INSULIN, CHOLESTEROL AND Aβ PRODUCTION:
Roles and Mechanisms in Alzheimer's disease

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Abstract

Alzheimer's disease (AD) is characterized by amyloid-β (Aβ) and tau pathologies, insulin resistance, neuro-inflammation and dysregulation of cholesterol homeostasis, all of which play a role in neuro-degeneration. The main aim of this study was to determine possible relationships between insulin signaling, cholesterol biosynthesis and their effects on Aβ, and inflammatory response *in vitro.* Insulin treatment increased cholesterol synthesis in human Neuroblastoma SH-SY5Y (SHY) and mouse neuroblastoma 2a (N2a) and N2a transfected with human APP (N2a-APP) by up-regulating biosynthesis enzymes including 24-dehydrocholesterol reductase (DHCR24) and 3-hydroxy-3methyl-glutaryl-CoA reductase (HMGCR) through sterol regulatory element binding protein-2 (SREBP2) up-regulation. Aβ caused insulin resistance in N2a-APP cells by phosphorylating IRS-1 at Ser612, inhibiting signaling to downstream targets. Aβ1-42-treated SHY exhibited similar IRS-1 phosphorylation at Ser612 and inflammatory response of JNK activation. Aβ1-42 caused down-regulation of neuro-protective/anti-inflammatory DHCR24, and an increase in HMGCR levels indicating dysregulation of cholesterol homeostasis in SHY cells. Insulin resistance, Aβ toxicity, neuro-inflammation and dysregulation of cholesterol homeostasis appear to be intertwined processes in AD that should be studied simultaneously.

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List of abbreviations

24-S-OH-Chol	(24S)-24-hydroxycholesterol
27-OH-Chol	27-hydroxycholesterol
ACAT	Acyl-CoA cholesterol acyltransferase
AD	Alzheimer's disease
AICD	APP intracellular domain
AMPA	α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
APOE	Apolipoprotein E
APOJ	Apolipoprotein J
APP	Amyloid precursor protein
APP23	Swedish mutation of APP
Aβ	Amyloid beta
BACE1	Beta-site APP cleaving enzyme 1
BBB	Blood-brain barrier
BSA	Bovine serum albumin
C83/99	Carboxy-terminal fragment
CEs	Cholesterol esters
CLU	Clusterin
CNS	Central nervous system
CSF	Cerebrospinal fluid
DHCR24	24-dehydrocholesterol reductase
DHCR7	7-dehydrocholesterol reductase
DMEM	Dulbecco's modified Eagle's medium
DMSO	Dimethyl sulfoxide
DS	Down's syndrome
ELISA	Enzyme-linked immunoSorbent Assay
EMEM	Eagle's minimum essential medium
EOAD	Early-onset AD
ER	Endoplasmic reticulum
ERK	Extracellular signal-regulated kinases

FBS	Fetal bovine serum
FPP	Isoprenoid farnesyl pyrophosphate
GGAs	Golgi-localized gamma-ear containing ADP-ribosylation factor binding proteins
GGPP	Geranylgeranly pyrophosphate
GLP-1R	Glucagon-like peptide 1 receptor
GSK-3	Glycogen synthase kinase-3
GTPases	Guanosine triphosphate-ases
GWAS	Genome wide association studies
hAPP	Human APP
HBSS	Hank's balanced salt solution
HDL	High density lipoproteins
HMGCR	3-hydroxy-3-methyl-glutaryl-CoA reductase
IDE	Insulin degrading enzymes
IGF	Insulin growth factor
IKK	Inhibitor KappaB kinase
IL-1β	Interleukin 1
INSIG	Insulin induced genes
IRs	Insulin receptors
IRS	Insulin receptor substrate
JNK	c-Jun N-terminal kinase
LDLs	Low-density liporproteins
LOAD	Late-onset AD
LRP	Lipoprotein receptor related protein
LTP	Long-term potentiation
LXR-β	Liver X receptor beta
MAPK	Mitogen-activated protein kinase
MCI	Mild cognitive impairment
mTOR	Mammalian target of rapamycin
N2a	Mouse neuroblastoma 2a
N2a-APP	Stably transfected N2a with hAPP695

NMDA	N-methyl-D-aspartate
NTFs	Neurofribrillary tangles
PI	Protein inhibitor
PI3K	Phosphatidylinositol 3-kinases
PKC	Protein kinase C
PPAR	Peroxisome proliferator-activated receptor
PSD	Postsynaptic density
PSEN1	Presenilin 1
PSEN2	Presenilin 2
QPCR	Quantitative polymerase chain reaction
RAGE	Receptor for advanced glycation end products
RXR	Retinoid receptor
sAPPα/β	Soluble APPα/β
SCAP	Sterol cleavage protein
Seladin 1	Selective AD indicator 1
SNPs	Single nucleotide polymorphisms
SPs	Senile plaques
SQLE	Squalene monooxygenase
SRE	Sterol regulatory element
SREBPs	Sterol regulatory element bin ding proteins
T2D	Type 2 diabetes
TBST	Tris-buffered saline Tween
TCA	Tricholoacetic acid
TNFα	Tumor necrosis factor alpha
TR	Thyroid-hormone receptor

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Introduction

Alzheimer's disease (AD) is a progressive age-related neurodegenerative disorder of the brain. It is the most common form of dementia that is incurable and terminal (Akiyama, Barger et al. 2000). The prevalence of the disorder is increasing at an alarming rate afflicting ~30 million people worldwide and is expected to quadruple by 2050 (Brookmeyer, Johnson et al. 2007). Dementia, and specifically AD, are among the most costly diseases for societies where in the US alone, total direct costs were estimated at $183 billion annually (Stefanacci 2011). Thus, AD is referred to as the pandemic of the 21st century with rising costs due to aging populations (Holtzman 2010). The disease course has progressive patterns of cognitive and functional decline where the mean life expectancy after diagnosis is approximately seven years (Zanetti, Solerte et al. 2009). The symptoms of AD include the impairment of memory, visuospatial skills, complex cognition, language, emotion and personality, as well as the progressive global deterioration in adaptive functioning ultimately leading to death (Iqbal, Flory et al. 2013).

Currently, there are no effective AD diagnostics due to the poorly understood etiology of the disease and the similarity to other dementias. Also, the exact cause of AD is unknown so that the available treatments remain palliative in nature (Ridge, Ebbert et al. 2013). There are two types of AD, Early-onset AD (EOAD) and Late-onset AD (LOAD). EOAD, or familial AD, constitutes ~7% of total AD cases; it begins at an earlier age and is believed to be inherited due to known mutations in three different genes encoding amyloid precursor

protein (APP), presenilin I (PSEN1) or presenilin 2 (PSEN2) (Ridge, Ebbert et al. 2013). LOAD, or sporadic AD, usually occurs after the age of 60 and its prevalence rises exponentially with age, such that ~40% of people over the age of 85 develop the disorder worldwide (Hebert, Scherr et al. 2003; Brookmeyer, Johnson et al. 2007). Numerous biomarkers and genetic risk factors have been identified for LOAD, but no causative gene has been identified (Ridge, Ebbert et al. 2013).

The neuropathological characteristics of AD include the presence of senile plaques (SPs), intracellular neurofibrillary tangles (NFTs), decreased glucose utilization, increased oxidative stress and neuro-inflammation leading to loss of synapses and neurons (Selkoe 2001). The NFTs are intraneuronal structures composed of hyperphosphorylated tau protein, which was shown to follow amyloid beta (Aβ) pathology, while the SPs are comprised of aggregated Aβ 40/42 peptides derived by proteolysis from APP (Gouras, Tsai et al. 2000; Soriano, Lu et al. 2001).

Recently, two other important features of AD pathology have been recognized; defective brain insulin signaling (De Felice 2013), and dysregulated cholesterol homeostasis (Xiong, Callaghan et al. 2008). Several biomarkers of peripheral insulin resistance are increased in the hippocampus of non-diabetic AD patients (Talbot, Wang et al. 2012). In addition, lower levels of insulin, insulin growth factor (IGF), and insulin receptors (IRs) are observed in AD brains (Hoyer and Nitsch 1989; Steen, Terry et al. 2005; Craft 2012). In animal models of AD, brain insulin signaling impairments are also described (Takeda, Sato et al. 2010;

Bomfim, Forny-Germano et al. 2012). Molecularly, Aβ oligomers bind to neurons and trigger the removal of IRs from the plasma membrane (Zhao, De Felice et al. 2008; De Felice, Vieira et al. 2009). Moreover, several key nuclear receptors involving cholesterol metabolism are significantly altered in AD brain, including decreased Liver X receptor beta (LXR-β), peroxisome proliferator-activated receptor (PPAR) and thyroid-hormone receptor (TR), and increased retinoid X receptor (RXR) (Xiong, Callaghan et al. 2008). Animal studies show that rabbits fed with high cholesterol diet develop hypercholesterolemia inducing Aβ accumulation in the brain (Sharma, Prasanthi et al. 2008). Aβ itself blocks cholesterol trafficking and changes cholesterol homeostasis by preventing it from binding to low-density lipoproteins (LDLs) leading to decreased cholesterol influx and intracellular cholesterol levels (Yao and Papadopoulos 2002).

It is evident that AD is a multi-factorial disease, and understanding the molecular relationships between the key pathological hallmarks is crucial for finding new therapeutic targets as well as new advances in medical care for AD patients.

1.1 APP and Aβ in AD

APP is a conserved, integral membrane protein that is expressed in both peripheral cells and brain cells such as astrocytes, microglia, endothelial cells and particularly neurons. APP695 is the dominant form in neurons, whereas other cell types express APP751/771 isoforms predominantly (Annaert and De Strooper 2002). APP has been proposed to function as a metalloprotein to bind metal ions such as iron, copper and zinc (Kong, Adams et al. 2007; Dahms,

Hoefgen et al. 2010). It has also been implicated in proliferation, motility (Gralle and Ferreira 2007), axonal vesicular trafficking, cell adhesion, cholesterol metabolism, gene transcription (Turner, O'Connor et al. 2003) and in developmental signaling pathways (Ridge, Ebbert et al. 2013). However, its exact function remains elusive (Ridge, Ebbert et al. 2013).

