Secreted Amyloid Precursor Protein Alpha Binds to and Mediates Neuronal Insulin Receptor Activities in Rat Brain

by

Zaid A. Aboud

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University of Manitoba

Winnipeg

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Abbreviation

AD	**Alzheimer's Disease**
T1D	**Type 1 Diabetes**
T2D	**Type 2 Diabetes**
T3D	**Type 3 Diabetes**
flAPP	**full length Amyloid Precursor Protein**
sAPPα	**Secreted Amyloid Precursor Protein alpha**
Aβ	**Amyloid Beta**
IR	**Insulin Receptor**
IRS	**Insulin Receptor Substrate**
Akt (PKB)	**Protein Kinase B**
ab	**Antibody**
ACID	**Amyloid Precursor Protein C terminal Intracellular Domain**
IGF	**Insulin-like Growth Factor**
IGFR	**Insulin-like Growth Factor Receptor**
DSS	**Disuccinimidyl Suberate**

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Abstract

Alzheimer's disease (AD) is the most reoccurring type of dementia, and remains incurable. Much work has been done to investigate the connections between AD development, type 2 diabetes and insulin receptor signaling abnormalities. Full length amyloid precursor protein (flAPP) is a large transmembrane protein that has significant physiological activities including *in utero* fetal development. Alpha secretase enzymes cleave flAPP, producing secreted amyloid precursor protein alpha (sAPPα), which has neuroprotective properties, including protection against neuronal apoptosis as well as the induction of neuronal outgrowth. There is no known dedicated receptor for the physiological action of sAPPα. Our data suggest that the physiological actions of sAPPα are a result of the physical interaction between sAPPα and the neuronal insulin receptor. We have shown that sAPPα phosphorylates, and thus activates, the neuronal insulin receptor as well as specific downstream proteins, including insulin receptor substrate (IRS), and protein kinase B (Akt). We have also shown that the observed interaction between sAPPα and neuronal insulin receptors is physical and that sAPPα competes with insulin for the insulin binding site.

These findings may have implications for therapies aimed at slowing down the progression of AD through the activation of the insulin receptor pathway, since in neurons, insulin and the insulin receptor pathway are critical to the neuronal health and plasticity.

General Introduction:

Alzheimer's Disease and Diabetes:

Over 100 years ago, a patient by the name of Auguste Deter was examined by the German neurobiologist Alois Alzheimer in which he identified characteristics of a disease that is now referred to as Alzheimer's Disease (AD). Deter's symptoms of paranoia, disorientation, and overall emotional and mental decline were also shown by other patients over the years and were considered the normal progression of aging. After Deter's death, Dr. Alzheimer identified what is now known as neurofibrillary tangles as well as amyloid plaques. Since then, over 100 years later, AD is still the most common form of dementia without any clear known causes of initiation and remains incurable (Ott et al., 1999).

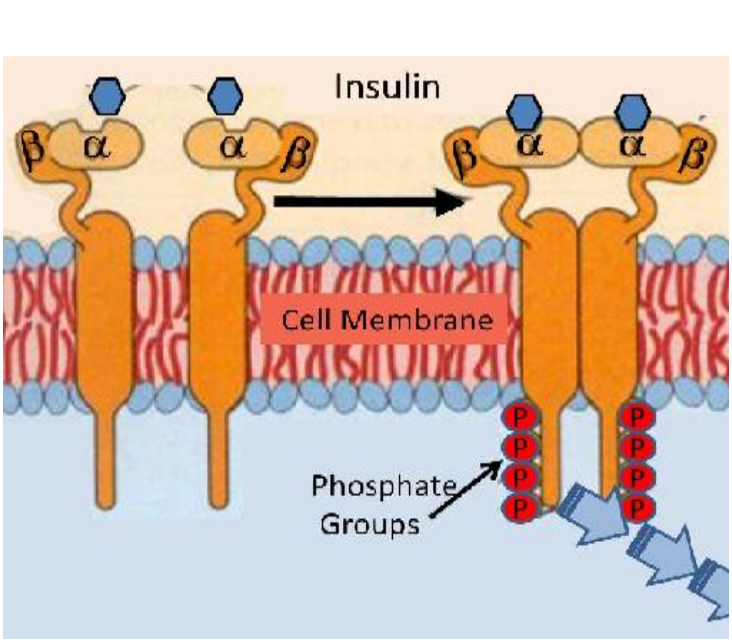
More research has indicated that AD is linked to other health problems and complications including diabetes, in particular type 2 diabetes (T2D) (Ott et al., 1999). T2D, a disease characterized by insulin resistance, is a well known risk factor for AD. When examining the brain tissue of an AD patient, and the pancreatic tissue of a T2D patient, both tissues show similar pathology. The strong correlation between the two disorders as well as the fact that brain insulin levels and insulin receptor activity are decreased in AD suggests that insulin signaling abnormalities contribute to the development of AD, and that AD is a brain specific form of diabetes referred to as "Type 3 diabetes" (Ritchie and Lovestone, 2002; Frolich et al., 1999; Rivera et al., 2005; Steen et al., 2005)

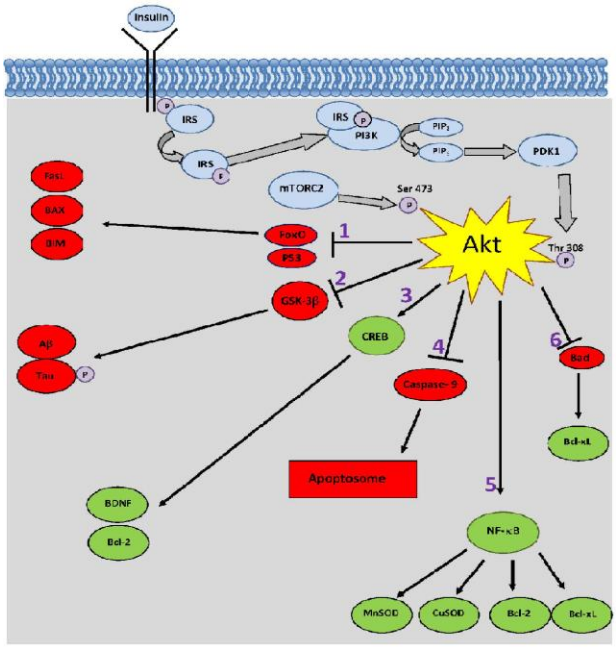
Adiposity (body fat content) may also have an effect on the development of diabetes and insulin resistance (Pi-Sunyer, 2002; Poirier et al., 2006). Patients with both elevated or below normal BMI are at increased risk of dementia (Atti et al., 2008; Kivipelto et al., 2005; Whitmer et al., 2005). Numerous cross sectional studies have demonstrated a link between

hyperinsulinemia and cognition (Kuusisto et al., 1997; Luchsinger et al., 2004). In women, fasting insulin levels which are a measurement of insulin resistance are related to cognitive performance (Okereke et al., 2005; van Oijen et al., 2008). T2D in postmenopausal women poses a 2 fold risk of mild cognitive impairment (MCI) (Petersen and Shulman, 2006).

Some studies demonstrated that T2D only causes vascular cognitive impairment not dementia (MacKnight et al., 2002). Others suggested that T2D is more related to vascular dementia (VD) than AD (Yoshitake et al., 1995). In the US, 39% of AD cases are T2D linked (Luchsinger et al., 2007), which according to FDPS (Finnish Diabetes Presentation Study) suggests that AD may be preventable with a healthy life style. People with T2D have an impaired neuropsychological function (Coker and Shumaker, 2003) and higher incidence of cognitive decline (Gregg et al., 2000). Increased oxidative stress is associated with diabetes, hyperinsulinemia, and obesity, and impacts normal function and survival of neurons in AD (Sasaki et al., 1998; Klein and Waxman, 2003; Craft and Watson, 2004; Stranahan et al., 2008). Diabetic patients that are carrying the apolipoprotein E (APOEε4), important molecule in the process of catabolism of triglyceride rich lipoprotein, and is a known genetic risk factor for AD) have higher risk of AD than non-diabetic carriers (Haan et al., 1999). There is no difference in hippocampal volumes among diabetic and non diabetic patients, however hippocampal tissue from T2D patients is more infarcted, additionally T2D patients have lower total brain volume, lower white matter volume, and lower gray matter volume (Saczynski et al., 2008). Defects in insulin signaling are associated with cognitive decline, and development of AD (de la Monte, 2009). Amyloid beta deposition in AD inhibits insulin signaling by interfering with insulin binding to its receptors (Zhao et al., 2007).

The Insulin Receptor and its Signaling Pathway:





(a) *(b)*

Figure 1: a representation of the subunit of the insulin receptor (a) and its downstream pathway upon the activation of the insulin receptor by insulin (b)

(a) Aulston et al., 2013.

(b) http://sphweb.bumc.bu.edu/otlt/mphmodules/ph/ph709_a_cellular_world/ph709_a_cellular_world7.html. February, 12, 2014.

As Figure 1, (a) shows, the insulin receptor (IR) consists of four subunits, two alpha subunits that are extracellular and two beta subunits, which extents intracellularly. Both the alpha and the beta subunits are connected by a disulfide bond (Hedo et al., 1981; Massague et al., 1981; Siegel et al., 1981). The binding of insulin to the insulin receptor alpha subunit results in a conformation change in the IR and activation of the tyrosine kinase in the intracellular component of the beta subunit (Kanzaki, 2006).

Figure 1 (b) shows the normal function of the insulin receptor pathway, the phosphorylation of the receptor by insulin leads to a downstream activation of intracellular proteins and kinases. Insulin receptor substrate (IRS) is the first intracellular protein to be phosphorylated and activated after the insulin receptor is activated as shown in Figure 1, a. IRS itself serves as a docking protein to another kinase called phosphatidylinostitol 3-kinase (PI3K), which generates phosphatidyl-3,4,5-triphosphate (PIP3). PIP3 in turn further phosphorylates 3-phosphoinositide-dependent-protein kinase 1 (PDK1), ending in the phosphorylation and activation of Akt which mediates much of the physiological response to insulin receptor activation (Khan et al., 1997; Bolotina et al., 1994).

In comparison to other organs, the brain consumes the largest amount of glucose. More than 30% of the body glucose is consumed by the brain (Zlokovic, 2008; Zlokovic, 2011). Normally, insulin in the peripheral tissue is required for the uptake of glucose. Glut4 is an insulin dependent glucose transporter, which upon the activation of the insulin receptor pathway, is translocated to the plasma membrane where glucose is transported into the cell by facilitated diffusion into the cell (Bondy and Cheng, 2004), as Figure 2 is showing.

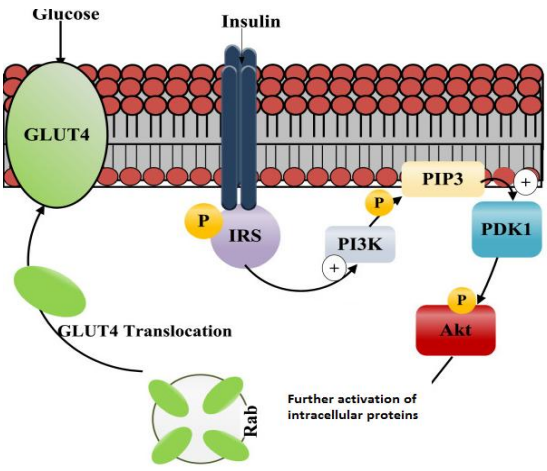


Figure 2: a representation of the translocation of the insulin dependent glucose transporter 4 as a result of the activation of the insulin receptor pathway by insulin. The activation of insulin receptor pathway ultimately results in the translocation of the glut 4 transporter to the plasma membrane aiding glucose to enter the cell by simple diffusion (Thorn et al., 2013).

The brain glucose uptake is not insulin dependent. Glut4 is not the main glucose transporter in the brain, but rather Glut1 and Glut3, which are insulin independent glucose transporters, are

highly expressed at all times. Insulin never the less plays a different and rather significant role as a neurotrophic factor for neurons (Bondy and Cheng, 2004). Many neuronal processes including neurite outgrowth, survival, pro-death gene inactivation and others are heavily influenced by insulin and insulin receptor signaling (Ishii, 1995). Insulin concentration in the brain is independent of peripheral insulin concentration (Havrankova et al., 1979). Loss of insulin increases GSK-3beta (Glycogen synthase kinase 3, which is responsible for the activation of many intracellular proteins that ultimately impact many cellular activities) activity leading to hyperphosphorylated tau, which is elevated in AD (Iqbal et al., 2009).

Early Work on Insulin influence on neuronal function:

There is a strong correlation between neuropathy, insulin abnormalities and diabetes (Ishii, 1995). The pathology of neuropathy is characterized by the loss of the axonopathy (Thomas et al., 1984). In diabetic axons, the number of axonal neurofilaments and microtubals is decreased (Yagihashi et al., 1990), which let many researchers to think that this reduction and nerve damage is related to the state of hyperglycemia, the increased in protein Glycosylation (Cerami et al., 1978), alternation of lipid metabolism (Field et al., 1965) as well as Schwann cell abnormality (Low et al., 1975). While others have suggested that the nerve damage is independent of hyperglycemia (Vinik et al., 1992).

These hypotheses were all proposed before it was known that insulin has a direct effect on the nervous system (Ishii 1995). The theory that the insulin and the IGF signaling pathways have a big influence on neurobiology which let to better understanding of diabetic neuropathy was formulated in the mid 1980's (Ishii et al., 1989; Carsten et al., 1992; Ishii et al., 1993). Anti IGF antibody decreases regulation of sensory and motor neurons (Glazner et al., 1993). Insulin

induces neuronal growth in embryonic sensory (Recio-Pinto et al., 1986), spinal cord (Glazner and Ishii 1988) as well as motor neurons (Caroni and Grandes 1990).

Insulin also plays a major role in increasing α and β tubulin (major structural proteins in axons) mRNA during neurite outgrowth (Fernyhough et al., 1989) by stabilizing these transcripts thus protecting them from degradation. Insulin also increases the neurofilaments mRNA during neurite outgrowth (Wang et al., 1992).

Since neurons are postmitotic, the loss of synaptic sensory as well as motor neurons is irreversible. Insulin and IFG provide a trophic support for the neurons at high risk of damage during diabetes (Ishii 1995), and increase survival rate (Bozyczko-Coyne at al., 1993). Intravenous administration of insulin can alter the firing frequencies of neurons (Anand et al., 1964), and influences neurotransmitter synthesis and uptake (Recio-Pinto and Ishii 1988). The severity of neuropathy in diabetes may dependents on the loss of insulin and IGF activities during diabetes leading to a decrease in production of axonal transport of tubulins and microfilaments (Ishii1995). The decline in the insulin and IGF with age in non-diabetic individuals contributes to the neuronal function loss (Hall and Sara 1984). Insulin can partially restore the loss of IGF1 activities (Scheiwiller et al., 1986) by inhibiting IGFBP, which itself inhibits IGF activity and is increased during diabetes (Luo and Murphy, 1991).

Generally, neuronal trophic factors like insulin enhance neuronal survival through the activation of Akt (Burent et al., 2001), which is considered one of the most critical pathways in inducing neuronal survival (Dudek et al., 1997). In the year 2000, Ginty and colleges have shown without a functioning Akt, neuronal survival cannot be promoted through the action of neurotrophins (Kuruvilla et al., 2000). There are many proteins, including transcription factors,

proteins that regulate programmed cell death, like Bcl-2 family members, cell growth and others have been shown to contain a protein motif that is Akt binds to, making these proteins substrates for Akt, which make them subjected to Akt regulation (Gingras et al., 1998). Critical enzymes that involve in mediating neuronal survival are also subjected to Akt regulations like nitric oxide synthase (Fulton et al., 1999), telomerase reverse transcriptase (Kang at al., 1999), IκB Kinase (Ozes at al., 1999), and others. Akt was shown to phosphorylate casing a deactivation of a class of major transcription factors known as the Forkhead box transcription factor class O (FOXO), which prevents them from expressing cell death genes. Akt also promote hippocampal neuronal survival by inhibiting the tumor suppressor p53, which when activated, it induces the expression of death genes (Yamaguchi et al., 2001).

