that sAPPα-mediated increases in neurite outgrowth of DRG neurons could be blocked with the competitive IR/IGF1R antagonist AG1024. This is in agreement with the data presented in Figure 6 demonstrating that insulin and sAPPα compete for ligand binding sites on neuronal IR. Although AG1024 does not discriminate between IR and IGF1R, the results obtained in Figure 4B confirm that sAPPα specifically activates IR. In summary, we found that sAPPα is protective in the diabetic PNS and that the neurotrophic effects of sAPPα in DRG cultures require insulin/IGF1 receptors.

sAPPα activates NFκB and increases MnSOD expression in diabetic DRG - In order to characterize neuroprotective expression changes in diabetic DRG, we determined *in vivo* levels of the NFκB transcription targets MnSOD, Cu/ZnSOD and calbindin. No difference in calbindin expression was found, however, consistent with previous reports MnSOD expression

was decreased by diabetes and interestingly, occurred with a concomitant increase in Cu/ZnSOD. Prior studies involving transgenic mice revealed that overexpression of MnSOD results in decreased Cu/Zn/SOD expression (Vincent et al. 2007) which suggests that the expression of the two SOD enzymes is linked and may represent an important evolutionary trait that ensures that the cell maintains some level of antioxidant protection when expression of either SOD enzyme is compromised.

Consistent with reports that sAPPα can stimulate NFκB activity (Barger et al. 1996), we found that sAPPα/insulin treatment of diabetic neurons increases nuclear localization of the NFκB subunits cRel and p50, but not p65 which was associated with enhanced MnSOD expression. Because MnSOD overexpression protects against the development of peripheral neuropathy in type 1 and type 2 diabetic mouse models (Vincent et al. 2007), we hypothesize that sAPPα-mediated activation of NFκB resulting in increased MnSOD underlies the protective of effects of sAPPα on DRG neurons.

7.4: sAPPα-OVEREXPRESSING NSCs IMPROVE MWM IN HEALTHY MICE

Wt-NSCs, in the absence of genetic modification, have been demonstrated to ameliorate cognitive deficits in APP mice without reducing tau or amyloid pathology (Blurton-Jones et al. 2009). Therefore, because sAPPα antagonizes both aberrant tau phosphorylation and amyloid accumulation sAPPα -NSCs may provide therapeutic advantages beyond Wt -NSCs. With this work, we found that sAPPα-overexpressing NSCs survive long term after implantation into the hippocampus and alter MWM performance of healthy mice.

The differentiation characteristics of NSC grafts have a significant impact on their therapeutic potential. While it has been established that sAPPα steers NSCs towards an astrocytic fate, unexpectedly, in our study, the majority of both sAPPα and Wt NSCs differentiated into

GFAP+ astrocytes. Because different classes of astrocytes can have varying protective effects on nervous tissue, the benefits from sAPPα -NSCs over Wt -NSCs we observed in our animals may not be due solely to elevated sAPPα levels. For instance, previous studies demonstrate that glial precursors differentiated with BMP release increased levels of GDNF, BDNF, IGF1 and IGF2 (Proschel et al. 2014) and are a more beneficial treatment than other GFAP+ cells in animal models of Parkinson's and spinal cord injury (Davies et al. 2008, Davies et al. 2011, Proschel et al. 2014). Interestingly, sAPPα has been found to activate BMP/Smad signaling in NSCs (Kwak et al. 2014) suggesting that sAPPα may drive NSCs toward an astrocytic lineage that releases elevated amounts of neurotrophic factors.

The MWM is a widely used procedure that is utilized to evaluate learning and memory capability of rodents. In our study, we found that sAPPα-NSC injected mice found the target platform significantly faster on day 2 of the acquisition trial phase However, additional analysis is required to confirm that the effects of sAPPα-NSCs on escape latency were not due to an increase in swim speed. Speculatively, because NSCs were injected in the hippocampi of our animals and both types of NSCs had limited migration from the injection site, it is unlikely that sAPPα-NSCs affected the motor centers of the brain resulting in an increase of swim speed, although additional studies are required.

Results obtained from the probe trial revealed that sAPPα-NSCs had significantly more platform crosses compared to control groups despite not spending an increased amount of time in the target quadrant. Further analysis will be required to understand this discrepancy.

Despite the therapeutic potential of sAPPα, a viable delivery method for sAPPα in human patients does not yet exist. At 110kDa sAPPα is too large for intranasal delivery (Ozsoy et al. 2009) and intra-cerberoventricular infusions may not be feasible in human patients due the

frequency in which sAPPα would need to be administered. Furthermore, cost of therapy remains a prohibitive factor for use of sAPPα in the clinic necessitating the development of practical, cost-effective methods. Given the long term survivability of NSC grafts in the brain, sAPPα-overexpressing NSCs may be able to meet this criterion.