The protein has a large N-terminal extracellular domain which constitutes the soluble APPα/β (sAPPα/β) domain, followed by a transmembrane region that contains the Aβ peptide sequence and a short C-terminal cytoplasmic tail domain called the APP intracellular domain (AICD) (Reinhard, Hebert et al. 2005) (Figure 1a). APP exists as a monomeric protein as well as dimers, and higher oliogmers structures (Dahms, Hoefgen et al. 2010).

APP can go under proteolysis in two processing pathways in all cell types: the amyloidogenic pathway and the nonamyloidogenic pathway (Haass, Kaether et al. 2012). In the nonamyloidogenic pathway, APP is first cleaved by α-secretase in the Aβ domain, preventing the generation and release of the Aβ peptide. Two fragments are released, the neuroprotective sAPPα fragment and a small carboxy-terminal fragment (C83) where C83 can be cleaved by γ-secretase to generate P3 (Kojro, Gimpl et al. 2001; Pearson and Peers 2006) (Figure 1b). In the amyloidogenic pathway APP is cleaved by β-secretase also known as β-site APP-cleaving enzyme 1 (BACE1) releases sAPPβ and retains C99 within the membrane. The γ-secretase complex containing PSEN1/2 then cleaves C99 to produce Aβ1-40 and Aβ1-42 at a ratio of 10:1 (LaFerla, Green et al. 2007) (Figure 1c).

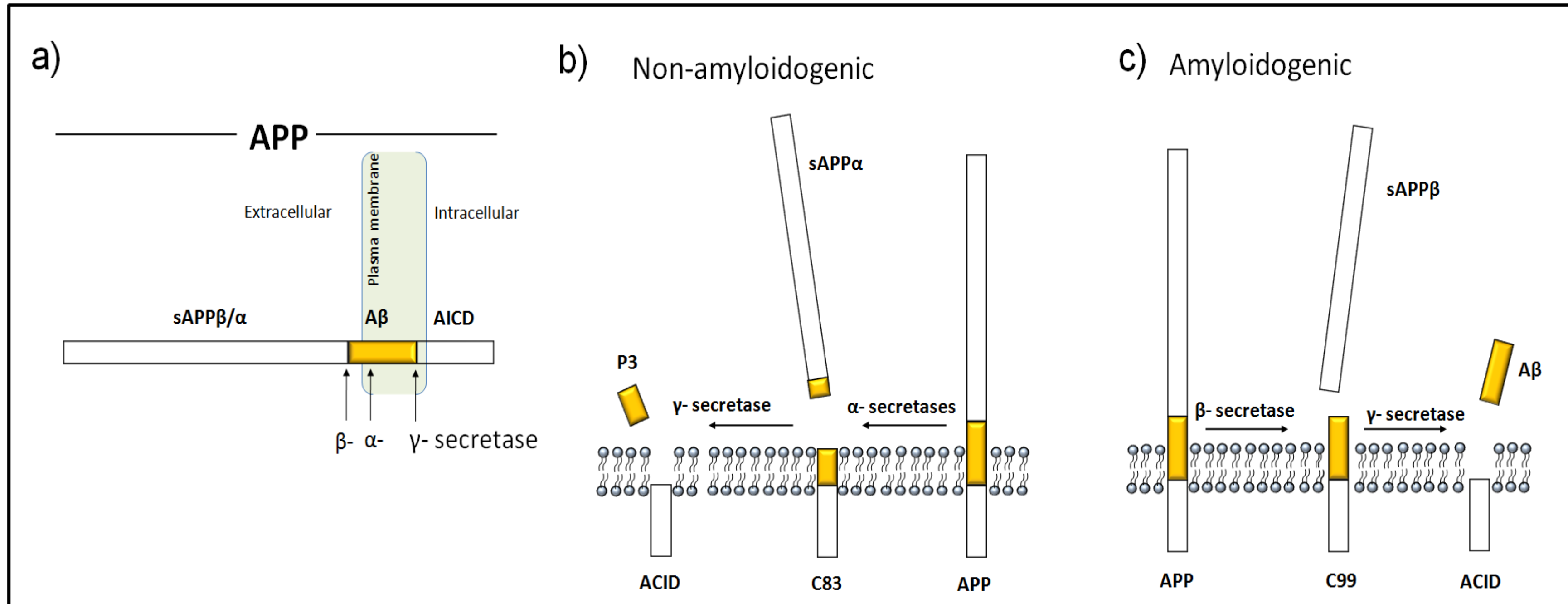

Figure 1: APP and its processing

a) A simplified depiction of the known APP protein structure which consists of N-terminal extracellular domain sAPPα/β, followed by a transmembrane region that contains the Aβ peptide sequence and a short C-terminal cytoplasmic tail domain AICD. The Aβ sequence is depicted in yellow colour and its length can vary, but it's usually 40 to 42 amino acids long. The arrows represent the relative position of cleavage sites for β-, α-, and γ-secretases respectively. **b)** The non-amyloidogenic pathway of APP is mediated by α-secretase where it cleaves within the Aβ domain releasing two fragments. The larger fragment is the sAPPα which is neuroprotective and the smaller fragment is the carboxy-terminal C83 that is further cleaved by γ-secretase to generate P3. **c)** In the amyloidogenic pathway, APP is cleaved by β-secretase (BACE1) releasing sAPPβ and C99 which is further cleaved by γ-secretase complex to produce predominantly Aβ1-40 and Aβ1-42.

Even though the processing of APP is predominantly through the non-amyloidogenic pathway (Vella and Cappai 2012), both pathways appear to occur in normal healthy brains where the cleavage products play a role in a variety of important physiological processes, including synaptic activity, neuroprotection and transcriptional regulation (Craft, Watterson et al. 2006).

Under healthy conditions, Aβ is cleared from the brain by both proteolytic digestion and transport across the BBB into the peripheries (Marques, Kulstad et al. 2009). The main enzymes that cleave Aβ include IDE, plasmin and neprilysin (Ling, Morgan et al. 2003; Nalivaeva and Turner 2013). Aβ is transported across the BBB via LRP1 and receptor for advanced glycation end products (RAGE). A major endogenous brain Aβ sinker is a soluble form of LRP, which can sequester 70-90% of plasma Aβ (Deane, Bell et al. 2009). In LOAD, Aβ production, accumulation and aggregation are considered the initial step to neurodegeneration and a critical part of the progression of the disease (Annaert and De Strooper 2002). Supporting scientific evidence comes from studies on the rare familial AD where early-onset of AD is observed with protein aggregates that are similar to those in LOAD due to mutations near the APP gene, PSEN1 and PSEN2 genes (Piaceri, Nacmias et al. 2013). Most mutations in EOAD increase the production of Aβ, especially the amyloidogenic Aβ1-42 that is neurotoxic, and is prone to aggregation (Pauwels, Williams et al. 2012). Furthermore, individuals with Down's syndrome (DS) (trisomy 21) have an extra copy of the gene encoding APP and universally develop AD pathology at an early age (Gouras, Tsai et al. 2000). The extra APP gene leads to ~150% increase in APP mRNA

levels in the brain of individuals with DS and Aβ deposition in the frontal lobe and cortex (Azizeh, Head et al. 2000; Zigman 2013). Interestingly, an individual with partial Trisomy 21 lacked the extra gene for APP and showed neither dementia nor AD pathology (Prasher, Farrer et al. 1998). Moreover, transgenic mice over-expressing human APP (hAPP) gene carrying the Swedish mutation (APP23) and another expressing hAPP23 and mutant human PSEN1 exhibited Aβ deposition in the brain (Maia, Kaeser et al. 2013). The mice also exhibited an age-related decrease of Aβ1-40 and an up to 80% decrease in Aβ1-42 in the mouse cerebrospinal fluid (CSF), which inversely correlates with the brain Aβ load (Lewis, Dickson et al. 2001). The same mice also showed an increase in total Tau in the CSF at the stages of prominent Aβ pathology mirroring the Aβ and Tau changes in the CSF of patients with LOAD (Maia, Kaeser et al. 2013). Multiple studies link different mutations in APP genes to altered processing of APP and altered modulation of secretase activities to increase Aβ production (Drouet, Pincon-Raymond et al. 2000). Finally, a mutation (A673T) in the APP gene was found to be protective against AD and cognitive decline in the elderly without AD by causing reduction in the formation of amyloidogenic peptides (Jonsson, Atwal et al. 2012).

The accumulated evidence supports the notion that Aβ is the initial pathological agent in AD where neurotoxicity of Aβ requires self-assembly of the peptide into aggregates of various sizes including Aβ fibrils that deposit as SPs and neurotoxic-soluble oligomers (Bjorklund, Reese et al. 2012). Aβ oligomers are thought to activate signaling pathways that lead to oxidative stress (Saraiva,

Seixas da Silva et al. 2010), abnormal tau phosphorylation and the formation of NTFs (De Felice, Wu et al. 2008; De Felice 2013). Moreover, a broad effect on synapses is observed when neurons are exposed to oligomers where N-methyl-D-aspartate (NMDA) and α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA)-type glutamate receptors are removed from the cell surface (Lacor, Buniel et al. 2007; Decker, Lo et al. 2010; Jurgensen, Antonio et al. 2011).

The biggest risk factor for developing sporadic AD is age. Other risk factors include plasma cholesterol level (Jick, Zornberg et al. 2000), Apolipoprotein E (APOE)4 status (Corder, Saunders et al. 1994), trauma (Jellinger 2004), deficiency in growth factors (Isacson, Seo et al. 2002) and other environmental factors. However, in spite of many advances in understanding AD, it is not yet known why these different risk factors should lead to neurotoxicity and why Aβ levels rise in the brains of sporadic AD patients who have no mutations in the APP or secretase genes (Annaert and De Strooper 2002; Gralle and Ferreira 2007).