Amyloid Precursor Protein and its Processing:

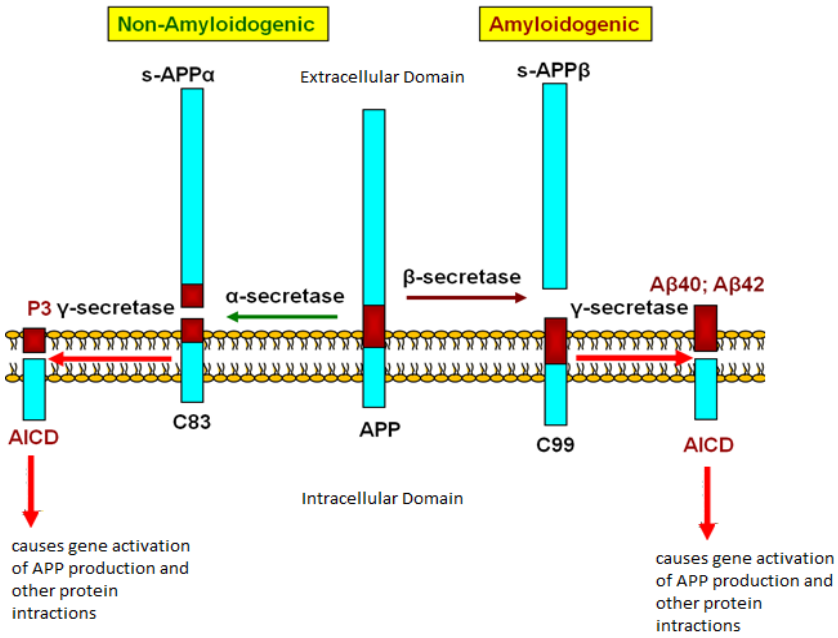


Figure 3: a representation of the full length amyloid precursor (APP) and its processing through both amyloidogenic and non-amyloidogenic pathways. Aplpha secretase cleaves APP producing sAPPα through the non-amyloidogenic pathway, while beta secretase cleavage starts the amyloidogenic pathway resulting in producing amyloid beta. Gama secretase cleaves the C-

terminal domain resulted from both pathways resulting in Amyloid precursor protein Intracellular C-terminal Domain (AICD), which acts as a transcription factor to activate genes encoding more APP production (Can Zhang, 2012).

Amyloid precursor protein (APP) is a transmembrane protein composed of 770 amino acids, with a large extracellular domain, which was first described as a type of receptor (Kang et al., 1987). Many signaling adapting proteins bind to the cytoplasmic domain of APP, like APP-BP1 (APP binding protein 1 which aids in the transduction of intracellular cascades mediated my APP) (Chow et al., 1996), and Fe65 (a transcription factor, which is present in the brain in high concentration and has many functions. McLoughlin and Miller, 2008) (Borg et al., 1996). The APP gene was first identified in 1987, the 240Kb gene is found on chromosome 21, and so far, over 25 disease causing mutations have been identified with different isoforms of APP (Mattson, 1997). APP can impact the activities of individual neurons and even enhances brain response to agonist. For example, APP increases the hippocampal neuron response to glutamate (Tominaga-Yoshino et al., 2001). The most conserved part of APP between human and other species is the 172 residues following the N terminal signal sequence (Daigle and Li, 1993). APP plays an important role in brain development by promoting neuron and glial cell adhesion, as well as assisting in the formation of long distance connection in the brain (Trapp and Hauer, 1994).

APP deficient mice have low body mass, low synaptic markers, and also display early postnatal lethality (Trapp and Hauer, 1994). APP concentrations are elevated in the prenatal period (Saitoh et al., 1989). In the adult brain, APP is expressed in the regions that undergo synaptic modification (Loffler and Huber, 1992). Figure 3 shows the processing of amyloid precursor protein. α-secretase cleaves the full length APP at the extracellular part and results in

the release of secreted APP alpha (sAPPα), which is referred to as the non-amyloidogenic processing of APP. sAPPα has many physiological functions including acting as a proliferative factor (Pietrzik et al., 1998; Caille et al., 2004), increasing keratinocyte motility (Kirfel et al., 2002), and promoting neuronal outgrowth (Milward et al., 1992).

sAPPα has many properties, but is best known for its neuroprotective properties (Smith-Swintosky et al., 1994; Furukawa et al., 1996; Mattson, 1997; Morimoto et al., 1998). The amyloidogenic processing of APP on the other hand starts with β-secretase cleaving APP at the N-terminal region of APP, which is referred to as the amyloidogenic sequence, resulting in the release of amyloid beta which when oligomerized is neurotoxic (De Strooper and Annaert, 2000; Turner et al., 2003). APP is also cleaved by γ-secretase (Cao and Sudhof, 2004). γ-secretase cleave in both the amyloidogenic and the non-amyloidogenic processing of APP and it results in the release of a 50 amino acid peptide called AICD (Amyloid Beta Intracellular C-terminal Domain), which is a signaling molecule and gene transcription molecule that induces further production of APP (Cao and Sudhof, 2001; Pardossi-Piquard et al., 2005).

The Amyloid Theory of Alzheimer's Disease:

In Alzheimer's patients, the degree of dementia in the patient correlates with brain amyloid beta levels. It is not understood why the concentration of the oligomer amyloid beta increases in AD patients that do not possess APP or secretase mutations. It has been suggested that a deficiency of amyloid beta clearance mechanisms results in amyloid beta accumulation (Jick et al., 2000), while others have related high cholesterol levels to the late onset of dementia by showing that high levels of cholesterol increase the affinity of APP to β-secretase enzymes which speeds the release of amyloid beta (Kojro et al., 2001). APLP1 (Amyloid Protein Like Protein 1) is expressed strictly in neurons (Lorent et al., 1995), while APP and APLP2 are

expressed in other types of cells (Yoshikai et al., 1990; Lorent et al., 1995). APP can be phosphorylated intracellularly and extracellularly at many sites by many kinases, like CDK5 (cyclin dependant Kinase 5, plays a role in sensory pathways, like pain pathways), and JNK3 (c-junction N-terminal Kinase 3, involved in inflammatory signaling), that help mediate cellular activities, communications and gene activation (Iijima et al., 2000; Kimberly et al., 2005; Muresan, 2005).

AICD has been shown to translocate to the nucleus as a transcriptional factor. It can form a transcriptional active complex with Tip60 and Fe65 (Cao and Sudhof, 2001; Gao and Pimplikar, 2001). The Amyloid beta region is conserved only in APP and APLP2 and not in APLP1 (Kimberly et al., 2001). APP contains two specific regions called E1, and E2 (which is the largest APP subdomain). These regions interact with heparin which causes APP/APP Cis-dimerization (through the E1 region) or Trans-dimerization (through the E2 region), which modulates γ-secretase activity. APP has an intracellular part which is a 47 amino acid long motif called YENPTY. A region on the YENPTY called the NPXY acts as a signal for endocytosis and is necessary for APP internalization (Lai et al., 1995).

Besides its toxic effect, the physiological role of amyloid beta is still unknown. Amyloid beta competes with lipids for the binding to apolipoprotein E APOE, which results in increased cholesterol levels (Lynch and Mobley, 2000). Amyloid beta regulates cell excitability extracellularly by facilitating membrane depolarization and calcium influx (Ekinci et al., 1999). Amyloid beta binds to the nicotinic receptor and impacts cognitive function (Wang et al., 2000).

Amyloid Beta, Insulin and the Insulin Receptor:

Amyloid beta peptides interfere with insulin binding to the insulin receptor (Xie et al., 2002), impair neuronal insulin receptor function (Zhao et al., 2008) and induce insulin resistance (Xie et al., 2002). Insulin regulates many enzymes like protein kinases and phosphatases, which if impaired, may contribute to many diseases including mental diseases (de la Monte, 2009). Insulin influences the synthesis and degradation of amyloid beta, and increases APP trafficking from Golgi network to the plasma membrane and thus impacts overall extracellular levels of amyloid beta (Gasparini et al., 2001).

Intranasal treatment with insulin reduces amyloid beta toxicity (Ott et al., 2012). The activation of Akt through insulin signaling induces cell-cell dissociation by deactivating amyloid beta induced glycogen synthase Kinase 3-B (Shineman et al., 2009). Amyloid beta oligomers cause decreases in surface insulin receptor number and reduction of insulin induced insulin receptor phosphorlyation in neurons (Zhao et al., 2008). Disruption of insulin signaling through PI3K inhibition decreases APP trafficking and secretion (Echeverria and Cuello, 2002).

Insulin deficiency increases β-secretase expression (Xu et al., 2004) and decreases the level of insulin degrading enzyme (IDE), which results in the decrease of the clearance of amyloid beta, since amyloid beta is a substrate for IDE (Qiu and Folstein, 2006). Increased amyloid beta levels lead to insulin resistance, results in increase in insulin levels which lowers amyloid beta clearance by inhibiting amyloid beta from binding to IDE (Devi et al., 2012).

Insulin receptor could be a common receptor for insulin and secreted amyloid precursor protein alpha. The focus of this thesis is to providing evidence that the physiological effects of sAPPα are mediated through the IR system, and furthermore that sAPPα accomplishes this by binding and activating the IR as an agonist.

Study Rational:

From the above introduction, it is reported that the family of the full length amyloid precursor protein may have interaction with the insulin receptor. For the 50 years, we have developed a concrete understanding of the insulin and insulin receptor involvement in neuronal health. This thesis will bring to the spot light new evidence to the possibility that the physiological effects of sAPPα reported may be through the insulin receptor pathway and that besides insulin, sAPPα can act as an agonist to the neuronal insulin receptor. This will combine our understanding of the insulin receptor function in the brain and the ability of sAPPα to induce neuronal survival into a bigger picture of inducing neuronal protection against neuropathy during diabetes and insulin resistance.

Global Hypothesis:

Secreted amyloid precursor protein alpha binds to, phosphorylates and thus activates the insulin receptor and its downstream pathway in neurons. Secreted amyloid precursor protein alpha competes with insulin over the insulin receptor, that is it binds to the insulin binding site.

Specific Aims:

- Determine if sAPPα is able to phosphorylate and activate the neuronal insulin receptor, and its downstream pathway (Chapter I and Chapter II)
- Determine if sAPPα is able to bind to the neuronal insulin receptor, if so, determine if it binds to the insulin binding site on the insulin receptor (Chapter II).
- Determine the physiological effect of sAPPα on neuron axonal outgrowth and overall survival, and the involvement of the neuronal insulin receptor in that physiological effect (Chapter I).

CHAPTER I

Secreted APPα directly interacts with and activates neuronal insulin receptors in vitro and in vivo

Secreted APPα directly interacts with and activates neuronal insulin receptors in vitro and in vivo

Jason Schapansky[1,2], [*]Zaid A. Aboud[1,2], Gary L. Odero[1], Brent D. Aulston [1,2], Benedict C. Albensi[1,2], Steven W. Barger[3] and Gordon W. Glazner [1,2]

[*] co-first author

From the [1]Division of Neurodegenerative Disorders, St Boniface Hospital Research Centre, Winnipeg, MB, Canada [2]Department of Pharmacology & Therapeutics, University of Manitoba, Winnipeg, MB, Canada, [3]Dep of Geriatrics, Anatomy, and Neurobiology, University of Arkansas for Medical Sciences, Little Rock, AR, USA.

Address correspondence and reprint requests to: Gordon Glazner, St Boniface Hospital Research Centre, R4046 – 351 Tache Ave, Winnipeg, MB R2H 2A6, Canada. Tel: (204) 235 3943; Fax: (204) 237 4092; E-mail: gordonglazner@gmail.com

Introduction

Secreted amyloid precursor protein α (sAPPα), which is cleaved and secreted from full-length amyloid precursor protein (flAPP), is a neurotrophic protein in the CNS with an unknown signaling pathway. sAPP induces neurite outgrowth in cortical neuronal cultures (Araki et al., 1991) and also reduces neuronal excitability thus preventing excitotoxic cell death (Mattson et al., 1993). sAPP also enhances synaptic plasticity in hippocampal slices by promoting long-term potentiation (LTP), a phenomenon believed to contribute to memory (Ishida et al., 1997). Although sAPP has several potential binding partners, including serum protein ApoE (Barger and Mattson, 1997) and guanylate cyclase at the plasma membrane (Barger and Mattson, 1995), a dedicated neuronal receptor has not been found.

Shared insulin receptor binding motif between insulin and flAPP-cleaved amyloid peptides (Yip, 1992; Kurochkin, 1998) indicates a physiological role for flAPP-cleaved peptides in insulin signaling. The flAPP cleavage product amyloid beta (Aβ) peptide, with which sAPP shares sequence homology, can directly activate the neuroprotective insulin/IGF1 pathway in neuronal cultures (Giuffrida et al., 2009). Homology between Aβ and sAPP, consisting of a 16 amino acid segment at the c-terminal end of sAPP and the N terminal of amyloid beta suggests that sAPP may also be involved in insulin signaling activation. Dimerized insulin or IGF1 receptor complexes are activated at the plasma membrane, leading to recruitment of kinases including PI3K and AKT to initiate insulin-induced activity. Insulin increases neuronal outgrowth *in vitro* in a dose-dependent manner (Mill et al., 1985; Wang et al., 1992), and prevents cell death through pro-apoptotic protein inhibition by PI3K induced AKT activation (Cross et al., 1995; Datta et al., 1997; Cardone et al., 1998; Turenne and Price, 2001). Insulin also modulates synaptic plasticity *in vitro* (Liu et al., 1995; Man et al., 2003), indicating a role

for insulin in memory formation. PI3K inhibition causes reduced memory formation in rats and mice (Lin et al., 2001; Chen et al., 2005). Similarly, intranasal insulin administration in humans improved memory in healthy adults (Craft et al., 1999; Craft et al., 2003), implying a potential therapeutic use for neurological diseases with a dementia component. Activation of insulin receptors by sAPP therefore may restore insulin signaling in the brain in order to treat or prevent the development of dementia.

In this study, our data demonstrates that sAPP specifically interacts with and activates insulin receptors *in vitro*, and also interacts with insulin receptors in APP overexpressing animals. Taken together, these data indicate that sAPP is a potential agonist for the insulin receptor.

Materials and methods

Embryonic Cortical Neuron Culture (performed by both Zaid Aboud and Jason Shapanky depending on the experiment)– Cortical tissue was isolated from day 16 embryonic CD1 mice. Cortices were triturated by fire-polished glass pipettes in HBSS buffer and seeded in 25 mm plastic dishes coated with poly-D-lysine (Sigma), at $8x10^5$ neurons/well. Neurobasal media with B27 supplement (Invitrogen) and 10% fetal bovine serum (FBS; Hyclone) was used at time of plating. Media was changed to Neurobasal+B27 supplement without FBS on the following day. Three days post-plating, half the volume of each dish was replaced with fresh Neurobasal+B27 containting 1 uM cytosine arabinoside (500 nM final), to limit astrocyte growth.

sAPP dose response experiment (performed by Jason Shapansky) and Immunoprecipitation (preformed by Jason Shapansky, confirmed, and repeated with controls by Zaid Aboud) - Experimental treatments were carried out 7 days *in vitro* (DIV), in Neurobasal

media alone. Cultures were incubated with 10, 30, or 100 nM sAPP for 15 minutes at 37°C and 5% CO2. Selected cultures were incubated with 1μM a selective insulin receptor blocker AG1024 (Santa Cruz Biotech) for two hours, and treated with 10nM sAPP. Following incubation, media was removed by aspiration; cells were lysed in 4%SDS/50 mM Tris-HCl, and diluted with 4x sample buffer for immunoblotting analysis.

For immunoprecipitation, selected cultures were treated with 10nM sAPP and/or 10nM insulin.