7. 5: POTENTIAL PITFALLS

The data presented here demonstrates that sAPPα can block the development of tau pathology and aberrant AKT signaling induced by diabetes, presumably by activating PP2A. Furthermore, we found that sAPPα-NSCs can improve cognition in healthy mice. Together these results suggest that sAPPα is an ideal therapeutic for AD. However, there are several potential pitfalls for a sAPPα-based therapy for the treatment of neurodegenerative disease. First, the mechanisms that underlie the effects of sAPPα on cognition remain unknown, however sAPPα-mediated activation of NMDARs may be involved (Taylor et al. 2008). Given the role of NMDARs in AD-associated excitotoxicity (Rammes et al. 2017), it's unclear whether or not sAPPα would be beneficial in the AD brain or if sAPPα modulation of NMDARs would accelerate cell death.

Secondly, although beyond the scope of this thesis, aberrant activation of inflammatory pathways may contribute to AD pathogenesis (Parbo et al. 2017). Inappropriately activated microglia may contribute to cell death in AD (Sarlus et al. 2017) and sAPPα is a microglia activator (Barger et al. 2001). While microglial activation as a result of sAPPα treatment has been suggested to be beneficial due to microglial-mediated clearance of amyloid beta (Fol et al. 2016) in AD mice, it is unknown whether or not sAPPα would exacerbate deleterious inflammation in human AD.

Finally, as previously discussed, the experiments described here were completed in type 1 diabetic, insulin-deficient animal models while AD is typically characterized by insulin resistance. Given the signatures of insulin resistance in the AD brain, it is uncertain whether or not sAPPα would be able to activate the insulin signaling pathway sufficiently to reverse neural pathology. Furthermore, if high doses of sAPPα are required to activate insulin signaling in the AD brain, this may an increase in the potential for the adverse effects described above.

7.6: DISCORDANT RESULTS

Although the work presented here expands on and is supported by the literature, there are number of discordant results with previous studies that will need to be explored in future studies.

AKT GSK3; in vivo vs in vitro: The mechanisms that underlie modulation of tau phosphorylation by the insulin signaling pathway are unknown and seem to vary between *in vitro* and *in vivo* paradigms. This is reinforced by previous studies that suggest sAPPα treatment *in vitro* reduces tau phosphorylation via a GSK3 dependent mechanism (Deng et al. 2015) while our data indicates that sAPPα reductions in phosphorylated tau *in vivo* are independent of GSK3. Similarly, a number of studies have found that sAPPα activates AKT in culture (Cheng et al. 2002, Jimenez et al. 2011) while our data show that sAPPα reduces AKT phosphorylation in diabetic mice. This discrepancy may be due to differences in PP2A activity as we and others have found decreased PP2A activation in the diabetic brain. Future work will need to focus specifically on how insulin signaling regulates PP2A in neuronal cultures in order to elucidate the differential effects of insulin on AKT/GSK3 signaling *in vitro* and *in vivo*.

The putative sAPPα receptor: Perhaps the biggest controversy addressed in this study is the identification of sAPPα as a ligand for neuronal insulin receptors. Although this work has demonstrated that sAPPα activates neuronal IR and can replace insulin *in vivo*, one potential weakness is that because of the antibodies used, it's unclear whether we observed APP or sAPPα binding to IR. However, given that exogenous sAPPα *in vitro* increases IR phosphorylation, and because sAPPα is released extracellularly from membrane bound APP, we believe that it is very likely that sAPPα, and not APP, is the binding partner for IR. These results, however, do not address the discrepancy between our data and previous studies that suggest the neurotrophic effects of sAPPα are mediated by an interaction with APP (Milosch et al. 2014). One possible explanation is that sAPPα interacts with multiple receptors and modulates multiple pathways. Therefore, future studies will need to identify which effects elicited by sAPPα are attributable to interactions with IR and APP. Although we demonstrated that the effects of sAPPα on DRG neurons could be blocked with the IR/IGF1R inhibitor AG1024, additional experiments analyzing the role of IR in sAPPα-mediated neuroprotection are needed.

7.7: FUTURE DIRECTIONS

Testing the effects of sAPPα on brain pathology in animal models of T2DM - Although this work demonstrates that sAPPα can prevent tau pathology and other diabetes-induced changes in the brain of STZ injected mice, the effects of sAPPα on brain health in T2DM remains unknown. In future studies, sAPPα overexpressing mice will be crossed with leptin receptor deficient mice (the obese, db/db^{+} model of T2DM) and tau phosphorylation/PP2A assessed. Concurrent experiments will evaluate tau phosphorylation and PP2A activity in the brains of high fat diet fed

mice. Because both of these models also develop peripheral neuropathy (O'Brien et al. 2014), we will also assess health of the PNS in these animals.