1.2 Insulin resistance in AD

Brain insulin originates from the pancreatic β-cells and it is also formed in pyramidal neurons found in the hippocampus, prefrontal cortex and the olfactory bulb but not in other neurons (Hoyer 2002). The brain’s insulin-independent glucose uptake capability led to the belief that it is an insulin-insensitive organ and less attention was given to insulin signaling in the brain in the past. Today, it is obvious that insulin is involved in many functions and signaling pathways in the brain, but its exact effect is not yet well defined (Ghasemi, Haeri et al. 2013). In

the peripheries, insulin is produced in the pancreas within the β-cells of the islets of Langerhans. It has many actions on global human metabolism including glucose uptake and utilization, protein synthesis, lipid and cholesterol synthesis and DNA replication (Najjar 2001). The blood content of insulin can be measured in international unit μIU/mL where 1 μIU/mL = 6.945 pmol/L. A typical blood level between meals is 8-11 μIU/mL (57-79 pmol/L) or 3 – 4.25 nM (Iwase, Kobayashi et al. 2001). The brain's main source of insulin is from the periphery where insulin enters the BBB through a receptor mediated transport system (Ghasemi, Haeri et al. 2013). A correlation between plasma and CSF levels of insulin is nonlinear and is evidence to a saturable transport system of insulin through the BBB (Ghasemi, Haeri et al. 2013). This is due to the observation that levels of insulin in CSF became proportionally smaller to increased plasma insulin levels (Ghasemi, Haeri et al. 2013). Insulin an important modulator of growth and metabolic function in the central nervous system (CNS) and its receptor is abundantly expressed in the brain (de la Monte and Wands 2005). In the hypothalamus insulin acts to regulate peripheral energy homeostasis (Marino, Xu et al. 2011; Scherer, O'Hare et al. 2011), and IRs is widely distributed in the hippocampus, which is a region involved in acquisition, consolidation and memory (Zhao and Alkon 2001). Insulin receptor signaling is also involved in postsynaptic neurotransmitter receptor trafficking suggesting a role in synaptic function and plasticity and involvement in learning and memory (Chiu and Cline 2010). For example, a powerful *in vivo* experiment that utilized the *Xenopus* visual circuit showed that reduced insulin receptor phosphorylation led to severe

decrease of glutamatergic synaptic input and reduced responses to natural light stimuli (Chiu, Chen et al. 2008). Moreover, insulin receptor signaling regulates dendritic outgrowth and involvement in neuronal survival since the insulin receptor has been shown to be a component of synapses at the postsynaptic density (PSD) (Chiu and Cline 2010; De Felice 2013).

Insulin receptors in the brain have similar pharmacological and kinetic properties to those in the peripheries but they differ in molecular size, carbohydrate composition and their regulation by insulin (Yip, Moule et al. 1980; Heidenreich, Zahniser et al. 1983). Unlike their peripheral counterparts, IRs in the brain do not show down-regulation in response to insulin excess indicating different regulatory mechanisms (Boyd and Raizada 1983; Zahniser, Goens et al. 1984). Insulin mediates its effect on cell growth, glucose transport, survival and homeostasis by signaling downstream through insulin receptor substrate (IRS) molecules (Hoyer 2002). IRS proteins transmit signals from insulin and IGF-1 receptors to several pathways including the phosphatidylinositol 3-kinases (PI3K) pathway and the mitogen-activated protein kinase (MAPK) pathway (De Felice 2013). PI3K signaling leads to cell survival, proliferation, and metabolic functions such as synthesis of lipids, and proteins. MAPK activation leads to mitogenic functions such as cell growth and gene expression (Figure 2) (Giovannone, Scaldaferri et al. 2000).

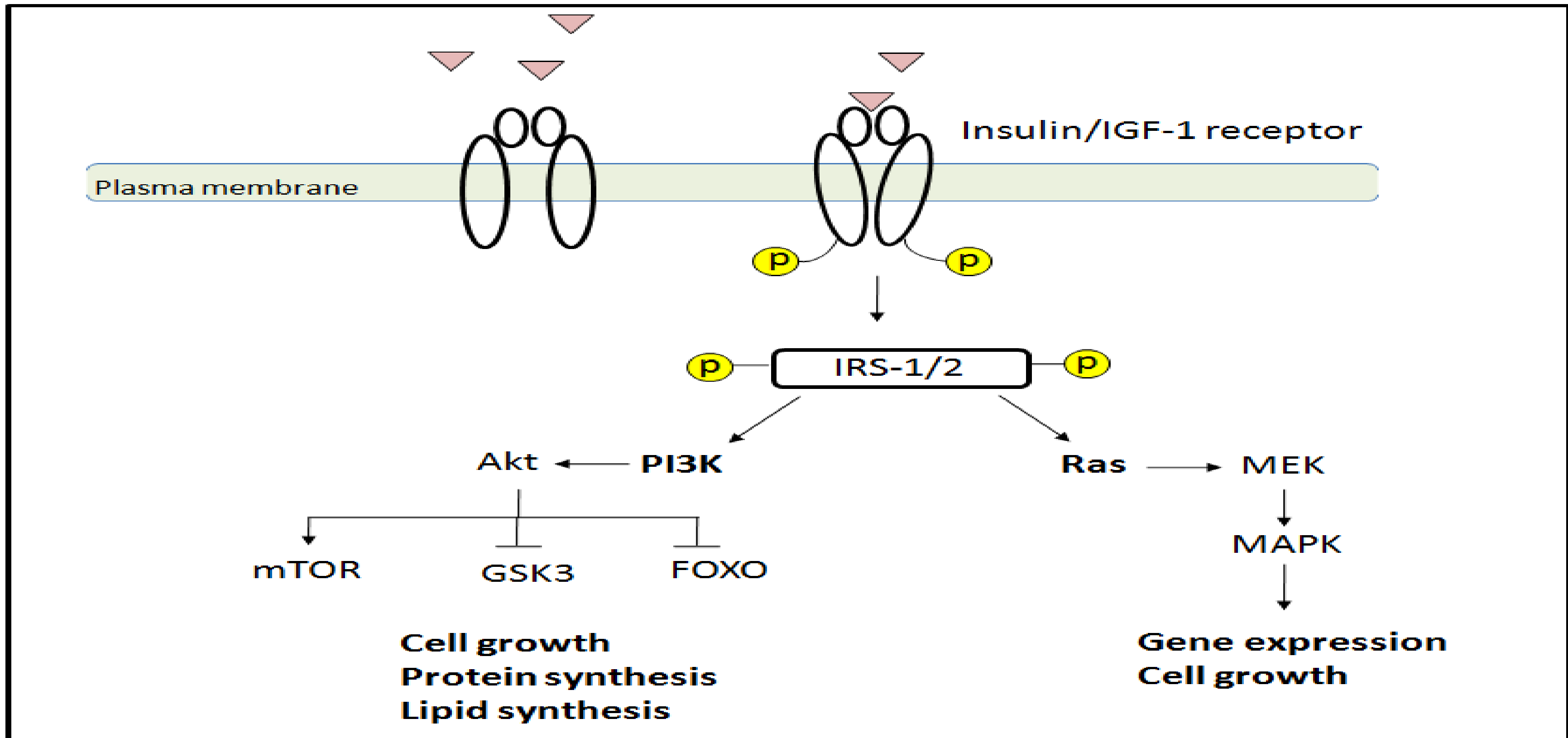

Figure 2: Simplified insulin signaling cascade

In this simplified depiction of insulin signaling, insulin binds to the extracellular domain of IR promoting autophosphorylation of the intracellular substrates and activating the receptor. Activation of IR phosphorylates intracellular substrates including IRS leading to recruitment and activation of multiple proteins and the initiation of several signaling cascades. PI3K mediates Akt signaling leading to metabolic and cellular survival activities including inhibition of glycogen synthase kinase 3 (GSK3) and neuronal survival. Ras mediates MAPK signaling which promotes gene expression and the mitogenic effects of insulin signaling.

The synapse is an important site for insulin signaling in the brain due to the highly enriched IRS localization in the synapses of the cerebellum, hippocampal neurons and cerebral cortex (Abbott, Wells et al. 1999; Ghasemi, Haeri et al. 2013). IRS-1 and IRS-2 are responsible for most of the diverse signaling effects of insulin and IGF-1 and both have been shown to be present in the brain (Boura-Halfon and Zick 2009). IRS-1 is present in neuronal cell bodies in forebrain and hypothalamus (Baskin, Sipols et al. 1993) nuclei and IRS-2 is abundant in the brain's nucleus (Torsoni, Carvalheira et al. 2003; Ghasemi, Haeri et al. 2013).

AD is characterized by both low insulin levels and insulin resistance in the CNS (Kroner 2009). Impairment of brain insulin signaling and brain insulin deficiency and resistance cause neuronal dysfunction and death due to deficits in energy metabolism, tropic factor withdrawal, and inhibition of insulin-responsive gene expression, such as the ones involved in acetylcholine homeostasis (de la Monte 2009). Several observations support the important role of insulin in AD. For example, patients at various stages of AD neurodegeneration have insulin receptor expression in the brain inversely proportional to the stage. An 80% decrease in the number of insulin receptors is observed in AD patients brains compared to normal subjects (Rivera, Goldin et al. 2005; de la Monte 2009). Another observation is that impaired insulin or IGF-1 signaling can result in hyper-phosphorylation of tau due to inhibition of PI3/Akt signaling, and increased activation of GSK-3β (Schubert, Brazil et al. 2003). Aβ oligomers and aggregates can bind to IRS-1 resulting in impaired insulin signaling and activation of GSK3β, which leads to tau pathology in AD (Takashima, Noguchi et al. 1993; Ma, Lim et

al. 2006). Also, Aβ has been shown to impair the extracellular signal-regulated kinases (ERK) response to growth factors and PI3K signaling, indicating resistance can occur beyond the receptor level (Chromy, Nowak et al. 2003; Tong, Balazs et al. 2004). Moreover, decreased inhibition of GSK due to low PI3K signaling can activate GSK3α subunit, which has been shown to stimulate generation of Aβ by γ-secretase (Phiel, Wilson et al. 2003; Ho, Qin et al. 2004). Clinical correlations indicate that individuals with type 2 diabetes (T2D) are twice more likely to develop AD than non-diabetics (Ott, Stolk et al. 1996). Impairment of memory and long-term potentiation (LTP) have been reported in diabetic animals where IRS-1 is important for proper brain function and is found to be inhibited in animal models and in AD brains (Wan, Xiong et al. 1997; Zhao and Alkon 2001; Haj-ali, Mohaddes et al. 2009; Kovacs and Hajnal 2009); alleviating IRS-1 inhibition improves cognition in transgenic mice (Bomfim, Forny-Germano et al. 2012). The mechanism of this inhibition maybe due to abnormal activation of tumor necrosis factor α (TNFα), mammalian target of rapamycin (mTOR), and c-Jun N-terminal kinase (JNK) signaling by Aβ oligomers (Ma, Yang et al. 2009; Moloney, Griffin et al. 2010). The activation of these cellular pathways by Aβ oligomers results in IRS-1 serine phosphorylation (pIRS-1 Ser); particularly pIRS-1 Ser312 and pIRS-1 S612 (Moloney, Griffin et al. 2010) leading to downstream insulin signaling inhibition and insulin resistance (Bomfim, Forny-Germano et al. 2012). Postmortem AD brain analysis revealed activated JNK and elevated pIRS-1 Ser (Zhao, De Felice et al. 2008). Insulin blocks both down-regulation of IR and the pIRS-1 Ser induced by Aβ (De Felice, Vieira et al. 2009). In patients with