Immunoblotting and immunoprecipitation (performed by Jason Shapansky, repeated with the controls by Zaid Aboud) – Cortical tissue from diabetic mice and controls were isolated, frozen on dry ice and stored at -80^{o}C until processed. RNA and protein was extracted using an All-prep kit (Qiagen), or by homogenization in 4% SDS buffer. Protein concentration was determined and samples were made up to 0.5 mg/ml in a 4x sample buffer. 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 TBST. The same solution was used for incubating with one of the following antibodies overnight at 4 ^{o}C: p-AKT [Ser473] (Cell signalling), p-insulin receptor [Tyr972] (Millipore), p-IRS[Tyr612] (abcam), Aβ1-16(6E10-Signet), and insulin receptor (Santa Cruz). The following day, membranes were incubated with an appropriate species-specific secondary antibody, washed in TBST, and exposed to a FluorSMax imaging system (Biorad).

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 4 ºC 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, or IgG control antibody (Santa Cruz Biotechnology), the amyloid beta 1-16 sequence (6E10; Signet), or IgG antibody (Santa Cruz Biotechnology) overnight at 4^{o}C. 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.

Neurite outgrowth analysis (performed by Zaid Aboud) – CD1 cortical neuronal cultures were seeded in 96 well plates in Neurobasal media containing B27 supplement without insulin (Gibco). 11 hours after the time of plating, cells were incubated with 1 μM of insulin receptor blocker AG1024 for one hour, after that, treatments including 10 nM sAPP and 10nM insulin. Cultures were incubated at 37 ^{o}C/5% CO_2 for a total of 48 hours from the time of plating to fixation with 2% paraformaldehyde. Cells were made permeable by 0.3% Triton X-100 (Sigma) prior to incubation with β-tubulin III neuronal specific antibody (rabbit, 1:200; Santa Cruz Biotechnology) overnight at 4^{o}C. Cultures were washed and incubated with anti-rabbit Alexa Fluor 568 antibody (1:250; Molecular probes) for 1 hour and washed again. Cells were imaged using LSM510 confocal microscope (Zeiss), fitted with a 40x air-immersion objective. Total pixal area was measure per well, subtracting cell body contribution, which represents total neurite outgrowth.

Animals and experimental treatments (performed by Jason Shapansky) – TgCRND8 is a transgenic mouse overexpressing a modified APP695 protein, the primary form of flAPP found in neurons. This modification includes two FAD APP mutations designed to promote Aβ cleavage (Swedish, K670N & M671L; Indiana, V717F). Gene transcription was driven by a cos Tet vector containing a Syrian hamster prion protein promoter that focalizes peptide expression

in neurons, with minor contributions to astrocytes. Mice were developed on a C57 background that maximizes survival despite elevated APP expression (Chishti et al., 2001). These animals gestate and are born normally, but develop Aβ plaque deposition and cognitive impairments by 3 months of age. Although cortical sAPP levels drop from >90% of all flAPP cleavage to 50-60% due to the mutation (Simons et al., 2001; Chauhan et al., 2004), gene overexpression ensures as much as 4x more APP is produced than in controls, maintaining high human sAPP expression In this study, 8-week old animals were i.p. injected with the drug streptozotocin (STZ), a compound that specifically targets and destroys insulin-producing β-cells in the pancreas. This leads to a severe reduction in insulin response, causing type 1 diabetes and hyperglycemia. A dose of 90 mg/kg was used on three successive days, with a 12 hour fast prior to injection. Blood glucose was determined prior to a fourth injection; if levels were higher than 18.0 mmol/l, the animal was considered diabetic and not reinjected. Otherwise, animals were re-injected once every three days until diabetic. Animals 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.

RNA extraction and reverse transcript PCR (RT-PCR)/PCR (performed by Jason Shapansky) - Total RNA was extracted from hippocampal tissue of STZ-injected animals and controls (14 weeks) using the RNAeasy Kit (Qiagen), and 200ng of RNA was used for first-strand cDNA synthesis with random hexamer primers using the iScript cDNA Synthesis Kit (BIORAD, Mississauga, ON). PCR amplification was conducted using the following cycle parameters: 94°C for 2 min (1 cycle), 94°C for 30 sec, 60°C for 30 s, 72°C for 1 min (30 cycles), 72°C for 7 min (1 cycle) and then held at 4°C. Primers used and sequences are listed in Table 1. PCR products were confirmed by agarose gel electrophoresis (1% gel, 100V for 25 min and

stained with ethidium bromide (2mg/ml)). RNA was used as a negative control to assess gDNA contamination. Serial dilutions of purified PCR products were generated to allow quantitation of the specific mRNA of interest.

Quantitative real-time RT-PCR (qRT-PCR) (*preformed by Jason Shapansky)* - A total volume of 1 µl of cDNA was used as the template in each 25 µl PCR reaction with iQSYBR Green (BIORAD). Cycling conditions were: 94°C for 10 min, followed by 40 cycles at 94°C for 15 s, T_m for primers (see Table 1) for 30 s, and 72°C for 30 s, 72°C for 7 min and held at 4°C. PCR assays were performed using the iCycler Thermal Cycler (BIORAD, Mississauga, ON). Crossing threshold (Ct) values for each sample were used to calculate the initial quantity of cDNA template by the standard curve method. Data was normalized to GAPDH cDNA to correct for variability in individual samples. Negative control reactions were also performed without template cDNA.

Results

sAPPα increases insulin receptor phosphorylation in neuronal cultures – To assess exogenous sAPP induced activation of the insulin/IGF1 pathway, insulin was removed prior to treatment by washing cultures in media containing no insulin before incubating for 2.5 hours to remove endogenous insulin signalling. Cortical neuronal cultures were treated with sAPP (10, 30 & 100 nM). After sAPP treatment (15 minutes), neurons were lysed and assessed for insulin receptor activation by immunoblotting for phosphorylated insulin receptor. The pIR [pTyr972] antibody detects phosphorylation of the insulin receptor at tyrosine 972 residue, required for insulin-mediated signalling. sAPPα increased Tyr972 phosphorylation, with a significant increase over controls at 10, 30, and 100 nM (Figure 1A).

***sAPP activates the insulin signalling pathway* in vitro -** Downstream of the insulin receptor, IRS and AKT are the major mediators of insulin pathway activation. AKT is responsible for anti-apoptosis and synaptic plasticity (de la Monte and Wands, 2005; van der Heide et al., 2006). The activation of IRS is an indication of the activation of the insulin receptor itself. Insulin signaling also increases neurite outgrowth, a process promoted by sAPP as well (Mill et al., 1985; Araki et al., 1991; Wang et al., 1992). Insulin pathway activation by sAPP was tested by analyzing phosphorylated IRS and AKT levels and neurite outgrowth following sAPP treatment. The IRS phosphorylation was increased compared to the control, as observed with phosphorylated insulin receptor, with a significant increase at 10 nM sAPP treatment (Fig. 1B). The AKT phosphorylation was increased compared to the control, as observed with phosphorylated insulin receptor, with a significant increase at 10 nM sAPP treatment (Fig.2A), and at 100nM sAPPα treatment (Fig.2C), and the phosphorylation was significantly decreased upon the addition of 1μM AG 1024 insulin receptor blocker (Fig.2A, 2C).

Neurite outgrowth and survival analysis were performed on neurons plated with 10 nM sAPPα, in the absence and presence of 1μM AG1024 inhibitor that prevents insulin receptor pathway activation. sAPPα significantly increased neurite outgrowth and survival in comparison to the control (Figure 2B, 2D). AG1024 inhibition prevented this increase, reducing outgrowth and survival. These results suggest that sAPPα promoted neurite outgrowth and neuronal survival through increased insulin receptor activity. Figure 2E, shows visual representation of the overall neuronal growth and survival.

In vivo *assessment of APP-induced insulin pathway activation*– Increased protein phosphorylaton *in vitro* indicated sAPPα could activate insulin signaling proteins in the absence

of insulin. Lowering brain insulin *in vivo* would require reduced peripheral insulin production, as most brain insulin is produced peripherally and transported across the blood brain barrier (Banks, 2004). To mimic low insulin conditions under which *in vitro* experiments were performed, overexpressing APP TgCRND8 transgenic mice were injected with streptozotocin (STZ). Four experimental groups were created; wild-type animals injected with saline (Wt), wild-type animals injected with STZ (WtSTZ), transgenic animals injected with saline (Tg), and transgenic animals injected with STZ (TgSTZ). Following the injection blood glucose measurements at tissue collection confirmed that both STZ-injected TgCRND8 mice (TgSTZ) and injected wild type animals (WtSTZ) were type 1 diabetic >18 mmol/L, with no difference in blood glucose between transgenic and wild-type animals (Table 2). Brain weights determined during tissue collection revealed no difference between wild-type and transgenic injected groups (Table 2).

Insulin depletion reduces insulin receptor activation in wild type but not transgenic animals- Insulin pathway activation in diabetic and control animals was assessed by immunoblotting with IR-[pTyr972] antibodies, as performed *in vitro*. Cortical pIR levels of diabetic wild type animals (WtSTZ) were decreased versus wild-type (WT) controls (Fig 3A), while the phosphorylation levels remained unchanged in hypoinsulinemic transgenics compared to transgenic controls (Fig 3B). A comparison of injected groups as percentage of their controls revealed a significant difference between the pIR level between the Wt STZ and Tg STZ.

sAPPα physically interacts with insulin receptors in Transgenic Mice– Potential physical interaction between the peptides and insulin receptor *in vivo* was assessed by co-immunoprecipitation (co-IP). Co-IP with insulin receptor antibody followed by IB with sAPPα

antibody in Tg and TgSTZ brain homogenates was followed by immunoblot with an anti-amyloid 1-16 antibody (6E10) capable of binding full length amyloid precursor protein (flAPP), sAPPα, and Aβ1-42. sAPPα was detected in the both Tg and TgSTZ pulldown samples (Fig 4B), but TgSTZ animals had significantly higher sAPPα levels than Tg animals alone. Neither Aβ monomers (ABm) or oligomers (ABo), two amyloid peptides that alter insulin signaling, were found in any insulin receptor co-IP samples (Fig 4B). Duplicating co-IP with 6E10 antibody followed by immunoblotting with insulin receptor antibody verified a physical interaction association between insulin receptor and sAPPα in both Tg and TgSTZ (Fig 4A). This experiment lacks the proper controls. The controls needed for this experiment are an IgG sample to indicate that this pulldown is specific and bands seen in Figure 4A are indeed the sAPPα band and the other is an input for sAPPα. These controls are shown in Figure 4C, which shows the absence of the sAPPα from the IgG control indicating that it is specific.

mRNA expression for insulin/IGF related protein differs between treatment groups. Insulin/IGF1 signalling can be initiated by multiple ligands and receptor dimer combinations (Belfiore et al., 2009). Subunits of insulin receptor, the structurally-similar insulin like growth factor 1 (IGF1) receptor, or the distinct insulin-like growth factor-2 receptor (IGF2R) can form hetero- or homodimers, changing ligand specificity. Insulin pathway gene expression was established to determine if the prevention of diabetes-induced insulin signaling depression in diabetic transgenic was due to increased expression of IGF2, IGF1, and insulin or their respective receptor. No significant differences were observed between any of the groups in mRNA expression of insulin, IGF1, and IGF2 receptor (data not shown).

A significant drop in IGF2 mRNA expression was observed in insulin-deficient wild-type animals versus wild-type animals (Fig 5C). However, IGF2 levels in insulin-deficient transgenic animals (TgSTZ) were unchanged versus transgenic controls (Fig 5C).

Hypoinsulinemic wild type animals had significantly higher IGF1R levels compared to the Wt, while levels in hypoinsulinemic transgenic did not change (Fig 5B).

Both hypoinsulinemic wild type and hypoinsulinemic transgenics animals had significantly higher InsR mRNA levels compared to the controls wild type and transgenics (Fig 6A).

Discussion

In central neurons, sAPPα promotes both neuronal activity and survival through an unknown mechanism. In this study, we discovered that sAPPα activated insulin receptors and the insulin signalling pathway, both *in vitro* and *in vivo*. In neuronal cultures, sAPPα increased insulin receptor phosphorylation in a dose-dependent manner, as well as stimulated neurite outgrowth through the activation of the insulin receptor. Also there was a physical interaction between insulin receptors and sAPPα resulting in receptor phosphorylation in APP overexpressing animals, supporting the hypothesis that sAPPα is a ligand for the insulin/IGF pathway. Insulin insufficiency in wild-type animals caused a reduction in the insulin signaling, which was prevented by insulin receptor activation by sAPPα in transgenic animals.

Insulin/IGF receptor stimulation is mediated by multiple ligands, including insulin, IGF1 and IGF2 (Belfiore et al., 2009), and amyloid peptides and insulin share an insulin-receptor binding motif that indicate sAPPα could directly stimulate insulin signalling as well (Yip, 1992; Kurochkin, 1998). Both sAPPα and insulin's actions on synaptic plasticity and LTP also suggest a commonality of function (Liu et al., 1995; Chen and Leonard, 1996; Ishida et al., 1997; Liao

and Leonard, 1999; Skeberdis et al., 2001; Man et al., 2003). In the current study, sAPPα stimulated insulin receptor phosphorylation in the absence of insulin, suggesting the hypothesis that sAPPα activation requires binding to the receptor active site. This hypothesis is also supported by the short time course of experimental treatment (15 min), as phosphorylation was unlikely to be due to activation of an alternate secondary messenger. In addition, insulin receptors, and not other receptors in the insulin/IGF pathway, were specific targets of sAPPα activation, as determined via immunoprecipitation with a specific insulin receptor antibody. Increased IRS and AKT phosphorylation downstream was consistent with receptor phosphorylation, suggesting that sAPP/insulin receptor interaction was functional.

At 100nM sAPPα, pAKT appeared to be double bands while at 10nM there is only a single band. One explanation may be that at a high dosage sAPPα, another kinase that is closely associated with AKT will also be phosphorylated which appears in the blot. The antibody itself may give different variation in the signal from one blot to another, or if the 10nM sAPPα blot (fig2,C) was to be left for a longer exposure, we might have seen the double band as well, either way there was a significant activation of this AKT by sAPPα treatments. Also, increased neurite outgrowth and overall cell survival by sAPPα were entirely mediated by insulin signaling, as inhibition of insulin receptor activation by AG1024 abolished the effect. Thus, sAPPα acted as functional agonist of the insulin receptor. The experiment lacks one control besides the no sAPPα treatment, which is the AG1024 blocker only treated cells. The absence of this control leaves out the possibility that the blocker itself could have had other effects on the cell itself which resulted in the reduction of AKT physophorylation levels. Figure 2B and 2D show the effect of sAPPα on both cell growth (fig 2B) and survival (fig 2D), which is that sAPPα treatments increase the neuronal axonal growth and overall survival in neuronal cells that lack the B27 growth media.

Figure 2B includes the control that is absent in Figures 2A and 2B which is the AG1024 blocker only treated cells, which shows that when added to starved cells (cells that have no B27), there is no significant decrease due to cell death indicating that the AG1024 has no significant effect on the cell survival during the time of incubation, which indicates that the decrease in phyosphorylation of AKT seen in both Figures 2A and 2B is due to the blockage of the insulin receptor by AG1024 preventing sAPPα binding.

Co-immunoprecipitation (co-IP) of sAPPα:insulin receptor complexes with separate antibodies to either insulin receptor or an amino acid sequence in sAPPα revealed a direct interaction between sAPPα and insulin receptors in both normoinsulinemic and hypoinsulinemic transgenic animals. The higher amounts of sAPPα bound to insulin receptors in insulin deficient transgenic animals may indicate a shared binding site on IR, however this remains to be shown definitively.

Aβ peptides are also capable of interacting with the insulin receptor, resulting in modifications to insulin pathway activation (Xie et al., 2002; Zhao et al., 2008). Therefore, as our model produces Aβ peptides, it was important to determine if Aβ may also bind to insulin receptor in immunoprecipitation experiments, as the 6E10 antibody used in these experiments interacts with a 16-mer sequence found in sAPPα, flAPP and Aβ. However, neither Aβ monomers nor oligomers were detected in any insulin receptor co-IP samples (Fig. 3B). This is likely due to the young age (8 weeks) at which transgenic animals were injected, as Aβ levels are not apparent at this age.