Testing the effects of sAPPα on vascular cells *in vitro* and *in vivo* - The effects of diabetes on the brain are not limited to nervous tissue as insulin signaling dysfunction also has profound effects on the cerebrovasculature. Although sAPPα is well documented as a neuroprotectant, emerging evidence suggests that endothelial cells may also utilize sAPPα for cytoprotection (d'Uscio et al. 2017). Furthermore, diseased endothelial cells may contribute to AD pathogenesis by overproducing Aβ that consequently gets spread throughout the brain via the vast network of the vasculature (Kitazume et al. 2010). Therefore, future of experiments will examine whether or not sAPPα can protect the cerebrovasculature *in vitro* and *in vivo* and prevent amyloidosis in animal models of vascular insufficiency.

Testing the effects of sAPPα-NSCs in animal models of neurodegeneration - In future work, we will test the hypothesis that direct brain delivery of sAPPα via sAPPα-NSCs will activate neuronal insulin pathways and ameliorate neuronal pathology associated with Aβ overproduction in animal models of diabetes and AD. In order to model human AD, we will be using the non-transgenic SAMP8 mouse model of AD. For controls, we will use the SAMR1 strain commonly used in parallel with the SAMP8. To model AD-associated insulin signaling impairment, some SAMR1 mice will be rendered diabetic by STZ injection. We will measure four classes of endpoint in this study 1: Function, in which the cognitive abilities of experimental animals will be assessed, 2: Neural Pathology, 3: APP processing and 4: insulin signaling. We expect that sAPPα-NSCs will ameliorate pathology in our models and that sAPPα-NSCs will exert benefits beyond that of Wt-NSCs due to a combination of insulin pathway activation and inhibition of Aβ

production. If successful, this work will provide preliminary evidence that sAPPα-NSCs are a potential therapeutic in the treatment of AD-associated insulin signaling dysfunction and Aβ accumulation.

7.8: CONCLUDING REMARKS

As of yet, the putative sAPPα receptor remains unknown. These studies, in combination with those done previously, strongly suggest that the neuroprotective effects of sAPPα are mediated by a direct interaction with IR. Moreover, our results demonstrate that sAPPα can inhibit the develop of pathology in the diabetic CNS and PNS without affecting glycemic control. Therefore, it is our belief that sAPPα, potentially as part of a NSC-based delivery system, should be pursued as a treatment for insulin signaling dysfunction in the diabetic and AD nervous systems.