early AD and mild cognitive impairment (MCI), intranasal insulin administration enhances verbal memory and improves performance (Benedict, Hallschmid et al. 2004; Nelson, Sun et al. 2008; De Felice, Vieira et al. 2009; Dhamoon, Noble et al. 2009). This protection seems to derive from IR dependent signaling and it might not work at later stages of AD where surface IRs decrease significantly (Schioth, Craft et al. 2012). However, analogs to insulin that can activate insulin-signaling pathways through G-protein-dependent signaling do exist and may offer a possible therapeutic benefit in AD as well as an alternative to repeat use of insulin at earlier stages (Vilsboll, Krarup et al. 2003; McClean, Parthsarathy et al. 2011). Glucagon-like peptide 1 receptor (GLP-1R) agonists are novel analogs to insulin and they require a functioning GLP-1 receptor (Holst, Burcelin et al. 2011; De Felice 2013). GLP-1 bypasses IRs and boost insulin related signaling pathways by up-regulating phosphorylated IRS-1, Akt and GSK3β (Gao, Wang et al. 2007; Holst, Burcelin et al. 2011; Bomfim, Forny-Germano et al. 2012; De Felice 2013).

Another observation in AD is that insulin-degrading enzyme (IDE) levels, activity and mRNA are decreased in the brain tissue (Cook, Leverenz et al. 2003; Zhao, Teter et al. 2004). As its name implies, IDE degrades insulin in a negative feedback control mechanism, and it also mediates Aβ degradation. Lower IDE levels affect Aβ clearance rates and it can lead to imbalance in homeostasis of Aβ (Farris, Mansourian et al. 2003; Cole and Frautschy 2007). Insulin itself directly increases Aβ secretion and decreases the intracellular levels of the peptide by stimulating its trafficking (Gasparini, Gouras et al. 2001).

Subsequently, Aβ can be cleared into the CSF mediated by Aβ carrier proteins albumin and transthyretin (Carro, Trejo et al. 2002). Interestingly, IGF-1 administration increases the entrance of those proteins to the brain through the choroid plexus and a parallel increase in levels of Aβ bound to these proteins in the CSF and blood is observed (Carro, Trejo et al. 2002; Gasparini and Xu 2003). TNFα blocks this IGF-1 effect on Aβ clearance (Pratico and Trojanowski 2000). It appears that insulin resistance and Aβ accumulation occur in a loop reinforcing each other leading to the progression of the disease (Zhao and Townsend 2009). A better understanding of normal physiological molecular pathways between insulin and Aβ signaling can shed light on potential areas of malfunction in a pathological state.

1.3 AD and cholesterol

The brain is the most cholesterol rich organ with ~25% of the total cholesterol in the body, and many pathways are involved in the storage and transport of cholesterol in the CNS (Mathew, Yoshida et al. 2011). Cholesterol is synthesized *de novo* in the brain by glial cells and neurons because it cannot cross the blood-brain barrier (BBB) from the periphery and is almost entirely independent of the plasma cholesterol (Mathew, Yoshida et al. 2011). It is mostly located at the specialized membranes of myelin, neuronal and glial cells and is important for neuronal plasticity. Cholesterol is required for the synthesis of neuronal synapses and it regulates the physiochemical properties of neural membranes and its bound enzymes, ion channels and receptors (Pfrieger 2003; Dante, Hauss et al. 2006; Valenza and Cattaneo 2006; Farooqui, Ong et al.

2010; Liu, Tang et al. 2010). It is involved in synaptic vesicle transport and exocytosis, and also in the formation, maintenance, turnover and homeostasis of synapses (Koudinov and Koudinova 2001; Dietschy and Turley 2004; Leoni and Caccia 2013).

Early on, it has been reported in AD that there is a higher occurrence of lipid granule accumulation in the glia (Alzheimer, Stelzmann et al. 1995; Foley 2010) suggesting abnormal lipid metabolism (Di Paolo and Kim 2011). Later, aberrant lipid composition was reported in post mortem brain tissue of AD patients, but it was the discovery of the ε4 allele of the APOE gene as the strongest genetic risk factor for sporadic AD that established the intimate link between lipid metabolism and the disease (Corder, Saunders et al. 1993; Bertram and Tanzi 2008). APOE is synthesized by astrocytes and microglia cells, is the main lipid carrier in the CNS, is a regulator of cholesterol metabolism in the brain, and it mediates the uptake of lipoprotein particles via the low density lipoprotein receptor-related protein (LRP) (Bu 2009; Di Paolo and Kim 2011). Also, APOE has a role in amyloid pathology where APOE-containing high density lipoproteins (HDL) particles inhibit the aggregation of Aβ (Kim, Basak et al. 2009). APOE has three common alleles; ε2 with 8% prevalence in the population and is associated with neuroprotection against AD, ε3 which is the most common form with 77% prevalence in the population, and ε4 with 15% prevalence in the population, but with 50% prevalence in AD patients (Mahley 1988; Corder, Saunders et al. 1994; Genin, Hannequin et al. 2011). APOE4 contributes to AD pathology by modulating amyloid metabolism and interfering with lipid

metabolism in the brain (Maxfield and Tabas 2005). This allele is associated with increased Aβ1-42 synthesis, SPs deposition, mitochondrial dysfunction, tau-protein phosphorylation and decreased receptor mediated Aβ clearance (Silva, Teixeira et al. 2013). Also, APOE4 is the least effective isoform with respect to neuron and synapse maintenance and repair and it binds to cholesterol at a lower affinity than other APOE isoforms resulting in altered cholesterol metabolism (Rapp, Gmeiner et al. 2006; Silva, Teixeira et al. 2013). Large independent genome wide association studies (GWAS) have implicated genetic variation in cholesterol metabolism to increased susceptibility to LOAD (Harold, Abraham et al. 2009; Lambert, Heath et al. 2009; Jones, Holmans et al. 2010). In addition to APOE, single nucleotide polymorphisms (SNPs) related to cholesterol metabolism have been recently found to be robustly associated with AD such as Rs3846662 that is associated with HMGCR exon 13 inclusion (Simmons, Zou et al. 2011). Another example is the loci clusterin (*CLU*) encoding apolipoprotein J (APOJ) that is associated with cholesterol transport and export of Aβ over the BBB (Corneveaux, Myers et al. 2010).

Hypercholesterolemia and altered cholesterol metabolism appear to play fundamental roles in AD, especially in amyloid plaque formation and tau hyperphosphorylation (Gamba, Testa et al. 2012). It is believed that increased levels of cholesterol can be a risk factor in developing AD based on several observations in the literature. First, a lower prevalence of AD related dementia is reported in patients under-going lipid-lowering therapy (Rodriguez, Dodge et al. 2002). Second, studies showed that an increase in intake of dietary cholesterol

increased Aβ levels and SPs deposition in brain tissue (Ghribi 2008). Third, hypercholesterolemia in middle ages is associated with higher risk of dementia and AD (Sambamurti, Granholm et al. 2004). However, other studies described contradictory findings (Li, Higdon et al. 2004; Mielke, Zandi et al. 2010). Dysregulation of cholesterol homeostasis and metabolism is observed in AD patients where it is characterized by lowered HDL, increased LDL, and hydroxycholesterol products of cholesterol degradation (Martins, Hone et al. 2006). (24S)-24-Hydroxycholesterol (24S-OH-Chol) is a hydrophilic cholesterol metabolite formed in the brain by cholesterol 24S-hydroxylase and is usually a marker of CNS cholesterol elimination in the CSF (Cibičková 2011). Higher levels of 24-S-OH-Chol along with SPs, NFTs are found in post-mortem analysis of AD brains. Since cholesterol synthesis and turnover in adult brain is very low, increased 24-S-OH-Chol suggests an altered cerebral cholesterol load (Lutjohann, Breuer et al. 1996; Fonseca, Resende et al. 2010; Silva, Teixeira et al. 2013). The increased levels of 24-S-OH-Chol can also be a marker for cognitive decline due to neuronal damage and higher turnover of neuronal membranes (Leoni and Caccia 2013). Certain polymorphisms in 24S-hydroxylase gene have been associated with a higher risk of dementia and AD (Wollmer 2010). An interesting link between hypercholesterolemia and AD is the 27-hydroxycholesterol (27-OH-Chol), another metabolite of cholesterol (Cibičková 2011; Shafaati, Marutle et al. 2011). Although, it is present at low levels in neurons and astrocytes in the brain, it is mostly formed in the periphery. However, it is hydrophilic and is capable of crossing the BBB from plasma to the

CNS and can diffuse into the brain at high plasmatic levels (Shafaati, Marutle et al. 2011; Leoni and Caccia 2013). In AD a 40-80% increase of 27-OH-Chol is observed in the temporal, parietal and occipital cortices of patients compared to controls (Heverin, Bogdanovic et al. 2004). *In vitro*, 27-OH-Chol has been shown to increase membrane expression of APP proteins, BACE1 and Aβ1-42 levels (Prasanthi, Huls et al. 2009). The increased activity of acyl-CoA cholesterol acyltransferase (ACAT), which cause an increased amount of cholesterol esters (CEs), provides further evidence for the dysregulation of cholesterol metabolism in AD (Martins, Hone et al. 2006). As much as 99% of cholesterol in the CNS is unesterified (Dietschy and Turley 2004) and CEs promote amyloidogenesis (Silva, Teixeira et al. 2013). Studies *in vitro* with ACAT inhibitors have shown complete abolishment of amyloidogenesis (Puglielli, Tanzi et al. 2003).