Insulin reduction via STZ had substantially different effects on insulin pathway activation in wild-type compared to transgenic animals. Following injection, wild-type animals had less insulin receptor phosphorylation than uninjected animals, in agreement with previous studies

(Jolivalt et al., 2008; Sugimoto et al., 2008; Jolivalt et al., 2009). Therefore, the insulin pathway activation in these animals reflected insulin removal, with receptors unstimulated due to reduced ligand, compared to wild-type controls. However insulin depletion did not cause a similar situation in the transgenic animals, as phosphorylation levels were unchanged in hypoinsulinemic transgenics compared to transgenic controls. Indeed, this suggests that reduced insulin signalling due to insulin depletion was completely prevented through activation of insulin receptors by sAPPα in transgenic animals. Thus, insulin-mediated receptor activation was replaced by sAPPα in hypoinsulinemic transgenic animals, a mechanism suggested by insulin-free *in vitro* experiments.

Insulin-like growth factor-2 (IGF2) activates the insulin signaling pathway through insulin receptor homodimers, mediating neurodevelopment and neuroprotection (Cheng and Mattson, 1992; Belfiore et al., 2009). In type 1 diabetic rats, a drop in IGF2 levels in the hippocampus precedes the onset of memory deficits in Morris water maze tests (Li et al., 2002). Insulin replacement therapy, however, can reverse reduced IGF2 mRNA levels in insulin deficient animals (Wuarin et al., 1996). Therefore, decreased IGF2 is a marker for loss of insulin action. In our study, diabetic wild-type animals had significantly less IGF2 expression than wild-type controls (Fig. 5C). However despite being as insulin deficient as hypoinsulinemic wild type animals, IGF2 levels did not decrease in hypoinsulinemic transgenic animals. Thus, activation of insulin receptors by sAPPα restored IGF2 expression, much as insulin does in wild-type insulin-deficient animals. Restored expression of IGF2, in addition to sAPPα, may have contributed to stimulation of insulin/IGF1 signalling in hypoinsulinemic transgenics.

Clinical studies have revealed that reduced insulin signalling increases the risk of developing dementia (Kalmijn et al., 1995; Ott et al., 1996; Stolk et al., 1997b; Stolk et al., 1997a).

Reduced insulin signaling can often result in Alzheimer's disease exacerbation in APP overexpressing animals, especially in older animals (Ho et al., 2004; Burdo et al., 2009; Jolivalt et al., 2009). However, the use of young animals (8 weeks) and a short time diabetic time course (6 weeks) in this study ensured activation of insulin receptors by sAPPα while reducing complications due to increased Aβ and insulin deficiency. We expect then an extended time course in our model would lead to a full reversal of neurotrophic sAPPα effect, as Aβ accumulation and poor metabolic health due to long term diabetes would enhance cognitive difficulties in injected animals compared to transgenics alone, resulting in the situation observed in older TgCRND8 animals of the Jolivalt et al 2009 study. Therefore, the aim of this study was to introduce insulin deficiency in asymptomatic animals, prior to formation of detectable Aβ, and terminate the study just as TgCRND8 animals began to display Aβ-induced impairments. Thus, we maximized exposure of insulin receptors to sAPPα and reduce the contribution of Aβ peptides to insulin signaling, a situation confirmed by high sAPP:insulin receptor ratios and absent Aβ:insulin receptor complexes in hypoinsulinemic transgenic co-IP experiments (Fig.4A and 4B). Traditionally, activation of the insulin/IGF1 pathway was initiated by only three ligands: insulin, IGF1 and IGF2. With sAPPα able to completely compensate for lost insulin signaling in transgenic diabetics, sAPPα could be as potent a ligand of the pathway as the aforementioned conventional agonists. In fact, this study may introduce the possibility that sAPPα is a normal contributor to basal insulin signaling in the brain, through the insulin/IGF1 pathway, and should be categorized as an insulin receptor activator. Further analysis will be

required to understand the conditions under which sAPPα normally contributes to neurotrophic signaling via the insulin/IGF1 pathway.

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Figure Legends

Figure 1 *– sAPP increases insulin receptor (IR) and IRS phosphorylation in cultured mouse cortical neurons.* (A) Mouse embryonic coritical neurons were cultured for 7 days, then treated with sAPP (10 nM, 30 nM, and 100 nM) in the absence of insulin (2 hours), for 15 minutes prior to collecting cell lysates. Immunoblotting was performed using an antibody to detect phosphorylated insulin receptor ([pTyr972], pIR). Treatment with sAPP increased IR phosphorylation in a dose-dependent manner. Error bars represent mean +/- SEM, One-way ANOVA was performed with a Tukey's post-hoc test. *$p<0.05$, **$p<0.01$ treatment verse control (not treated). Each treatment was performed in triplicate. (B) Mouse embryonic cortical neurons were cultured for 7 days, and then treated with 10 nM sAPP in the absence of insulin (2 hours), for 15 minutes prior to collecting cell lysates. Immunoblotting was performed using an antibody to detect phosphorylated insulin receptor substrate (IRS). Treatment with 10 nM sAPP significantly increased IRS phosphorylation. Error bars represent mean +/- SEM, Student t-test was performed. **$p<0.01$. Each treatment was performed in triplicate.

Figure 2 – *Insulin receptor inhibitor blocks sAPP-mediated AKT phosphorylation and neurite outgrowth, and overall cell survival.* Mouse embryonic cortical neurons were cultured for 7 days, and then exposed to vehicle or 1μM AG1024, an insulin receptor blocker which binds to and blocks the insulin binding site on the receptor, for one hour, followed by vehicle or 10 nM sAPPα (A), and 100nM sAPP (C). After an additional 15 min, cells were scraped, protein isolated, and pAKT levels determined by Western blotting. Treatment with 10nM and 100nM sAPPα increased phosphorylation of AKT relative to untreated control cells (**$p<0.01$). Co-treatment with 10 nM sAPP and 1μM AG1024 inhibited the increase in AKT phosphorylation at

both10 nM and100 nM sAPPα. Error bars represent mean +/- SEM, One-way ANOVA was performed with a Tukey's post-hoc test. *p<0.05, **p<0.01 in comparison to the control. Neurite outgrowth and total survival were analyzed by immunofluorescence of neuron specific β-tubulin Type III after 48 hours *in vitro* (Figure 2B and 2D). Treatments with sAPPα (10 nM) and insulin (10 nM) resulted in significant increases in neurite outgrowth and over all cell survival relative to the control –B27 (B27 is the growth media that was removed in order to starve the cells. Cells were starved for a total of 12 hours) (p<0.01, p<0.01, respectively). Co-treatment with sAPP (10 nM) and AG1024 (1μM) resulted in a significant decrease in neurite ouotgrowth relative to APP treatment alone (p<0.01). Similarly, co-treatment with insulin (10 nM) and AG1024 (1μM) resulted in a significant decrease in neurite outgrowth relative to insulin treatment alone (p<0.01). Error bars represent mean +/- SEM, One-way ANOVA was performed with a Tukey's post-hoc test. Figure 2E shows visual representation of the neuronal cell outgrowth and survival. Cells were treated with insulin or sAPPα after starving them for 12hr. AG1024 was added for 2hr. These pictures were taken 36hr after treatment. The arrows pointing to cell bodies and axonal growth.

Figure 3 *– Insulin receptors are phosphorylated significantly more in diabetic APP-CRND8 than in diabetic WT brain.* Cortical tissue from control and insulin-deficient animals was homogenized probed for activated insulin proteins using immunoblot. Representative lanes are shown of blots using an antibody to the phosphorylated insulin receptor at Tyr972 (pIR). Insulin deficient transgenic animals (TgSTZ) showed significantly higher pIR than in insulin-deficient wild type animals (WtSTZ). (Mean +/- SEM, t-test, n=2 per group, *p<0.05).

Figure 4 *– Physical interaction between sAPPα and insulin receptor is greater in insulin-depleted APP-CRND8 mouse brain.* Proteins were isolated from cortical tissue from either 8 week diabetic or age-matched non-diabetic APP-TgCRND8 mice. Proteins were immunoprecipitated using 6E10 antibody (A), or total insulin receptor antibody (B), then the precipitate was separated by SDS-PAGE protein levels determined by western blotting. (A) Immunoblotting precipitate using anti-insulin receptor shows a significantly greater (~60%) level of insulin receptor in precipitate from the diabetic vs non-diabetic brain. (B) Immunoblotting using 6E10 shows a significant (~250%) increase in sAPP relative to total insulin receptor in the precipitate from diabetic vs non-diabetic brain. (C) Immunoblotting using pIR antibody includes the IgG control indicating that the interaction between sAPPα and the insulin receptor is specific. The sAPPα band is absent from the IgG treated sample as indicated in the blot. The input (IN) shows no sAPPα band while the Pull down sample (PD) shows a clear sAPPα band (upper band) indicating that the sAPPα interacts with the insulin receptor. The second band (bottom band) in the PD sample is the IgG portion of the insulin receptor. (Mean +/- SEM, Student t-test, n=3,4/group, *$p<0.05$).

Figure 5 *– Gene expression is altered by STZ injection in both wild type and TgCRND8 mice.* qRT-PCR was used to analyze gene expression in hippocampal tissue from experimental groups. Both injected groups demonstrated (A) increase insulin receptor gene expression versus control (Wt STZ vs WT, $p<0.05$; Tg STZ vs Tg, $p<0.05$) (B) IGF1R mRNA expression was elevated in Wt STZ versus control ($p<0.05$), but unchanged in transgenic tissue. (C) IGF2 mRNA levels were significantly decreased in injected wild type animals versus control ($p<0.001$). Wt, n=6; Wt STZ, n=4; Tg, n=5; Tg STZ, n=7 (Mean +/- SEM, one way ANOVA, Tukey's post hoc, $p<0.05$, $p<0.001$)

Figure 1: sAPPα increases insulin receptor (IR) and IRS phosphorylation in cultured mouse cortical neurons.

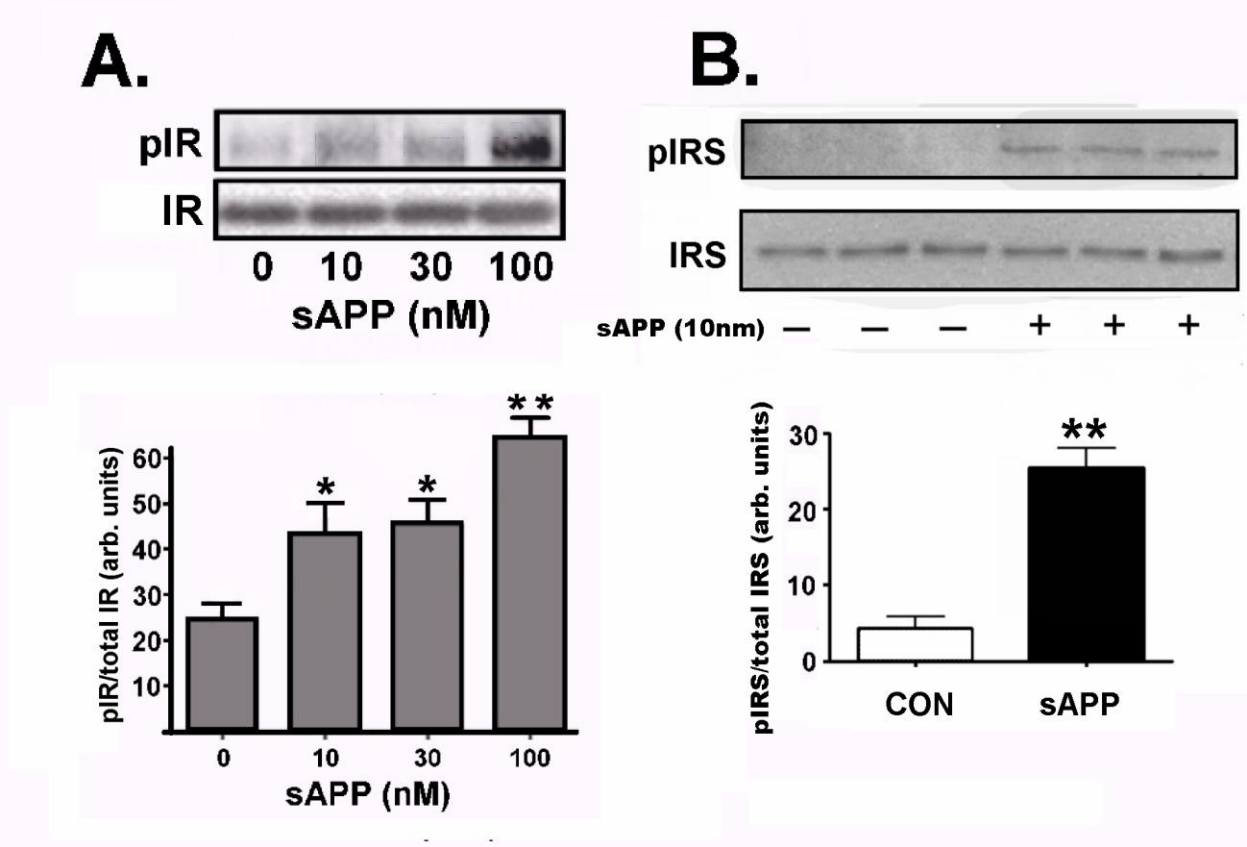
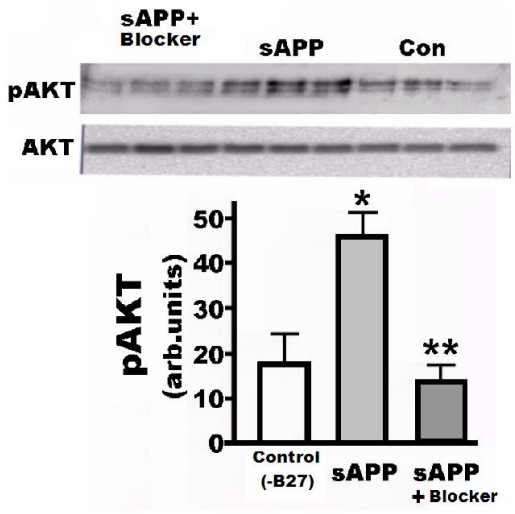


Figure 2: Insulin receptor inhibitor blocks sAPP-mediated AKT phosphorylation and neurite outgrowth. The blocker represents AG1024.

A.