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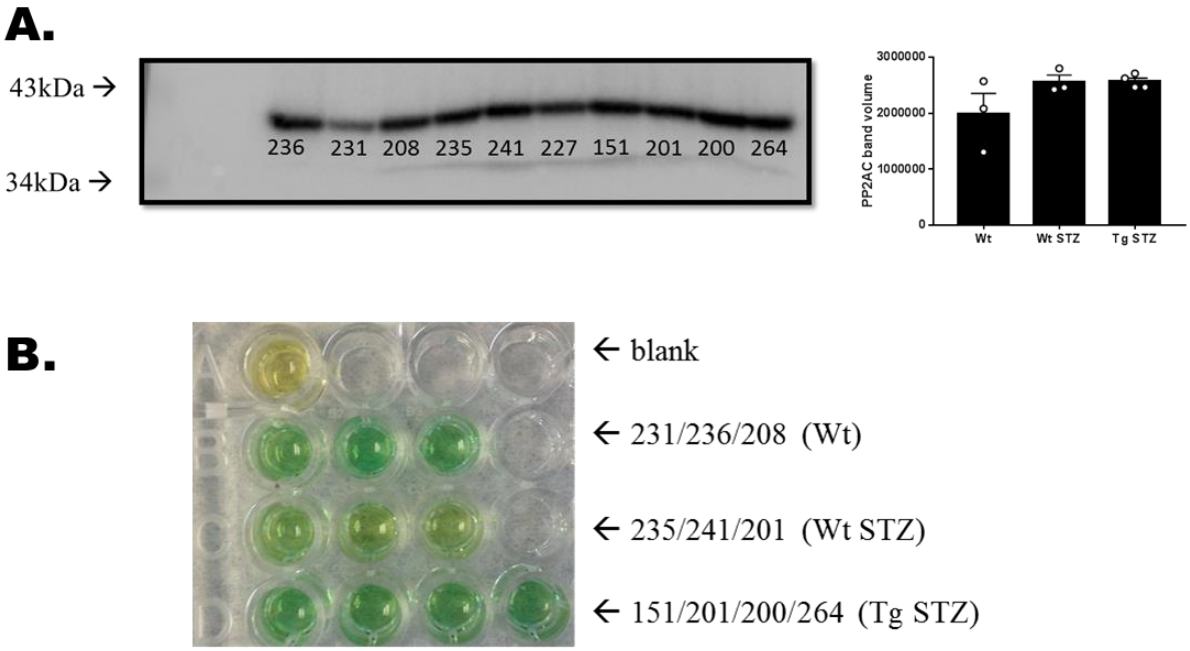
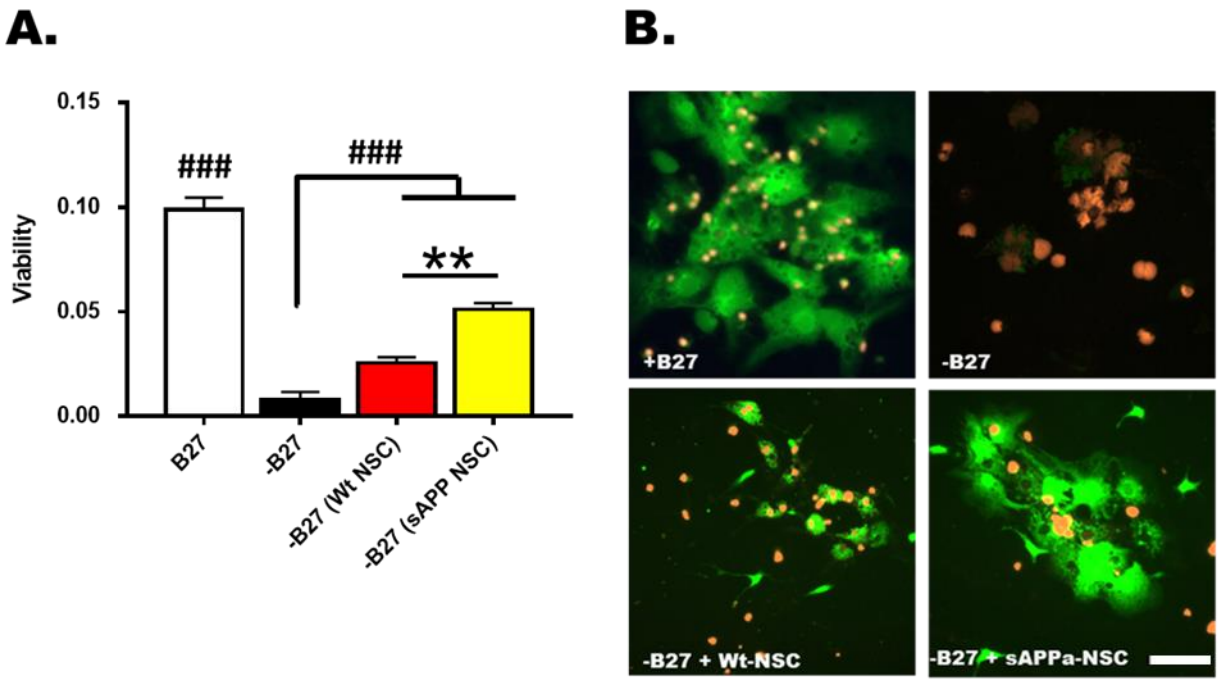
SUPPLEMENTARY FIGURES

Supplementary Figure 1: PP2AC viability assay



Supplementary Figure 1: PP2AC viability assay – *A. immunoprecipitated PP2A-C proteins were blotted and band density quantified. Number below bands represent animal IDs. Data is mean density +/- SEM. No differences in average band density were detected following analysis with 1-way ANOVA and Tukey's post hoc test. B. image of the plate used for the colormetric assay which quantified PP2AC activity in brain homogenates.*

Supplementary Figure 2: Culture media conditioned by sAPPα-NSCs increases neuronal survival compared to media conditioned by Wt-NSCs



Supplementary Figure 2: Culture media conditioned by sAPPα-NSCs increases neuronal survival compared to media conditioned by Wt-NSCs – *NSCs were placed into starvation media (neurobasal -B27) for 24 hours then cultures were centrifuged and supernatant (conditioned media) was collected. Cultures of cortical neurons (see Materials and Methods) were treated for 24 in neurobasal +/- B27 or media conditioned by Wt – NSCs (-B27 (Wt NSC)) or sAPPα-NSCs (-B27 (sAPP NSC)). A. Viability of neuronal cultures was assessed by presto blue assay. Data is mean viability (Arb unit) +/- SEM. Data was analyzed by a 1-way ANOVA and Tukey's post hoc test. N = 5-8 wells/ treatment. ### indicates p<0.001 compared to -B27 group. ** indicates p<0.01. B. Cultured neurons were stained for a viability marker (hydrolyzed calcein AM: green) and a apoptotic marker (ethidium homer dimer – 1: red) following treatment. Scale bar is 20μm.*