The activity of enzymes involved in the APP metabolism is influenced by cholesterol where the post translational processing of APP occurs in cholesterol-rich membrane domains called lipid rafts, which are small heterogeneous domains enriched in steroids and sphingolipids (Xiong, Callaghan et al. 2008). Both β- and γ-secretases reside in cholesterol-rich lipid rafts of plasma membrane, and so the altered levels of cholesterol could affect their enzymatic activities based on the fluidity of the membranes and determine preferential APP processing (Di Paolo and Kim 2011). Studies *in vitro* and *in vivo* have shown enhanced β- and γ- secretase activities and promoted APP metabolism by the amyloidogenic pathway in response to cholesterol level increase (Puglielli, Tanzi et al. 2003; Florent-Bechard, Desbene et al. 2009; Zhang, Fan et al. 2013).

Interestingly, APP seems to control cholesterol turnover needed for neuronal activity (Pierrot, Tyteca et al. 2013).

Cholesterol binds to APP at the α-secretase cleavage site, favoring β-secretase activity leading to increased production of Aβ (Refolo, Malester et al. 2000; Shobab, Hsiung et al. 2005). Analysis of *In vivo* brain samples showed that neurons with NFTs contained more free cholesterol than NFTs free neurons and cholesterol concentrations in plaques were significantly increased (Distl, Meske et al. 2001; Panchal, Loeper et al. 2010). Free cholesterol in the cytoplasm has been found to affect the aggregation of soluble Aβ peptides into fibrils and tangles, and it can induce intraneuronal accumulation of Aβ oligomers (Umeda, Tomiyama et al. 2012; Zhou and Xu 2012). In a recent study, cholesterol was found to increase β-sheet formation in Aβ peptide by 4-fold which led to fibril formation (Zhou and Xu 2012). In another study, Aβ is found to be capable of modifying the cellular levels and distribution of cholesterol as well as its esterification rate (Fonseca, Resende et al. 2010). Overall, there seems to be many conflicting reports on the levels of cholesterol in AD brain at different stages of the disease. What is clear is that cholesterol dysregulation plays critical role in AD pathology and progression, but despite the increasing amount of evidence from genetic, epidemiological and biochemical studies an exact mechanism still remain to be elucidated (Maulik, Westaway et al. 2013).

A potential connection between dysregulation of insulin signaling and cholesterol homeostasis in relation to Aβ metabolism remains largely uninvestigated. Insulin resistance causes increased cholesterol synthesis and

decreased cholesterol absorption (Hoenig and Sellke 2010) which could alter cholesterol metabolism in the brain. Insulin activates the transcription factors SREBPs that are involved in cholesterol and fatty acid synthesis (Figure 3) (Suzuki, Lee et al. 2010). Three members of SREBPs regulate the expression of genes needed for synthesis of cholesterol, fatty acids and phospholipids; SREBP1a and SREBP1c regulate fatty acid synthesis and SREBP2 is specific for cholesterol biosynthesis (Brown and Goldstein 1999; Goldstein, Rawson et al. 2002; Horton, Goldstein et al. 2002; Bonzon-Kulichenko, Schwudke et al. 2009).

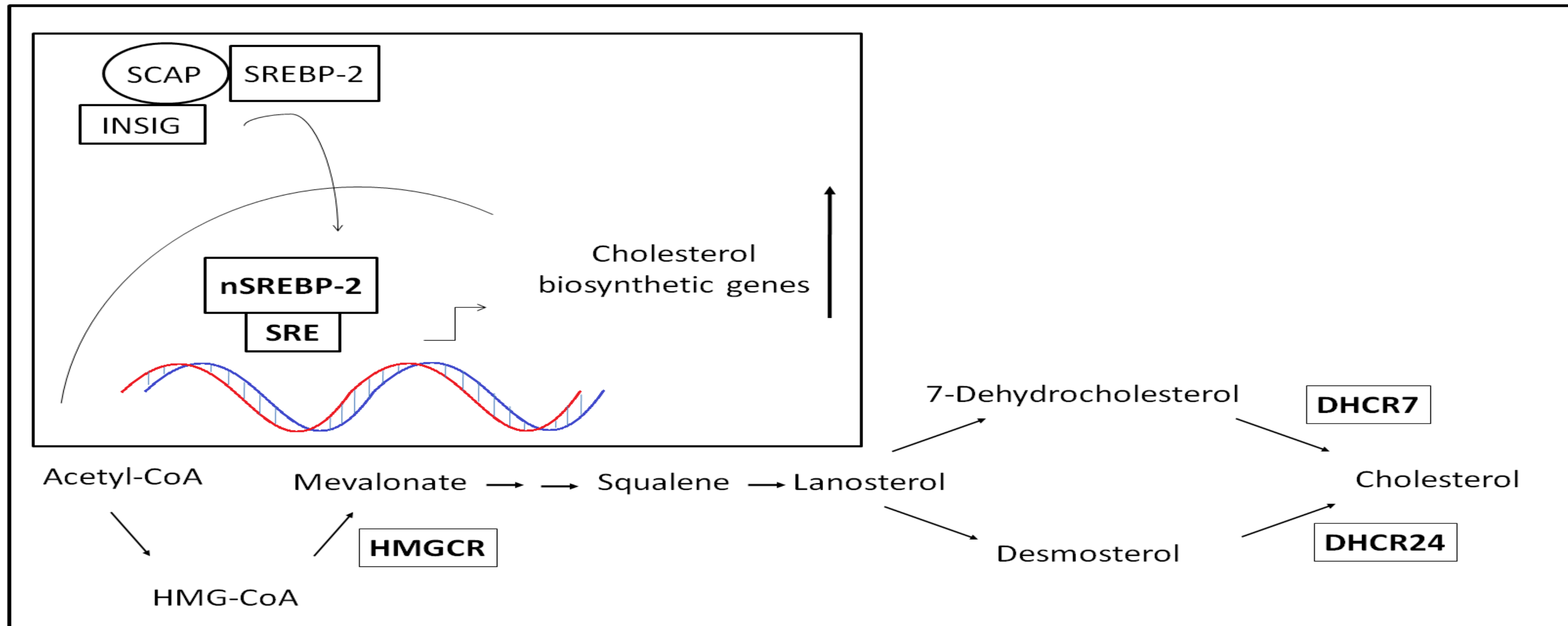

Figure 3: Simplified schematic of cholesterol synthesis pathway

SREBP2, a 126 kDa protein, is associated with sterol cleavage activating protein (SCAP) that is in turn bound to insulin induced genes (INSIG). This complex is retained at the endoplasmic reticulum (ER) under homeostasis. Insulin signaling causes the degradation of INSIG, which allows the SCAP/SREBP complex to be delivered to the Golgi where SREBP2 is cleaved to a 55 kDa water soluble N-terminal domain (nSREBP2) that translocates to the nucleus and binds to specific sterol regulatory element (SRE) upregulating the synthesis of enzymes involved in sterol synthesis. Cholesterol synthesis starts at the rate limiting step of converting HMG-CoA to mevalonate by HMGCR. Mevalonate is converted to several other products in the biosynthesis pathway including squalene, lanosterol, desmosterol and 7-dehydrocholesterol. DHCR24 catalyses the reduction of desmosterol to cholesterol and 7-dehydrocholesterol reductase (DHCR7) catalyzes the production of cholesterol from 7-dehydrocholesterol.

It has been reported that there is a decrease in SREBP2 levels in the brain of diabetic mice due to a decline in insulin levels, which results in reduced cholesterol biosynthesis, and altered neuronal function (Suzuki, Lee et al. 2010). It is important to note that this reduction of cholesterol is observed in the synaptosomal membrane rather than total brain cholesterol, where synaptosomal cholesterol pool turns over more quickly and can reflect short-term changes in cholesterol synthesis (Okabe, Kim et al. 1999; Bjorkhem and Meaney 2004; Trudeau, Gagnon et al. 2004; Barres 2008). These observations of increased cholesterol synthesis due to insulin resistance and decreased cholesterol due to low insulin levels add another layer of complexity to the disease because both insulin resistance and low levels of insulin have been observed in AD brain (Steen, Terry et al. 2005; Talbot, Wang et al. 2012). Multiple molecular pathways downstream of insulin signaling cascade are implicated in the regulation of SREBP2 including mTOR, PI3K, and MAPK pathways (Suzuki, Lee et al. 2010). On the other hand, hypercholesterolemia has been shown to induce insulin resistance where higher cholesterol synthesis disrupts the fluidity of plasma membranes causing IRs to localize to lipid rafts where they are unresponsive to insulin stimulation (Taghibiglou, Bradley et al. 2009). Moreover, the increased activity of β-secretase leads to inhibition of IR transcription and reduced IR expression mediated by increase in the intracellular Ca^{2+}. Under normal condition, inhibition of IR and reducing its expression by Aβ can be a feedback mechanism by which excess cholesterol regulates insulin signaling through controlling IR expression (Maesako, Uemura et al. 2011). One of the toxic events

of Aβ accumulation is catalyzing cholesterol oxidation to 7β-hydroxycholesterol, a highly toxic oxysterol that is implicated in AD and is a potent inhibitor of enzymes critically involved in memory consolidation and synaptic plasticity (Nelson and Alkon 2005). 7β-Hydroxycholesterol, as well as some low density lipoproteins, inhibit insulin-dependent phosphorylation of the signaling kinases ERK and Akt by acting as a second messenger for insulin (Nelson and Alkon 2005).

1.4 Aims and hypothesis of this study

It is clear that the regulation of cholesterol, insulin, and Aβ is complex and intimately intertwined and the exact network of interactions and metabolism in these systems is not well understood. One of the major goals of this research is to determine and understand the molecular events occurring under physiological insulin signaling state, and insulin resistant state in normal cells and in cells challenged with Aβ treatment. The main objectives are to investigate the roles and mechanisms of insulin and insulin signaling in regulating cellular cholesterol, its synthesis, APP processing and Aβ production in neural cells. Also, the roles of Aβ in insulin resistance and cholesterol synthesis dysregulation will be investigated in neural cells. The information obtained will help to determine the merit of the hypothesis: dysregulation of insulin signaling in AD may affect the expression of the enzymes involved in cholesterol regulation/synthesis, and may lead to alteration of APP processing and Aβ production in neural cells; while accumulation of Aβ may cause insulin resistance and disruption of insulin signaling.