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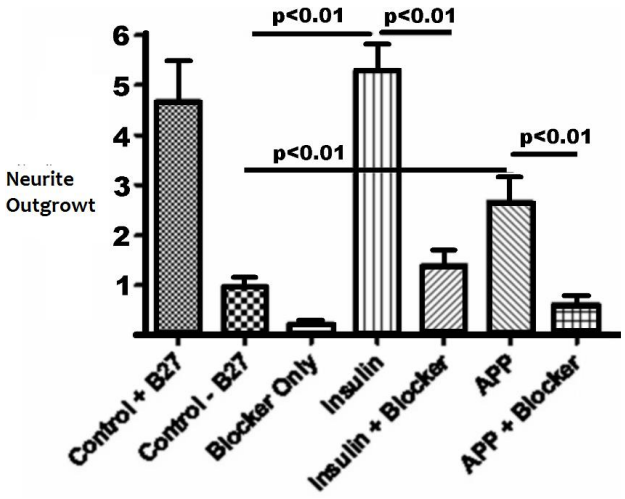

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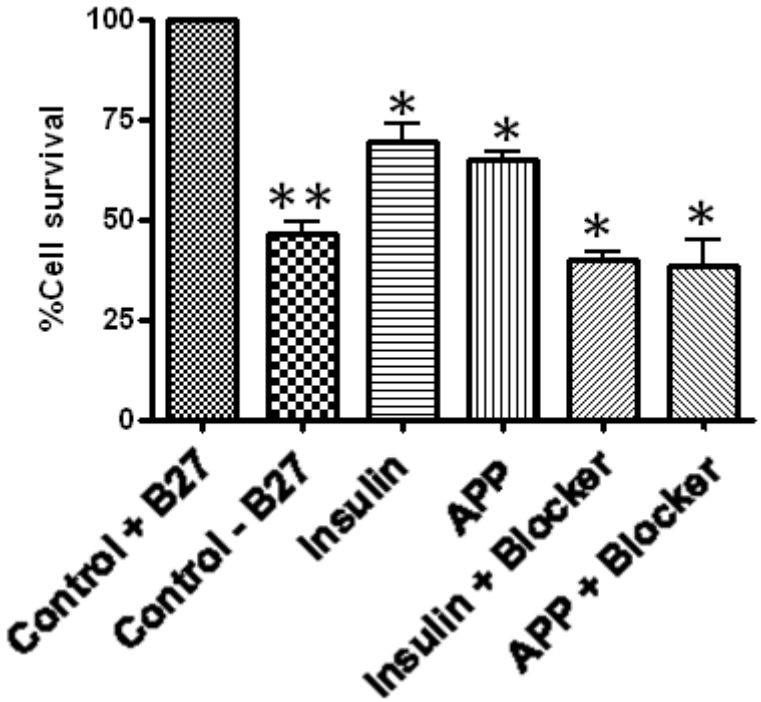

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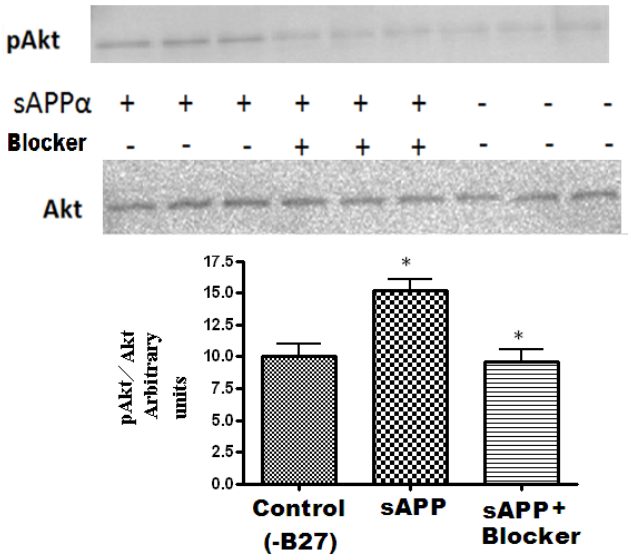

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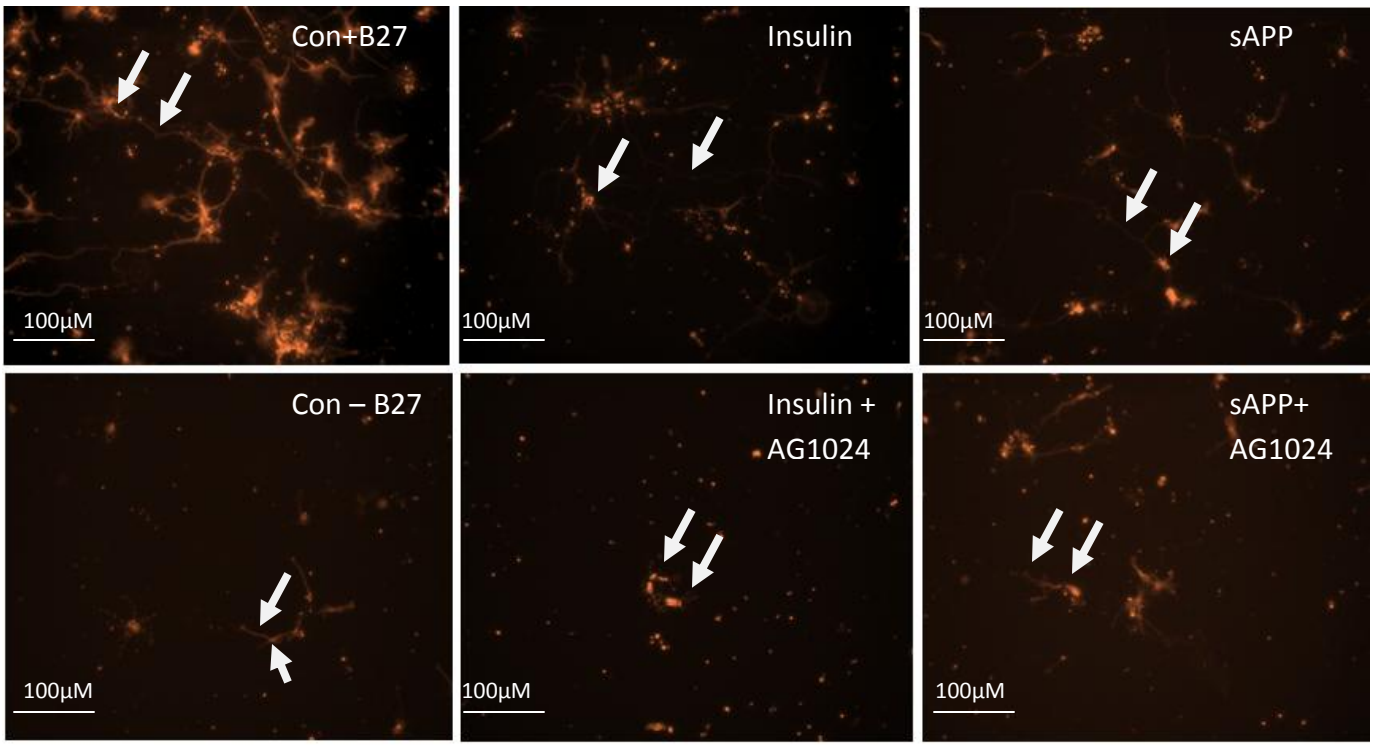
Con+B27
Insulin
sAPP
100µM
100µM
100µM
Con – B27
Insulin +
AG1024
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Figure 3: Insulin receptors are phosphorylated significantly more in diabetic APP-CRND8 than in diabetic WT brain.

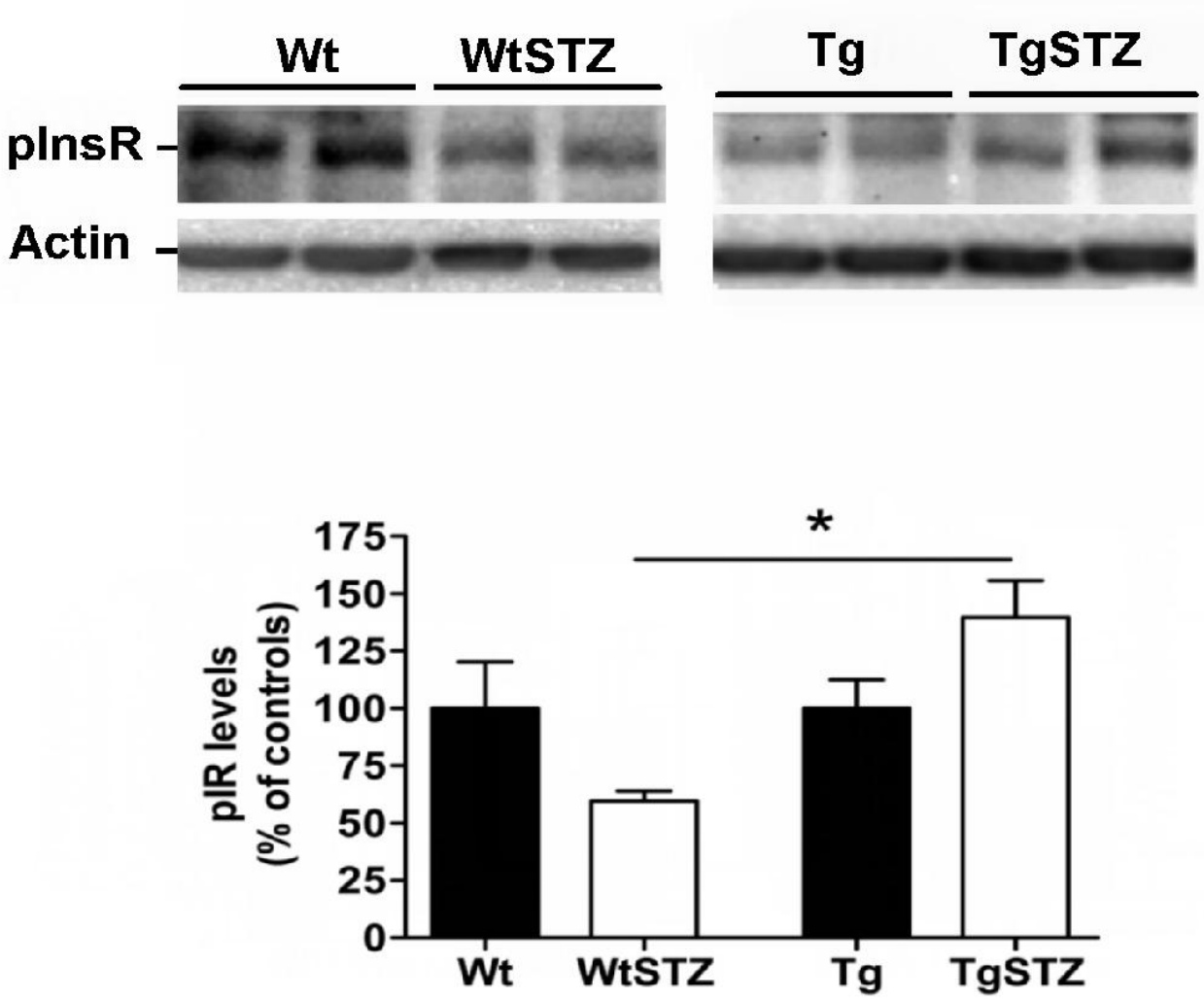


Figure 4: Physical interaction between sAPPα and insulin receptor is greater in insulin-depleted APP-CRND8 mouse brain.

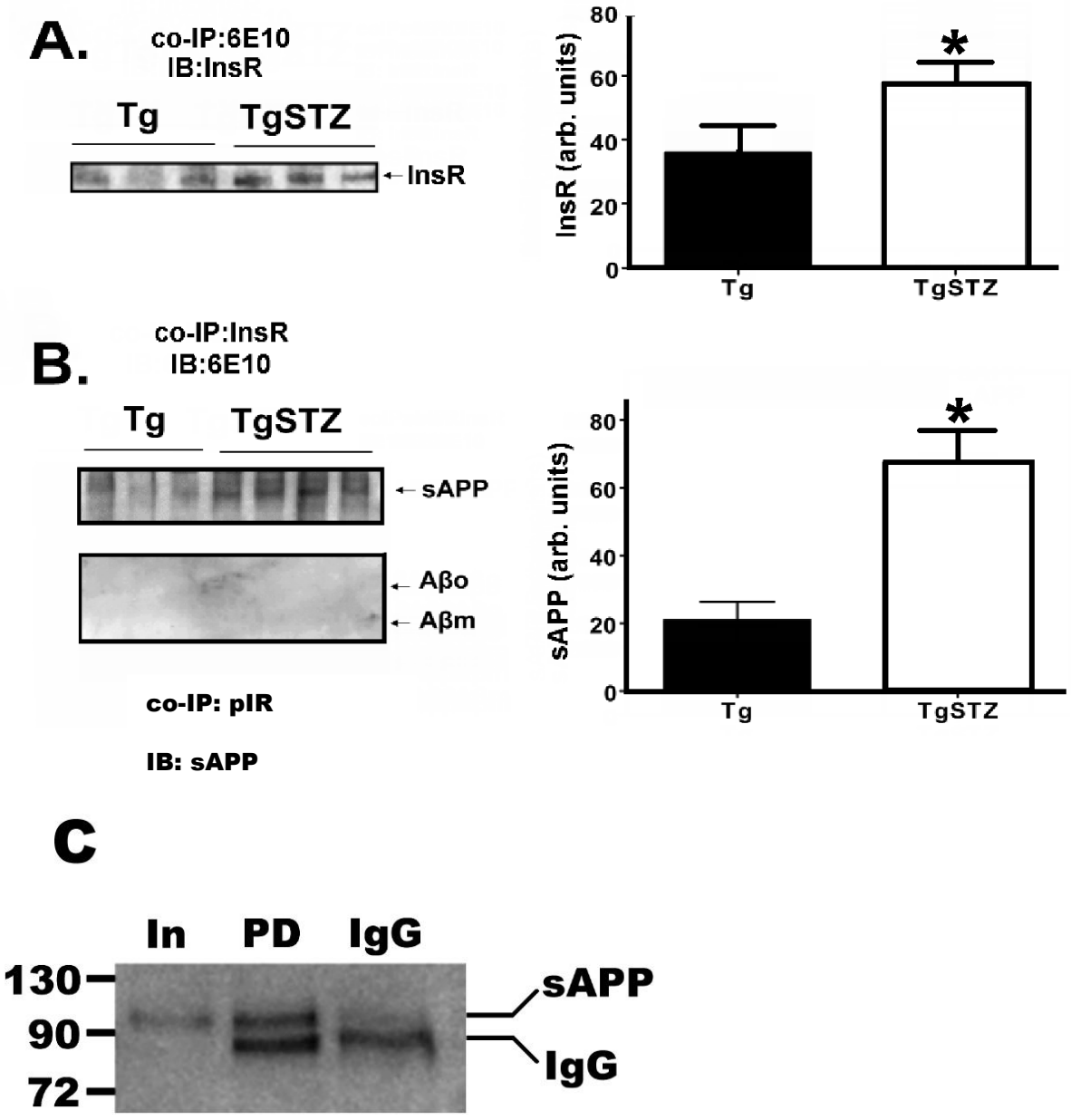


Figure 5: Gene expression is altered by STZ injection in both wild type and TgCRND8 mice