Materials and Methods

2.1 Chemical reagents

Dulbecco's modified Eagle's medium (DMEM), TRIzol, geneticin, 0.25% trypsin/EDTA and antibiotic gentamicin were purchased from Invitrogen Life Technologies Inc. (Burlington, ON, Canada). Fetal bovine serum (FBS) was purchased from Hyclone (Logan, UT, USA). Hank's balanced salt solution (HBSS) was purchased from Wisent Multicell (Montreal, QC, Canada). Dimethyl sulfoxide (DMSO), recombinant human insulin, protease inhibitor cocktail (PI), bovine serum albumin (BSA), and antibiotic penicillin-streptomycin were purchased from Sigma Aldrich Canada Ltd. (Oakville, ON, Canada). Eagle's minimum essential medium (EMEM) was purchased from ATCC (Manassas, VA, USA). Ham's F12 media was purchased from Cellgro (Manassas, VA, USA). The BSA standard, Experion RNA StdSens analysis kit, SsoFast EvaGreen Supermix, iScript cDNA synthesis kit and PVDF membrane were purchased from Bio-Rad (Hercules, CA, USA). Recombinant Aβ1-42 and a scrambled peptide, featuring the same amino acids in a randomized order, were purchased from r-Peptide (Bogart, GA, USA) (Table 1). Actin, total c-Jun, phospho c-Jun ser-63 and 73, total GSKα/β, phospho GSK-3 α/β, phospho IRS-1 (Ser612), phosho IRS-1 (Y895), total IRS, total AKT, phospho AKT (Ser473) and DHCR34/Seladin-1 antibodies were purchased from New England Biolabs (Pickering, Ontario, Canada). SREBP2, HMGCR and DHCR7 antibodies were purchased from Abcam (Cambridge, MA, USA). Squalene monooxygenase (SQLE) antibody was

purchased from Proteintech (Chicago, IL, USA). Western-Lighting Plus-ECL was purchased from Perkin Elmer Inc. (Waltham, Massachusetts, USA). Autoradiography film was purchased from Mandel Scientific (Guelph, Ontario, Canada)

Table 1: Amino acid sequences of Aβ1-42 and scrambled Aβ1-42 peptides

Peptide	Amino Acid sequence
Aβ 1-42	DAEFRHDSGYEVHHQKLVFFAEDVGSNKGAIIGLMVGGVVIA
Scrambled Aβ 1-42	KVKGLIDGAHIGDLVYEFMDSNSAIFREGVGAGHVHVAQVEF

2.2 Cell culture

Immortalized, undifferentiated human SHY cells were maintained in EMEM:Ham's F12 (1:1) with 10% FBS, and 1% gentamycin at 37°C with media change every 2 days and passage once a week. The cells were maintained from passage 18 to passage 40 before being discarded. Immortalized mouse neuroblastoma 2a cells (N2a) and the N2a cells stably transfected with human APP695 gene (N2a-APP) were grown in EMEM:DMEM (1:1) with 5% FBS, and 1% penicillin-streptomycin, with media change every 2 days, and passage twice a week starting at passage 8 to passage 25 before the cells were discarded. Geneticin was added only to N2a-APP to select for cells carrying the APP gene.

2.3 Insulin and Aβ treatments

The three cell lines were plated in separate 60mm dishes at a cell density of 70% in serum-deficient media. Human recombinant insulin was solubilized in sterile water at pH 2. Cells were treated 24 hours after plating with insulin

concentrations 0.1 nM, 0.5 nM, 1 nM, 5 nM and 1.72 μM for 30 min, 1, 2, 4, 6, 8, 10, and 12 hours. Negative control cells were treated with insulin vehicle.

Aβ1-42 and scrambled Aβ1-42 were dissolved in 25% sterile acetic acid to 400 mM concentration. The prepared Aβ includes small peptides, oligomers, low weight complexes and higher weight aggregates (as determined from previous experiments in the laboratory). SHY cells were treated with 5 μM Aβ1-42 or scrambled Aβ1-42 for 1, 2, 4, 6, 8 and 10 hours. Negative control cells were treated with Aβ 1-42 vehicle.

2.4 Protein isolation and western blotting

After treatment, the cells were washed 2 times with HBSS and lysed with western loading buffer (25% glycerol, 25% β-mercaptoethanol, 15% SDS, 0.25% bromphenol blue, 50 nM Tris-HCl). The cell lysates were boiled at 100°C for 10 minutes, cooled on ice for 5 minutes and spun at 14,000 rpm for 15 minutes. The supernatant containing the solubilized proteins was transferred to new tubes and total protein levels were determined by tricholoracetic acid (TCA) assay. Briefly, BSA was used to generate a standard curve on a clear 98 well plate (Costar). Samples and standards were mixed with 60% TCA and incubated for 30 minutes at 37°C, and then read by Spectra MAX 240 spectrophotometer at 570 nm wavelength using SoftMax PRO software. Proteins were loaded on 10% SDS-PAGE gels at 15 μg and the gels were run for one hour at 125 V. The proteins were then transferred onto PVDF membrane overnight at 150 mA with cooling running water. The blots were incubated for 1 – 2 hours at room temperature in blocking buffer [5% skim milk powder in 1X Tris-buffered saline Tween 20

(TBST) or filtered 3% BSA powder in 1X TBST)]. The blots were incubated with a primary antibody at 1:1000 dilution over night at 4°C, washed with 1X TBST, incubated with an appropriate secondary antibody at 1:5000 dilution, and the protein bands were visualized with ECL Plus solution on X-ray film. The bands were analyzed using UN-SCAN-IT gel 6.1 software (Silk Scientific Inc.) to obtain densitometry and the treatment samples were compared to controls by obtaining the fold-change of treated sample to vehicle.

2.5 Enzyme-Linked ImmunoSorbent Assay (ELISA)

Commercial Aβ1-40 and Aβ1-42 ELISA kits were purchased from Invitrogen (Camarillo, CA, USA). Media from N2a-APP cells treated with insulin at 5 nM and 1.72 μM concentrations were harvested and stored at -80°C. Media samples were spun at 200 rpm to remove cells and diluted 1:2 using provided diluents buffer and PI was added to prevent protein degradation. Standards and samples were prepared as per kit's instructions. In summary, 50 μL of standards and samples were added to a clear 98 well plate and incubated in detection antibody for 3 hours at room temperature. After the incubation period, the wells were washed with provided washing buffer, IgG HRP was added to wells and incubated for 30 minutes at room temperature, followed by a second washing step and finally stabilized chromogen was added and incubated for 30 minutes at room temperature in the dark. The plate was read at 450 nm by Spectra MAX 340 spectrophotometer using SoftMax PRO software. Results were normalized to protein content of cells and presented relative to vehicle.

2.6 Cholesterol assay

Amplex Red Cholesterol Assay Kit was purchased from Invitrogen (Camarillo, CA, USA). Cells were treated, washed with HBSS and scraped in methanol. Following harvest, the cells were sonicated for 5 minutes 3 times and spun to remove debris at 200 rpm. A 50 µL aliquot was used to determine protein concentration using BioRad protein assay. Chloroform was added to remaining solution in a 1:1 ratio and the samples were vortexed for 30 seconds and they were allowed to settle for 1 hour. The samples were then spun at 10000 g for 10 minutes and the bottom organic phase was transferred to a new tube and vacuum evaporated for 45 minutes. 100 µL of methanol was added to residue left in tube to solubilize cholesterol and the cholesterol assay was carried out as per kit instructions in a 98 well black plate. In summary, cholesteryl-esters in samples were hydrolyzed by cholesterol esterase into cholesterol, which was then oxidized by cholesterol oxidase to yield hydrogen peroxide that was detected by an Amplex red reagent producing fluorescent resorufin. Fluorescence was read using a fluorescence microplate reader at excitation range of 530 – 560 nm and emission detection at 590 nm. Results were normalized to protein content presented relative to vehicle.

2.7 RNA isolation and quantitative polymerase chain reaction (QPCR)

Total RNA was isolated from treated cells using TRIzol reagent. The harvested samples were collected into tubes and chloroform was added at 1:1 ratio with TRIzol. The samples were vortexed for 15 seconds, allowed to settle at

room temperature for 2 minutes, and then spun at 14000 rpm for 15 minutes at 4°C. The clear aqueous phase containing RNA was transferred to new tubes and mixed with isopropanol at 1:1 ratio. Samples were mixed, incubated at 4°C overnight to precipitate the RNA, then incubated at room temperature for 10 minutes and then spun at 14000 rpm for 10 minutes at 4°C. The isopropanol was decanted off and the pellet was washed with 1 mL of 70% ethanol in DNase/RNase free distilled water. Samples were spun at 10000 rpm for 5 minutes at 4°C, ethanol was decanted and the pellet was left to dry for 30 minutes. The pellets were resuspended in DNase/RNase-free water and heated at 55°C for 10 minutes.

The samples were then cleaned up of genomic DNA using Ambion DNA-*free* kits (Life Technologies, Burlington, ON). In summary, recombinant DNase I enzyme (1 µL) and 0.1 volume of 10X DNase I buffer were added to each sample and were incubated at 37°C for 30 minutes. After incubation, 0.1 volume of DNase inactivation reagent was added to each sample, vortexed and was allowed to sit for 2 minutes at room temperature. The samples were then spun at 10000 g for 1 minute and the supernatant containing clean RNA was transferred to a new tube.

The concentration of RNA in each sample was determined using NanoDrop 1000 UV-Vis Spectrophotometer (Thermo Scientific Inc, Nepean, ON). Also, the RNA integrity was checked using Experion Automated Electrophoresis System from Bio-Rad (Berkeley, CA, USA). The mRNA was transcribed into cDNA using iScript kits where 2 µg of RNA was combined with 5x iScript reaction mix and 1x

reverse transcriptase enzyme and ran for 5 minutes at 25°C, 30 minutes at 42°C and 5 minutes at 85°C to produce cDNA.

Commercially available QPCR primers were ordered from IDT (Coralville, IA, USA) (Table 2). Mixtures consisting of 10 µL Ssofast Eva Green Supermix (Bio-Rad), 2 µL of cDNA sample at 1/10 dilution (determined using standard curves for each primer), 2 µL of primers at 500 nM final concentration and 6 µL DNase/RNase-free water at a final volume of 20 µL were prepared for qPCR and run using a CFX96 Real-time PCR detection system (Bio-Rad). The reactions were carried out using 2stepAmp+melt program with conditions 95°C/0:10 min, 55°C/0:30 sec, go to step 2 39x, 95°C/0:10 min. The PCR efficiency was assessed by performing standard curves using pooled cDNA material by plotting the log of the starting quantity of the template against the Cq values to determine the equation of the linear regression line. Quantification of each target gene was normalized against β-actin house keeping gene and fold change for each gene was calculated using vehicle as controls.

Table 2: qPCR primer sequences

Gene			Sequences
Hs. DHCR24	Human	Forward	5'-CTTGCTACCCTGCTCCTTC-3'
		Reverse	5'-CGCTCTCGCTTATCTTCGAT-3'
Hs. HMGCR		Forward	5'-CTGACATGCAGCCAAAGC-3'
		Reverse	5'-GTTTACCCTCGATGCTCTTGT-3'
Hs. SREBF2		Forward	5'-GGACACACAGAAGAATCCGT-3'
		Reverse	5'-TCCCTACTCCATTGACTCTGAG-3'
Mm. DHCR24	Mouse	Forward	5'-CGAAGAGGTAGCGGAAGATG-3'
		Reverse	5'-AGAACTACCTGAAGACAAACCG-3'
Mm. HMGCR		Forward	5'-ACTGACATGCAGCCGAAG-3'
		Reverse	5'-CACATTCACTCTTGACGCTCT-3'
Mm. SREBF2		Forward	5'-GACACATAAGAGGATTCGAGAGC-3'
		Reverse	5'-CCCTATTCCATTGACTCTGAGC-3'

2.8 Statistical analysis

All results were reported as mean +/- SEM. Statistical significance was analyzed by unpaired two-tailed t-test for two group comparisons or by one-way ANOVA with Bonferroni post-hoc tests for multiple group comparisons. Analysis for all experiments was done using GraphPad Prism from GraphPad Software (La Jolla, CA, USA). Statistical significance was defined as $p<0.05$.

Results

3.1 Insulin treatment affects cholesterol levels and synthesis in neural cells

3.1.1 Insulin signaling occurs in SHY at physiological and higher levels as measured by western blotting

SHY cells were treated with various doses of insulin 0.1 nM, 0.5 nM, 1 nM, 5 nM and 1.72 μM (10 μg/mL) for 1 hour. Insulin signaling was determined by analyzing downstream targets of IRS, specifically phosphorylation of Akt at serine 473 and GSK-3β (Figure 4a). Phosphorylation levels were normalized to cellular β-actin levels. Analysis by two-tailed t-test showed a significant treatment effect on phosphorylation of Akt and GSK that showed increased phosphorylation relative to vehicle control (Two-tailed t-test, $p<0.05$, $N=3$) (Figure 4b & c) . There was no significant difference between total Akt or in total GSK compared to vehicle. Insulin signaling was occurring in the treated cells (Figure 4b & c).

Since the dose of insulin that had the most significant response was 1.72 μM, this concentration was used to treat SHY for a time course starting at 30 minutes to 12 hours to monitor for how long insulin signaling takes place. Insulin signaling was confirmed once again by looking at phoshporylation of Akt and GSK (Figure 5a). All phosphorylation levels were normalized to β-actin and analysis by two-tailed t-test showed a significant increase in phosphorylation relative to vehicle control, which lasted up to 6 hours for pAkt and 4 hours for pGSK (Two-tailed t-test, $p<0.05$, $N=3$) (Figure 5b & c). There was no significant

difference between total Akt and GSK to vehicle, signifying insulin signaling response in treated cells (Figure 5b &c).

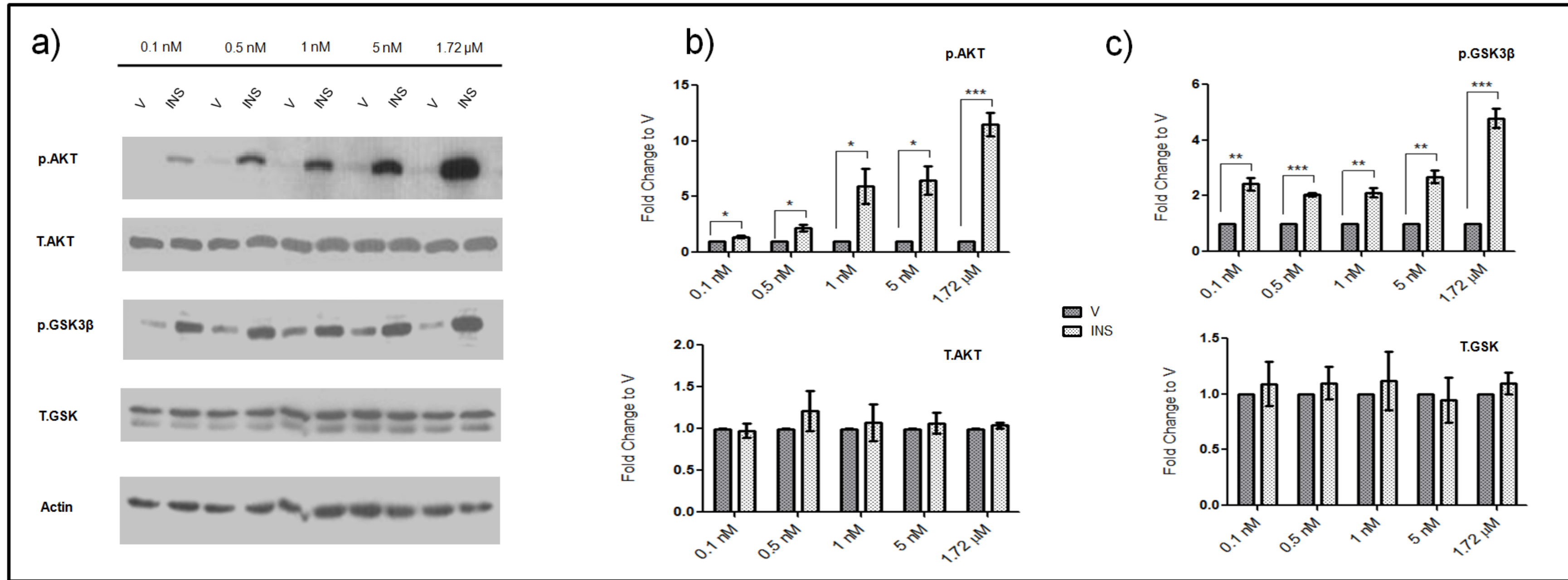

Figure 4: Insulin signaling occurs in SHY treated with insulin at physiological levels and higher

a) SHY cells were treated for 1 hour with insulin (INS) at concentrations 0.1 nM, 0.5 nM, 1 nM, 5 nM, and 1.72 μM and pAkt at Ser473, pGSK-3β, total Akt, and total GSK protein levels were detected by western blotting, normalized to β-actin, and presented relative to vehicle (V) (set to 1). Two-tailed t-test comparison was carried out in GraphPad Prism ($p<0.05$, N=3). **b)** There was a significant increase in pAkt Ser473 in response to all concentrations of insulin used and no significant change in total Akt protein was observed indicating insulin signaling downstream of IRS. **c)** pGSK3β was significantly increased at all insulin concentrations compared to controls without significant change in total GSK protein levels further indicating insulin signaling and inhibition of GSK.

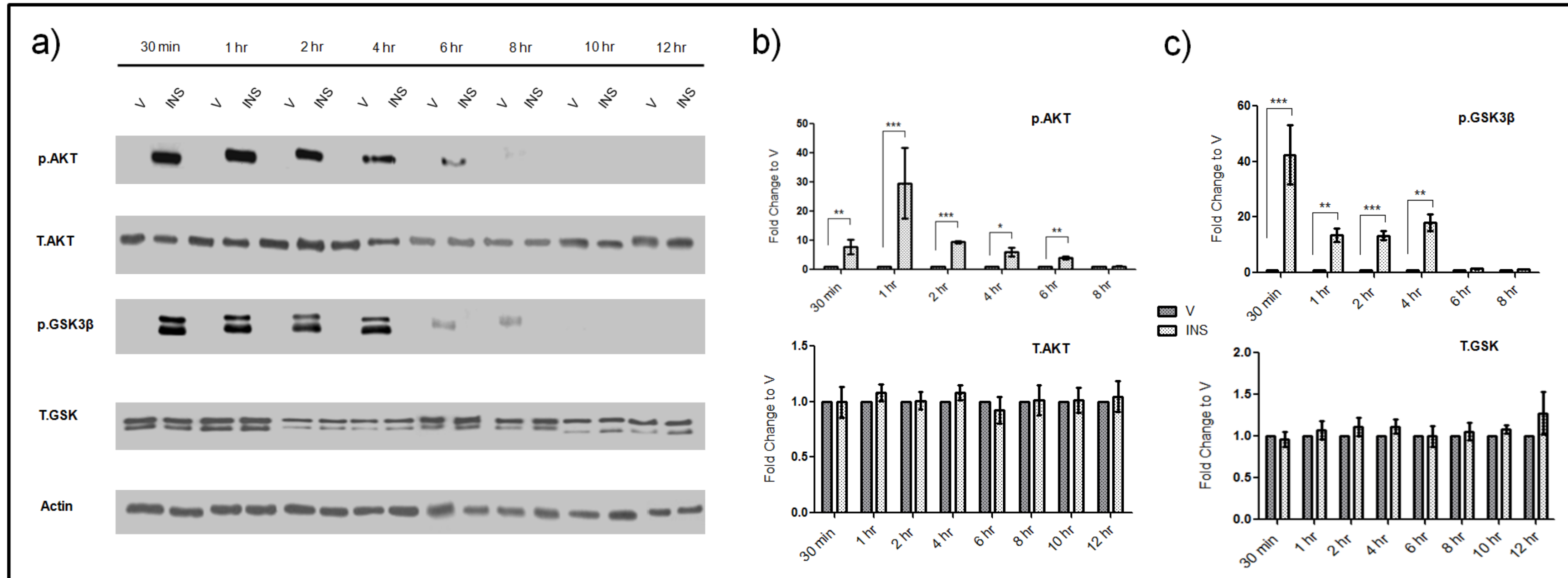

Figure 5: Time course of Insulin signaling in SHY treated with 1.72 μM insulin

a) SHY cells were treated with 1.72 μM insulin for 30 min, 1, 2, 4, 6, 8, 10, and 12 hours. pAkt at Ser473, pGSK3β and total protein levels were detected by western blotting, normalized to β-actin, and presented relative to vehicle (Two-tailed t-test, $p<0.05$, N=3). **b)** There was a significant increase in pAkt at 30 minutes and up to 6 hours and no significant change in total Akt was observed indicating insulin signaling started well before 30 minutes time frame and can last up to 6 hours. **c)** A significant increase in pGSK3β follows a similar trend to pAkt starting at 30 minutes and up to 4 hours with no significant increase in total GSK.