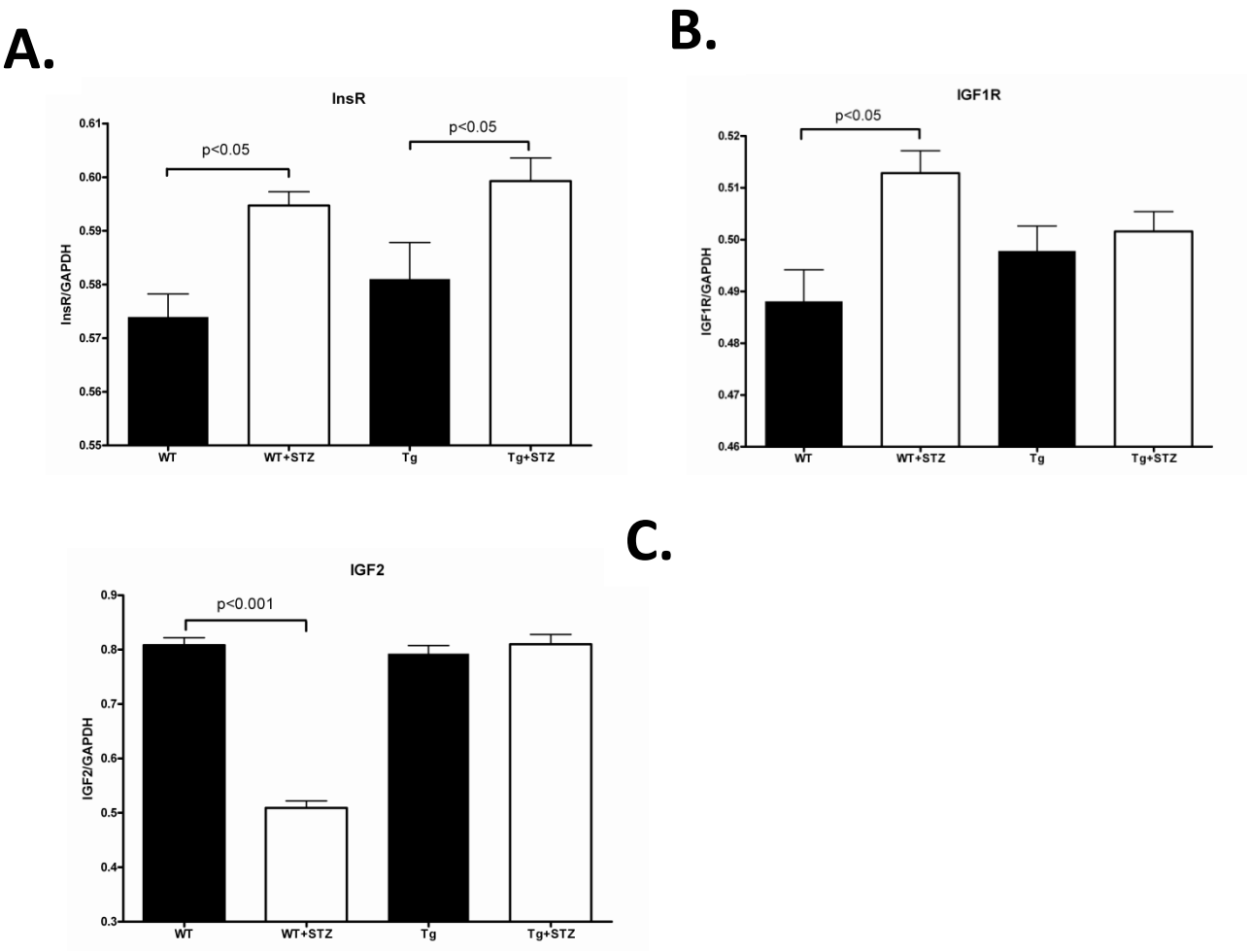


Table 1. **Primer Sequences and qRT-PCR Conditions**

Gene	Sequence (5' to 3')	Position (mRNA)	Size (bp)	T_m (°C)	Primer Conc. (uM)
Ins	TTC TAC ACA CCC AAG TCC CGT C(Forward)	145	135	62	2.0
	ATC CAC AAT GCC ACG CTT CTG C (Reverse)	279			
InsR	TGA CAA TGA GGA ATG TGG GGA C (Forward)	875	129		1.0
	GGG CAA ACT TTC TGA CAA TGA CTG (Reverse)	1003			
IGF1	GAC CAA GGG GCT TTT ACT TCA AC (Forward)	65	127		0.75
	TTT GTA GGC TTC AGC GGA GCA C (Reverse)	191			
IGF1R	GAA GTC TGC GGT GGT GAT AAA GG (Forward)	2138	113		0.75
	TCT GGG CAC AAA GAT GGA GTT G (Reverse)	2250			
IGF2	CCA AGA AGA AAG GAA GGG GAC C (Forward)	763	95		0.5
	GGC GGC TAT TGT TGT TCA CAG C (Reverse)	857			
IGF2R	TTG CTA TTG ACC TTA GTC CCT TGG (Forward)	1066	91		0.5
	AGA GTG AGA CCT TTG TGT CCC CAC (Reverse)	1156			

IGF2R	CTG GTT CCT ACC CCC AAT G \(Forward)	1066	91		0.5
	CTC AGA TGC CTG CTT CAC CAC CTT C (Reverse)	1156			
GAPDH	CGT GTT CCT ACC CCC AAT GTG TCC (Forward)	766	186	69.4	1.0
	GAA GGT GGT GAA GCA GGC ATC TGA G (Reverse)	843			

Table 2 – Blood glucose and brain weights were unchanged between injected animals.

	Blood glucose (mmol/L)	**Brain Weight (mg)**
Wt	13.1 +/- 0.54	449.7 +/- 6.8
Tg	12.3 +/- 0.67	431.6 +/- 3.5
WtSTZ	31.9 +/- 0.77	403.3 +/- 6.7
TgSTZ	30.8 +/- 1.24	407.5 +/- 9.5

Levels were in the diabetic range for all injected animals at tissue collection (>20 mmol/L). Based on blood glucose, both control groups were not considered to be diabetic. Whole brain weight was taken following euthanasia. (n=5-11/group)

Chapter II

Investigation into the physical interaction between sAPPα and insulin receptors in cultured embryonic cortical neurons

Introduction

Amyloid precursor protein cleavage products interact with the insulin receptor:

We have shown that secreted amyloid precursor protein alpha (sAPPα) can phosphorylate, and thus activate the insulin receptor and its downstream pathway. This activation caused an increase in both neuronal survival and axonal growth. Maintaining protein homoeostasis through the activation of the insulin receptor by sAPPα can be beneficial in a state of insulin resistance, since insulin resistance will shift homoeostasis to produce a more toxic form of amyloid beta. However, the question remains as to whether sAPPα is a competitive or non-competitive agonist at the IR. In this chapter we explore this question.

Both biochemical activation of the insulin receptor (by phosphorylation) by sAPPα and the physiological result seen on the neurons were inhibited by the use of the insulin receptor blocker AG1024. Since the AG1024 binds to the insulin binding site blocking the action of sAPPα, this could indicate that sAPPα may bind at the site of insulin on the insulin receptor (that is AG1024 is a competitive inhibitor). But if sAPPα binds at a different site than the insulin binding site, then, another explanation would be that upon the binding of the AG1024, the conformational changes in the insulin receptor prevents the binding of the sAPPα at whatever site that may be.

The idea that amyloid beta (and its sister proteins produced from the full length amyloid precursor protein) interacts with insulin receptor and its pathway has been suggested before (Arias et al., 2012). The insulin receptor is expressed in regions of the brain that are impacted by AD, like the hippocampus, so its signaling pathway activities can impact the progression of the

disease (Livingston et al., 1989; Luchsinger and Mayeux, 2004; de la Monte, 2005; Crafts, 2007). It has been shown that amyloid beta itself modulates the insulin receptor activities in the presynaptic nerve endings (Arias et al., 2012).

Some suggested that amyloid beta physically interacts with the insulin receptor (Klein et al., 2008; Martin et al., 2002), which explains the activity modification of the insulin receptor by amyloid beta, and also explains the significant effect of amyloid beta on the presynaptic terminal in locations with higher density of insulin receptor expression (Fallon et al., 1999). Intra neuronal amyloid beta prevents AKT activation (Querfrth et al., 2009). Others found a small exposure of amyloid beta leads to increased activities of both AKT and IRS-1 (Arias et al., 2012). However this increase in the phosphorylation levels of AKT is at the Ser473 position which is an inhibitory phosphorylation site contributing to insulin resistance (O'Neill et al., 2005), and thus the physiological implications are unknown.

Insulin degrading enzyme (IDE) degrades amyloid precursor protein cleavage products along with insulin:

Insulin degrading enzyme (IDE) is a zinc metallo peptidase that is another common link between amyloid beta and insulin, since it degrades both proteins. Its ability to interact with amyloid beta has captured the attention of many researchers looking into neurodegeneration caused by amyloidoses. Besides insulin and amyloid beta, IDE can degrade other proteins like amylin, a pancreatic islet amyliodosis that is associated with T2D *in vivo* (Guenette et al., 2003). A significant role of IDE shown in mice is its ability to degrade not only insulin and amyloid beta that is associated with AD but also the amyloid precursor protein intercellular C-terminal domain (AICD), which is the product of the gamma-secretase enzyme activity when processing

the full length APP, both in the amyloidogenic and the non-amyloidogenic pathway. This is significant because the AICD acts as a transcription factor by inducing further transcription of the full length APP. In the case of insulin resistance, then, the large amount of circulating insulin will compete with amyloid beta over the IDE allowing more amyloid beta to be available to become toxic and also prevents the breakdown of AICD, which allows for more full length APP to be produced and the cycle gets more vicious, since insulin resistance promotes the breakdown of the full length APP into the amyloidogenic pathway, and thus more amyloid beta will be produced (Guenette et al., 2003). This can explain why IDE knockout mice showed elevated levels of both amyloid beta and AICD in the brain (Guenette et al., 2003, Thiele et al., 2003). The levels of activity of IDE decrease in AD brain especially in the regions of the brain which contain elevated levels of amyloid beta (Castano et al., 2000; Castano et al., 2004). This has been explained by the fact that when oxidized, the level of activity of IDE decreases, and that oxidation occurs through the interaction of IDE with 4-HNE (4-hydroxynonenal a product of lipid peroxidation, which is found in animals in high concentrations during oxidative stress), an organic molecule present in the AD brain where there are high levels of amyloid beta (Hersh et al., 2005; LaFerla et al., 2005).

Amyloid beta is not only a substrate for IDE, but rather it remains bound to it in an irreversible matter (Castano et al., 2008). This interaction is so strong that low pH, SDS, and heat are unable to break it, and it is described as a non-disulfide covalent binding. Although amyloid beta interacts with many other enzymes, IDE is the only one that shows such interaction. There is a unique physiological function to this newly formed amyloid beta-IDE complex, which when formed is marked for degradation that ultimately prevents the formation of a toxic amyloid beta oligmer (Castano et al., 2008).

Insulin impacts the production of amyloid beta by accelerating the translocation of the full length amyloid precursor protein to the plasma membrane (Wang et al., 2001). Investigating the physical interaction between the amyloid beta family (and sAPPα) is critical in understanding this physiological relationship, for example, if amyloid beta promotes more amyloidogenic processes of the full length amyloid precursor protein, does sAPPα promote the non-amyloidogenic processes? Is the insulin receptor involved in these processes?

Our hypothesis is that sAPPα binds to the insulin receptor causing its activation. In this chapter, we will deal with the physical interaction between sAPPα and the insulin receptor. The technique that will be used is referred to as cross-linking immunoprecipitation. Unlike normal immunoprecipitation, cross linking provides a different interaction between proteins. It is an SDS-resistant covalent binding. Disuccinimidyl suberate (DSS) is used to perform the cosslinking process. DSS is a noncleavable bi-functional cross linking agent that contains an amine-reactive N-hydroxysuccinimide (NHS) ester at each end of the molecule (separated by 8 carbon atoms). This procedure is a reliable tool for showing physical interaction between proteins. Generally, if two proteins have physical interaction with one other, they can be cross-linked covalently forming a heavier protein complex.

This chapter revolves around the crosslink of sAPPα to the insulin receptor. Upon the addition of DSS, non-specific cross linking will occur, hence the amount of time to treat with DSS will only be 20 minutes in order to minimize the amount of non-specific cross linking. Furthermore, an IP will be performed to pull down the sAPPα/insulin receptor cross linked complex. This will show more evidence of the physical interaction between sAPPα and the insulin receptor.

Methods and Materials

Cross linking: Cortical tissue was isolated from day 16 embryonic CD1 mice. Cortices were triturated by fire-polished glass pipettes in HBSS buffer and seeded in 25 mm plastic dishes coated with poly-D-lysine (Sigma), at 1.5million neurons/well. Neurobasal media with B27 supplement (Invitrogen) and 10% fetal bovine serum (FBS; Hyclone) was used at time of plating. Media was changed to Neurobasal+B27 supplement without FBS on the following day. Three days post-plating, half the volume of each dish was replaced with fresh Neurobasal+B27 containting 1 μM cytosine arabinoside (500 nM final concentration), to limit astrocyte growth. On the seventh day the culture was starved my removing the B27 portion of the media by replacing it with neurobasal without B27, for 12h, and then treated with 10nM sAPPα and 10nM insulin for 15 minutes. 5mM DSS (Sigma) was added to some sAPPα and insulin treated cells for 20 minutes, while others had only the sAPPα or the insulin treatment only, which were used as control treatments.

Cells were then homogenized after treatment 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 4 ºC 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 of antibodies to insulin receptor, IgG control antibody (Santa Cruz Biotechnology) or the amyloid beta 1-16 sequence (6E10; Signet) overnight at 4°C. 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.

AG1024 insulin receptor blocker testing: Cortical tissue was isolated from day 16 embryonic CD1 mice. Cortices were triturated by fire-polished glass pipettes in HBSS buffer and seeded in 25 mm plastic dishes coated with poly-D-lysine (Sigma), at 800,000 million neurons/well. Neurobasal media with B27 supplement (Invitrogen) and 10% fetal bovine serum (FBS; Hyclone) was used at time of plating. Media was changed to Neurobasal+B27 supplement without FBS on the following day. Three days post-plating, half the volume of each dish was replaced with fresh Neurobasal+B27 containing 1 μM cytosine arabinoside (500 nM final concentration), to limit astrocyte growth. On the seventh day the culture was starved my removing the B27 portion of the media by replacing it with neurobasal without B27 for 10h, then was incubated with the selective AG1024 for 2h. After the 2 hour incubation, 10nM insulin or 10nM sAPPα was added to some wells for 15 minutes. Treatment was then terminated, protein concentration was determine via protein assay and was carried on for a western blotting using pIR antibody, or pIRS antibody.

Results

Physical interaction between sAPPα and InsR in cultured embryonic cortical neurons is inhibited by insulin. Rat embryonic cortical neurons were cultured for 7 days, and then exposed to vehicle, 10 nM sAPPα, 10 nM insulin, or co-treatment of 10nM of both insulin and sAPPα for 15 minutes. An IP was performed by using an anti- insulin receptor antibody (Santa Cruz), and then samples subjected to western blotting using anti sAPPα antibody to determine level of sAPP in each sample. Figure 1 shows that sAPPα treatment to cultures significantly increased the abundance of sAPPα bound to IR in comparison to the non- sAPPα treated group (i.e. the control), as hypothesized. However, co- treatment with insulin significantly reduced the level of sAPPα precipitated using anti-IR antibody, indicating the possibility that insulin and sAPPα may compete for the same binding site.

AG1024 Blocks sAPPα phosphorylation and activation of IRS in neuronal cell culture. Rat embryonic cortical neurons were cultured for 7 days, and then exposed to 10 nM sAPPα for 15 minutes, after two hour pre incubation with 1μM AG1024. A western blot was performed using pIRS antibody (abcam). Figure 2 shows upon the addition of the insulin receptor blocker there is a significant decrease in the level of IRS phosphorylation in comparison to the sAPPα only treated cells, indicating that sAPPα failed to activate the insulin receptor itself, since IRS is the first intracellular protein to be phosphorylated after the insulin receptor activation.

Cross-linking shows physical proximity between sAPP and IR in cultured cortical neurons. Neuronal cortical neurons were starved of B27 for 12 hours then treated with sAPPα. Some cultures were then treated with DSS for 20 minutes to induce crosslinking (Figure 3). Protein isolates were precipitated using an anti-sAPP antibody and analyzed by western blotting using and anti-IR antibody. The first three lanes show immunoprocepitation in the absence of

cross-linking while the next three are cross-linked. The appearance of three high-MW bands in the cross-linked samples are evidence that IR and sAPPα have been cross-linked in the short exposure to DSS, indicating very close physical proximity between the two proteins. In other cultures, insulin was added instead of sAPPα, and the proteins analyzed in the same manner. As shown in Figure 4, in those cultures in which insulin was added in place of sAPPα, the large MW bands in the cross-linked samples are changed from three bands to only two, indicating that the bands in the sAPP cross-linked samples are both specific and due to sAPPα interaction.

Cross-linking shows sAPP is part of the IR-IRS complex. We have presented evidence that sAPPα physically interacts with the IR, and that the physiological actions of sAPPα are dependent upon IR activation. These observations support a hypothesis that sAPPα binds directly to the IR and activates it. However, they are not direct evidence of this phenomenon. Upon binding of insulin or IGF to IR, the IRS protein physically binds to IR, forming an activated complex. Therefore, if sAPPα were binding to and activating IR, as opposed to simply being in proximity, DSS should cause cross-linking between IR and IRS due to the sAPPα activation (and being crossed linked to) of IR. In order to determine if sAPPα is in fact bound to the activated IR, the protein samples isolated in the experiment described above were also probed for IRS. Figure 5 demonstrates that when sAPPα is added to neuronal cultures and then exposed to DSS, there are unique and specific high MW bands that appear. These bands are not present in cultures treated with insulin nor in cultures not exposed to DSS. This demonstrates that sAPPα in neuronal cultures physically interacts with the activated IR-IRS complex.

No evidence that sAPP binds to IGF-R. Because there is often cross-binding of IR and IGF-R using commercial antibodies, the samples described above were probed using anti-IGF R. Figure 6 demonstrates there was no appearance of high-MW bands when the blot was probed for

anti-IGFR, which may suggest that sAPPα binds specifically to IR. However, since this blot showed no bands, and we had no positive control, this conclusion remains tentative, and further experiments are needed.

AG1024 blocks insulin and sAPPα from activating the insulin receptor. Figure 7 shows that an incubation with 1μM of AG1024 for 2hr showed a significant decrease in the insulin receptor phosphorylation level by insulin, and by sAPPα in comparison to the non-AG1024 incubated group. This indicates that AG1024 inhibits the insulin receptor since it blocks the action of insulin; the natural agonist for the insulin receptor. AG1024 also blocked the action of sAPPα from phosphorylating the insulin receptor in comparison to the sAPPα only treated group. The antibody used is insulin receptor specific and does not bind to the IGF receptor indicating that it is unlikely for the detection of a phosphorylated IGF receptor.

Error bars represent mean +/- SEM, One-way ANOVA was performed with a Tukey's post-hoc test. These results suggest that AG1024 can be used in these experiments as a reliable blocker for the insulin receptor.

Figure Legends

Figure 1 – *Physical interaction between sAPP and InsR and it is inhibited by insulin.*

Rat embryonic cortical neurons were cultured for 7 days, and then exposed to vehicle, 10 nM sAPP, 10 nM insulin, or co-treatment of 10nM sAPP and insulin for 15 minutes. Proteins were isolated and immunoprecipitated using anti-pIR antibody, then separated by Western blot and probed using anti-sAPP antibody. The amount of sAPP was quantified. (Mean +/- SEM, n=3 per group, $p<0.05$)

Figure 2–*AG1024, a competitive insulin receptor blocker blocks sAPPα phosphorylation and activation of IRS in neuronal culture cells.*

Rat embryonic cortical neurons were cultured for 7 days, starved for 12hrs by removing B27 and then treated with 10nM sAPP with or without 1μM AG1024, an insulin receptor antagonist. Levels of pIRS were determined by Western blotting, and values reported relative to total actin. Error bars represent mean +/- SEM, n=3. One-way ANOVA was performed with a Tukey's post-hoc test.

Figure 3– *sAPPα binds (cross linked) to the insulin receptor*

Rat embryonic cortical neurons were cultured for 7 days, starved for 12 hrs by removing B27, and then treated with 10nM sAPP for 15 min. In some cultures, 5mM DSS was added to cross-link proteins for 20 minutes. Proteins were isolated and immunoprecipitated using anti-sAPP antibody. The precipitate was then separated and analyzed by Western blotting for levels of pIR

Figure 4 – *High-MW crosslinked bands are different in insulin- vs sAPP-treated cultures*

Rat embryonic cortical neurons were cultured for 7 days, starved for 12 hrs by removing B27, and then treated with vehicle, 10nM insulin, or 10nM sAPP for 15 min. In some cultures, 5mM DSS was added to cross-link proteins for 20 minutes. Proteins were isolated and immunoprecipitated using anti-sAPP antibody. The precipitate was then separated and analyzed by Western blotting for levels of pIR

Figure 5 – *sAPP is part of the activated IR-IRS complex*

Rat embryonic cortical neurons were cultured for 7 days, starved for 12 hrs by removing B27, and then treated with vehicle, 10nM insulin, or 10nM sAPP for 15 min. In some cultures, 5mM DSS was added to cross-link proteins for 20 minutes. Proteins were isolated and immunoprecipitated using anti-sAPP antibody. The precipitate was then separated and analyzed by Western blotting for levels of pIRS

Figure 6 – *No evidence that sAPP binds IGF-R*

Rat embryonic cortical neurons were cultured for 7 days, starved for 12 hrs by removing B27, and then treated with vehicle, 10nM insulin, or 10nM sAPP for 15 min. In some cultures, DSS was added to cross-link proteins for 20 minutes. Proteins were isolated and immunoprecipitated using anti-sAPP antibody. The precipitate was then separated and analyzed by Western blotting for levels of IGF-R.

Figure 7 – *AG1024 blocks insulin and sAPPα from activating the insulin receptor*

Rat embryonic cortical neurons were cultured for 7 days, starved for 12 hrs by removing B27 and then treated with 10nM insulin or 10nM sAPPα with or without 1μM AG1024, an insulin receptor antagonist. Levels of pIR were determined by Western blotting through the use of an anti-pIR antibody, and values reported relative to total IR. Error bars represent mean +/- SEM, n=3. One-way ANOVA was performed with a Tukey's post-hoc test.

Figure 1: Physical interaction between sAPP and InsR is inhibited by insulin addition.

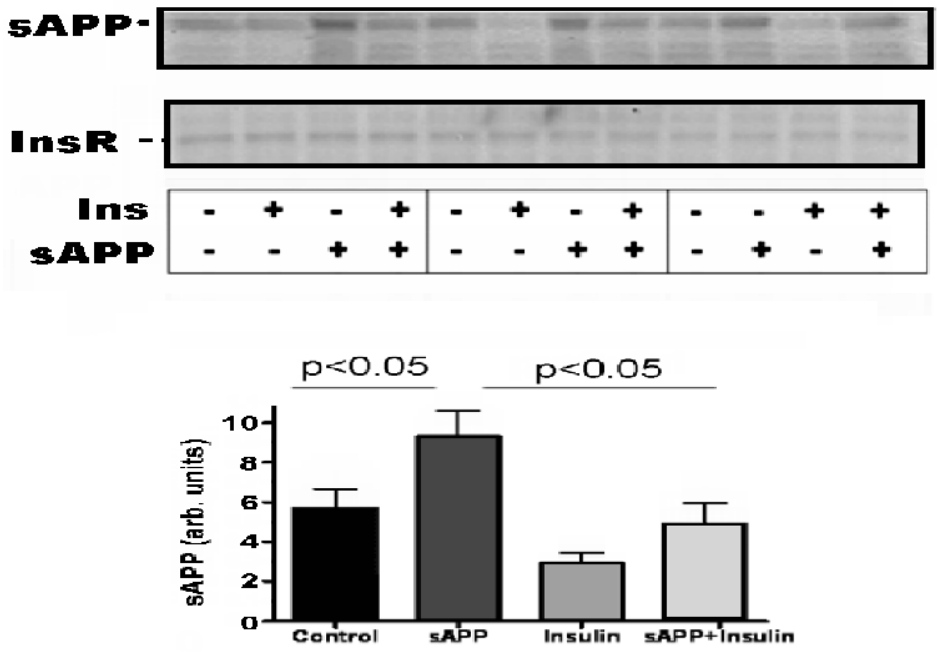


Figure 2: AG1024, a competitive insulin receptor blocker blocks sAPPα phosphorylation and activation of IRS in neuronal culture cells.

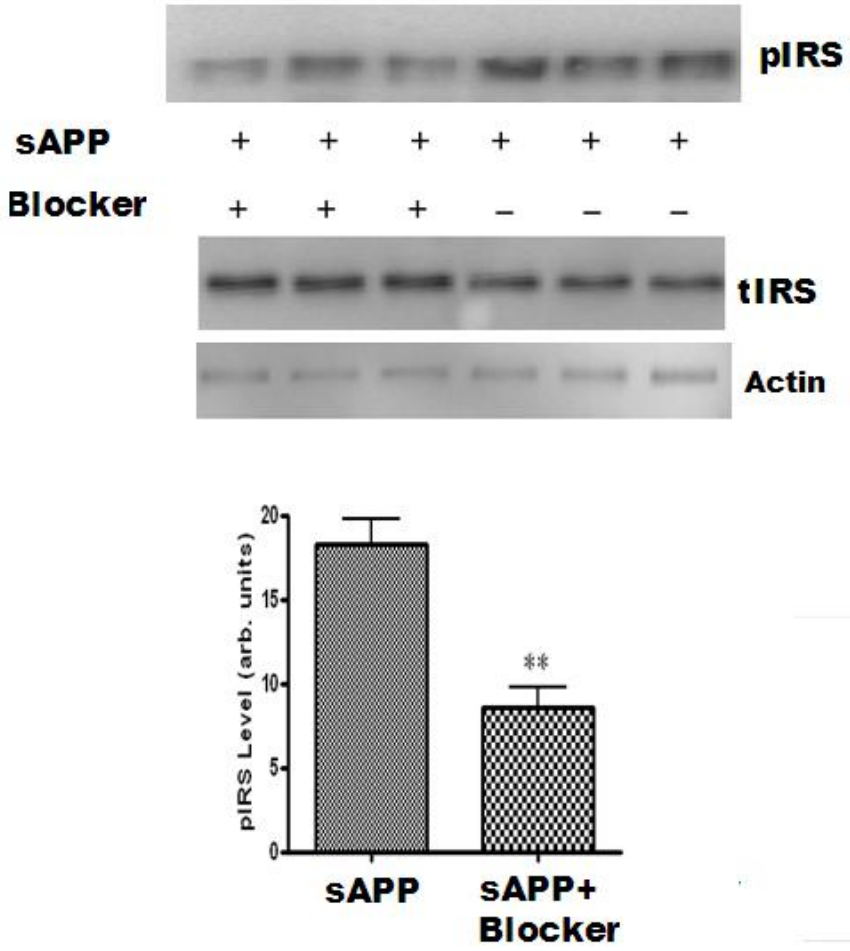


Figure 3: sAPPα binds (cross linked) to the insulin receptor

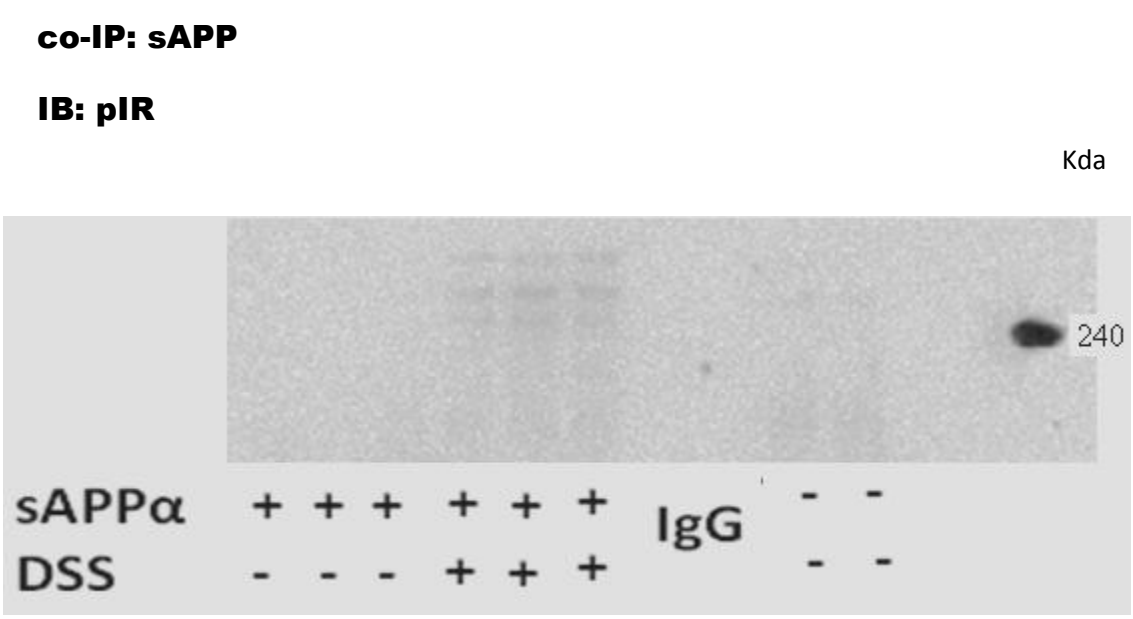


Figure 4: High-MW crosslinked bands are different in insulin- vs sAPP-treated cultures

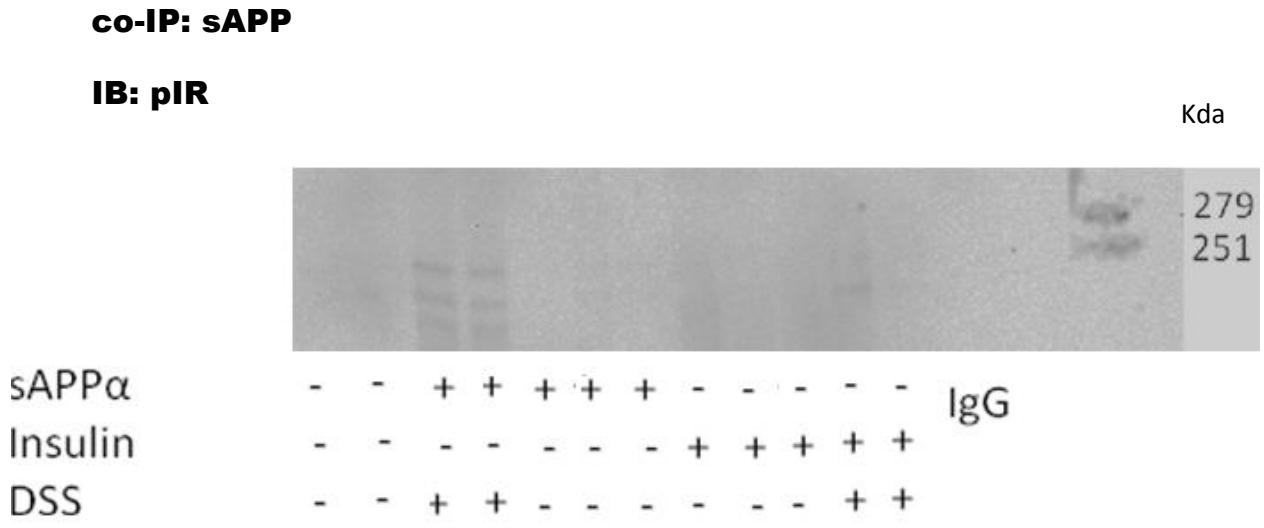


Figure 5: sAPP is part of the activated IR-IRS complex

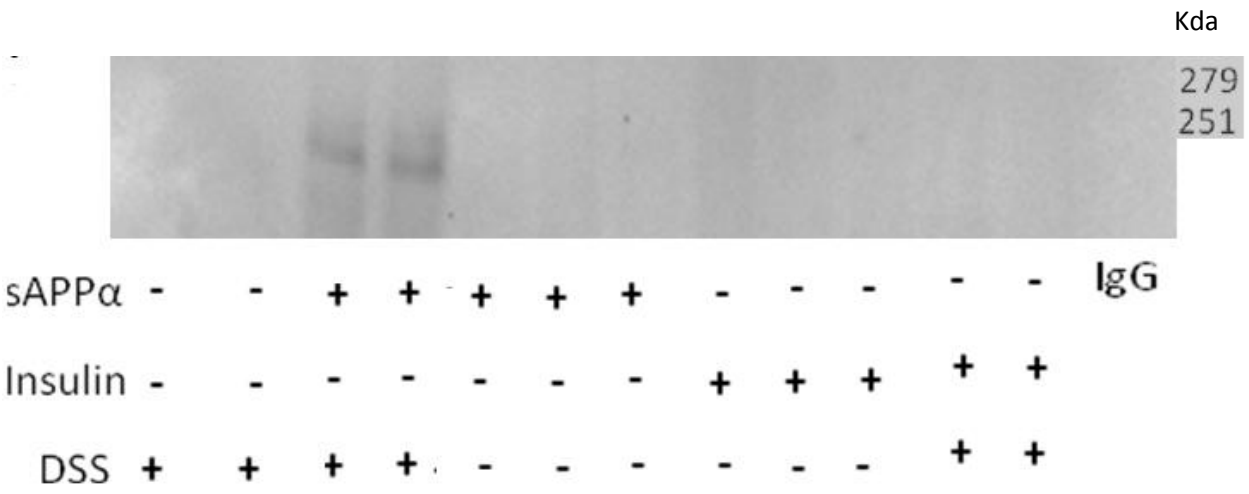


Figure 6: No evidence that sAPP binds IGF-R

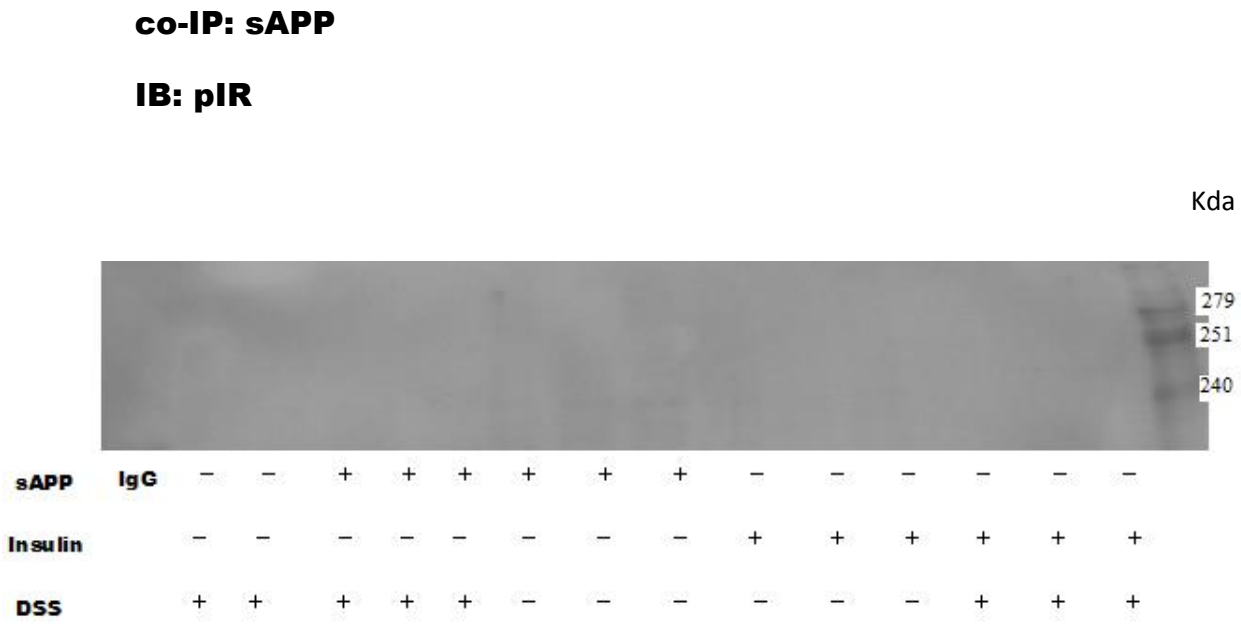
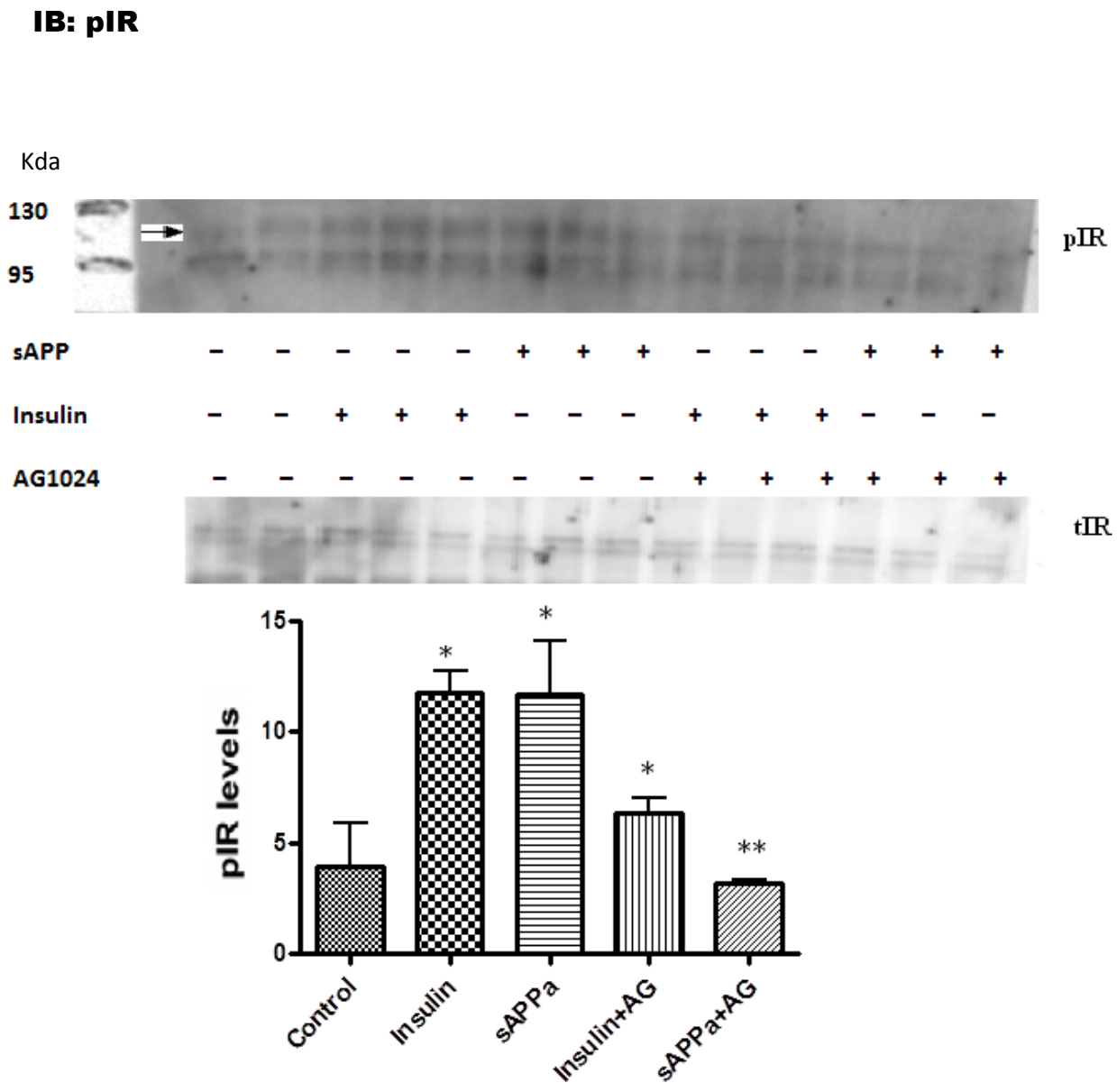


Figure7: AG1024 blocks insulin and sAPPα from activating the insulin receptor



General Discussion

The insulin receptor pathway is activated by sAPPα according to our data. This activation raises the question whether sAPPα itself binds and activates the insulin receptor or whether sAPPα increases the insulin receptor sensitivity to insulin. According to our data, sAPPα activates the IRS molecule. IRS is the first molecule phosphorylated and activated after the activation of the insulin receptor, which is another indication of the activation of the insulin receptor itself.

Figure 1 shows a pull down using a pIR antibody after a co-treatment of both insulin and sAPPα. The Figure shows that upon the addition of sAPPα and insulin together, there is a reduction in the amount of sAPPα binding to the insulin receptor, which may indicate that both sAPPα and insulin both compete for the binding site, this explanation suggest a competitive agonistic behavior of sAPPα to the insulin receptor. This conclusion is not exclusive, since more experiments may be required to further verify this relation. Insulin binding to the insulin receptor causes internalization of the insulin receptor, which can explain the fact that the reason the sAPPα levels are decreased upon insulin addition is simply due to the decrease number of expression insulin receptor as a result of internalization after insulin binding. sAPPα may also work in a non-competitive matter, or increase insulin receptor sensitivity to insulin by binding to another site than the insulin receptor site. One way to test the binding pattern is by performing kinetic studies. The insulin receptor can be isolated from the cell membrane and treated with different concentrations of insulin and sAPPα, and measuring concentration change over time for both substrates. A graphic representation can provide the values of Km, and Vmax which determine the type of inhibition that insulin has over sAPPα if any.

We have shown that a 10nM sAPPα treatment can activate the IRS molecule following the activation of the neuronal insulin receptor (fig 2, chapter 1). The blockage of the insulin

receptor by AG1024 prevented the phosphorylation of IRS which indicates that the insulin receptor itself was blocked and not activated. The control for this experiment was the sAPPα without the AG1024. A control with no treatment of either sAPPα or AG1024 and a control with only AG1024 treatment would make a better overall presentation of the experiment, but Figure 1B in chapter one showed that sAPPα significantly increased the levels of IRS phosphorylation in comparison to the vehicle, which makes the argument for Figure 2 still standing.

The addition of AG1024 not only inhibited the IRS molecule but also increased its expression in comparison to the sAPPα treated cells. Jena J. Steinle et al showed that silencing the IRS expression in retinal Müller cells increases cell death (Steinle et al., 2012), which might occur in neuronal cells, since AG1024 is a blocker to a receptor that is critical in neuronal survival and insulin is a neuronal growth factor, it may be expected for the neuron to elevate IRS expression to resist apoptosis and stress, but further investigation is required.

AG1024 is a synthetic tyrosine kinase inhibitor belonging to a family of molecules called tyrphostins, which inhibit receptor autophosphorylations (Levitzki and Gazit, 1995). They resemble the phenolic group of tyrosine due to their derivation from benzylidene malononitril nucleus. This family of inhibitors has the same backbone structure with different substituents that give each member slightly different inhibitory characteristics (Marcelina et al., 1997). Some tyrphostins against neuronal growth factors show their ability to block PLC-γ phosphorylation, PI3K activities, as well as neurite outgrowth (Ohmichi et al., 1993), while other tyrphostins prevent DNA synthesis in porcine aorta endothelial cells by blocking platelet derived growth factor receptors (Kovalenko et al., 1994). Tyrphostins also block epidermal growth factor ability to induce cellular proliferation (Osherov et al., 1993).

The effect of this class of inhibitors on cellular proliferation is specific to the IGF and the insulin receptor pathway inhibition (Marcelina et al., 1997). Most of these inhibitors show no specificity for IGF over the insulin receptor except AG1024 and AG1034. AG1024 inhibits the insulin receptor (Marcelina et al., 1997).

The AG1024 is a selective inhibitor for tyrosine kinase receptors like the insulin receptor and the IGFR family, with high affinity for these kinds of receptors (Yao et al., 2011). Figure 7 shows that AG1024 inhibited the insulin activation of the insulin receptor; this experiment was performed in order to confirm the validity of this blocker. Although this experiment is missing a second control which is vehicle with AG1024 alone, which tells us if the blocker itself is causing other effects that contribute to the decrease of the phosphorylation level of the insulin receptor, Figure 2, B in chapter one shows that AG1024 treatment by itself did not cause a significant decrease in cell death, which could indicate that by itself, AG1024 is unable to cause significant decrease in the insulin receptor phosphorylation level by other effects like causing cell death, for example. AG1024 can also block IGF receptor (Marcelina et al., 1997). If sAPPα binds to the IGF receptor, similar physiological responses will be observed, including the phosphorylation of IRS and Akt. The antibody used to test the pIR site and for IP however is specific to insulin receptor alone and not to IGF receptor, indicating that the decrease seen in the phosphorylation levels and neuronal out growth when AG1024 was added may be due to the blockage of the actions of sAPPα on the insulin receptor rather than the IGF receptor.

AG1024 did block the action of sAPPα which indicates that by doing so, it blocks the insulin binding site of the insulin receptor, suggesting that sAPPα action requires the insulin binding site on the insulin receptor.

In order to determine a physical interaction between sAPPα and the insulin receptor without the sample denaturing and molecular dissociation that occurs during the coarse of a standard IP when proteins are exposed to DSS, we performed a molecular cross link of the covalent bonds binding proteins to one another. The addition of the cross linking agent Disuccinimidyl Suberate (DSS) to the culture will cause covalent bonds among many proteins, proteins and receptors, two receptors and even transcription factors, which is why DSS exposure was only 20 minutes, to ensure minor crosslinking had occurred, followed by an IP.

When bound and cross linked, unlike a normal IP, the sAPPα and the insulin receptor become one much larger molecule that remains bound together when subjected to the SDS in WB. The combination molecule appears at a much larger molecular weight marker position than either the insulin receptor or sAPPα alone. The results from that experiment showed three bands appearing close to one (a triplet) another rather than one band. The experiment was repeated with the addition of more controls, in particular insulin treatment groups as shown in Figure 4. Figure 4 shows differences between the sAPPα treated cells and the insulin treated cells, both Figure 3 and Figure 4 show that triplet appearance were consistent when DSS is added and they are absent in the DSS untreated group, suggesting that the triplet is a product of crosslinking. The insulin treated cells only showed two bands.

When sAPPα binds to the insulin receptor it causes insulin receptor phosphorylation and activation driving further proteins to be associated with the insulin receptor which also get phosphorylated. When added, DSS might have crossed linked more than just the sAPPα and the insulin receptor. There can be many combinations, including IRS, another insulin receptor, or even an IGF receptor. Through the use of various desired antibodies, we have identified that these bands are sAPPα, insulin and IRS molecule although further investigation is necessary.

When the IRS antibody was used to examine these bands, a single band in the sAPPα sample appeared which is missing from the insulin treated and crossed linked samples, which may indicate that IRS is attached to the insulin receptor when sAPPα binds to the insulin receptor, and since the activation of the insulin receptor causes IRS to be the first intracellular protein in the insulin receptor pathway to be activated, this explanation is logical.

Another possibility is an IGF receptor dimerized with the insulin receptor, which as shown in Figure 6, both groups, the insulin treated and the sAPPα treated groups, IGF was not dimerized with the insulin receptor in this case. Further investigation may be required since this experiment lacked a positive IGFR cross linked control.

This data may suggest that in the treatment of sAPPα, both the insulin receptor and IRS are bound together, since sAPPα activates the insulin receptor, which in turn activates IRS. The addition of DSS, therefore, would link the insulin receptor with its ligand and any proteins, such as IRS, that are in contact with the insulin receptor during activation of insulin signaling.

This thesis has addressed the global hypothesis (as well as addressed all the specific aims) which is that sAPPα binds to and activates the insulin receptor. The data presented shine the light on the possibility that the neuronal insulin receptor has another agonist, and it may be a partial agonist, but it is nonetheless, an effective one when it comes to the health of the neurons and their plasticity. The data presented in this thesis involved many different techniques, which showed the effect of sAPPα not only on the insulin receptor itself but also on the overall neuronal growth and survival through the insulin receptor. Some of the data did lack some controls that could have made the argument stronger, which is one weakness of this work. Further directions for this work is further investigating the *in vivo* effect of sAPPα on neuronal health through the insulin receptor. One approach can be established through generating stem

cell that over express that sAPPα and investigate the effect of sAPPα on slowing down neurodegeneration and restore the overall neuronal health and survival. Another thing that could make the data argument stronger is using another insulin receptor blocker along with AG1024, like S961 to further show the specificity studies. For years, it has been suggested that there has always been a connection between the neuronal insulin receptor and the amyloid precursor protein family, this work has provided evidence of sAPPα does in fact interact with the insulin receptor, which can be the receptor for sAPPα, since there is no dedicated receptor for it yet.

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Conclusion

With the rise of Alzheimer's disease (AD) and the limited ways to treat it, understanding insulin receptor signaling abnormalities and insulin resistance in the brain during AD may be critical in understanding the development and progression of AD.

An agonist for the insulin receptor during this insulin resistance state may be beneficial in slowing down the progression of AD, restoration of memory and promotion of neuronal survival and growth. Our data demonstrates that secreted amyloid precursor protein alpha (sAPPα), a product of alpha secretease enzyme cleavage of full length amyloid precursor protein (flAPP), is a natural agonist of the neuronal insulin receptor, with less efficacy than insulin itself. Additionally, our data indicates that sAPPα increases cell survival and axonal growth through the biochemical activation of the insulin receptor and its downstream pathway, and it competes with insulin over the insulin binding site.

One of the consequences of developing T2D is the increase risk of AD development, and since insulin receptor signaling abnormalities are common link between both disorders, sAPPα can be used in the case of insulin resistance during T2D, as a preventative against AD by enhancing neuronal activities and lengthens their survival, or even slows the progression of AD.

This work brings new evidence and understanding of how sAPPα affect neuronal cells, and can be applied as new pharmacological approach to disorders that involve insulin resistance and insulin signaling abnormalities like T2D and AD.

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