3.1.2 Cholesterol synthesis enzymes are up-regulated in SHY cells in response to insulin treatment

After confirming that insulin signaling occurs in SHY cells, the effects on cholesterol synthesis were investigated. Protein levels of HMGCR and DHCR24, enzymes in the cholesterol synthesis pathway, were determined by western blotting after treating SHY cells with various doses of insulin (0.1 nM, 0.5 nM, 1 nM, 5 nM, and 1.72 μM) for 8 hours (Figure 6a). The levels of HMGCR protein were significantly up-regulated at all insulin concentrations including physiological levels (0.1 – 1 nM) (Li, Barrett et al. 2005) (Figure 6b). Similarly, the levels of DHCR24 protein were significantly up-regulated at all insulin concentrations (Figure 6c) (Two-tailed t-test, $p<0.05$, N3). All of the protein bands were normalized to cellular β-actin protein levels.

Since all concentrations of insulin seem to have similar effects in SHY cells, the concentration of 1.72 μM with the most robust effect on pAkt (Figure 4a) was chosen to treat SHY cells in a time course from 30 min to 12 hours and monitor the levels of cholesterol synthesis enzymes. Several proteins were probed including HMGCR, DHCR24, DHCR7 and SQLE (Figure 7a). The levels of HMGCR protein started to significantly increase at 8 up to 12 hours, DHCR24 levels increased significantly starting at 6 up to 12 hours, DHCR7 levels increased significantly at 12 hours and SQLE levels started increasing significantly at 4 to 12 hours (Two-tailed t-test, $p<0.05$, N=3) (Figure 7b). This indicated that the cholesterol synthesis pathway was activated and cholesterol synthesis was occurring in SHY cells in response to insulin stimulation.

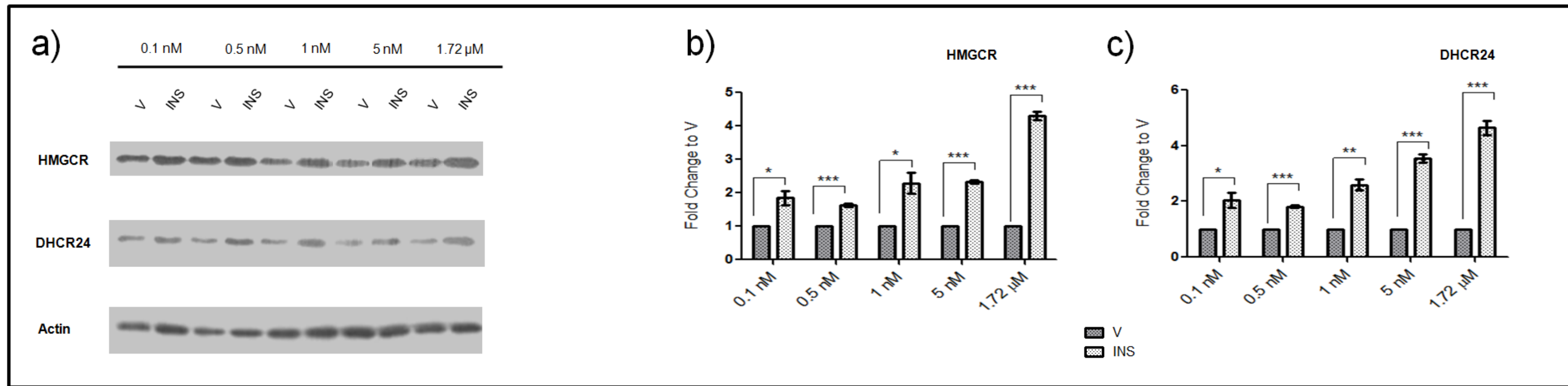

Figure 6: Cholesterol synthesis enzyme levels increases in SHY treated with insulin at physiological levels and higher

a) SHY cells were treated for 8 hours with insulin at concentrations 0.1 nM, 0.5 nM, 1 nM, 5 nM, and 1.72 µM and cholesterol synthesis enzymes HMGCR and DHCR24 protein levels were analyzed by western blotting, normalized to β-actin, and presented relative to vehicle (Two-tailed t-test, $p<0.05$, N=3). **b)** There was a significant increase in HMGCR protein levels at all insulin concentrations used. **c)** Similarly, a significant increase in DHCR24 protein levels was observed at all insulin concentrations used indicating cholesterol synthesis pathway in SHY was activated in response to insulin treatment.

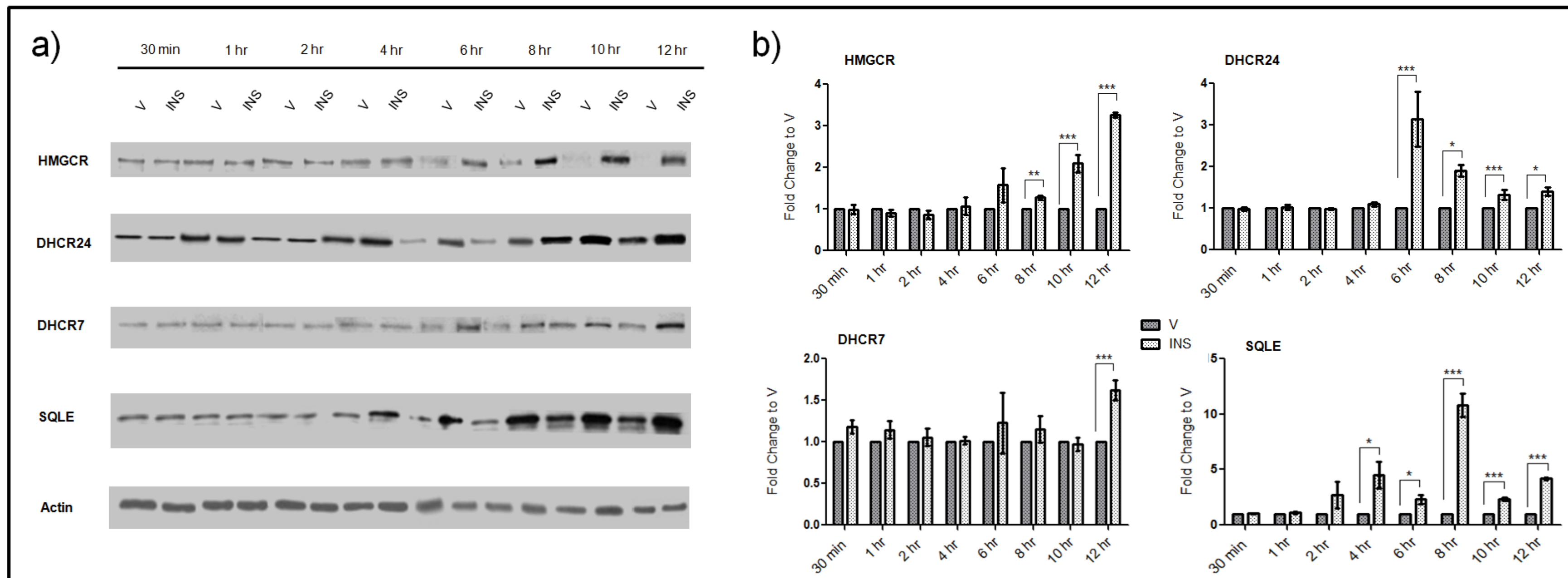

Figure 7: Cholesterol synthesis enzymes levels increase in SHY treated with 1.72 μM insulin

a) SHY cells were treated with 1.72 μM insulin for 30 min, 1, 2, 4, 6, 8, 10, and 12 hours. HMGCR, DHCR24, DHCR7 and SQLE protein levels were analyzed by western blotting, normalized to β-actin, and presented relative to vehicle (Two-tailed t-test, $p<0.05$, N=3). **b)** There was a significant increase in HMGCR protein levels starting at 8 hours and lasting up to 12 hours, similarly a significant increase in DHCR24 was observed starting at 6 up to 12 hours, DHCR7 was significantly up-regulated at 12 hours with increasing trends starting at 6 hours and finally SQLE was up-regulated significantly starting at 4 up to 12 hours. Cholesterol synthesis pathway in SHY was activated in response to insulin treatment at concentration 1.72 μM.

3.1.3 SREBP2 protein levels change in SHY cells in response to insulin treatment

Following the observed increases in cholesterol synthesis enzyme levels in SHY cells treated with 1.72 μM of insulin, the regulatory protein of cholesterol homeostasis, SREBP2, was evaluated by western blotting to see if it was involved as well. Cells were treated in a time course from 30 minutes to 12 hours and levels of the protein were evaluated by western blotting including the 126 kDa of SREBP2, considered to be associated with the ER, and the 55 kDa, considered to be the part of the protein that is cleaved and translocated to the nucleus to up-regulate cholesterol biosynthesis genes (Figure 8a). The 55 kDa subunit of SREBP2 was increased significantly at 1 hour only (Two-tailed t-test, $p<0.05$, $N=3$) (Figure 8b) indicating the involvement of SREBP2 in up-regulation of cholesterol synthesis enzymes. Moreover, there was an observed increase in the 126 kDa subunit at 1 hour, but it was not significant and it was followed by significant decreases in protein levels starting at 4 up to 8 hours (Figure 8b). This indicated that SREBP2 protein levels were changing in response to insulin treatment and the down-regulation of the 126 kDa of SREBP2 may be a negative feedback control mechanism in SHY cells.

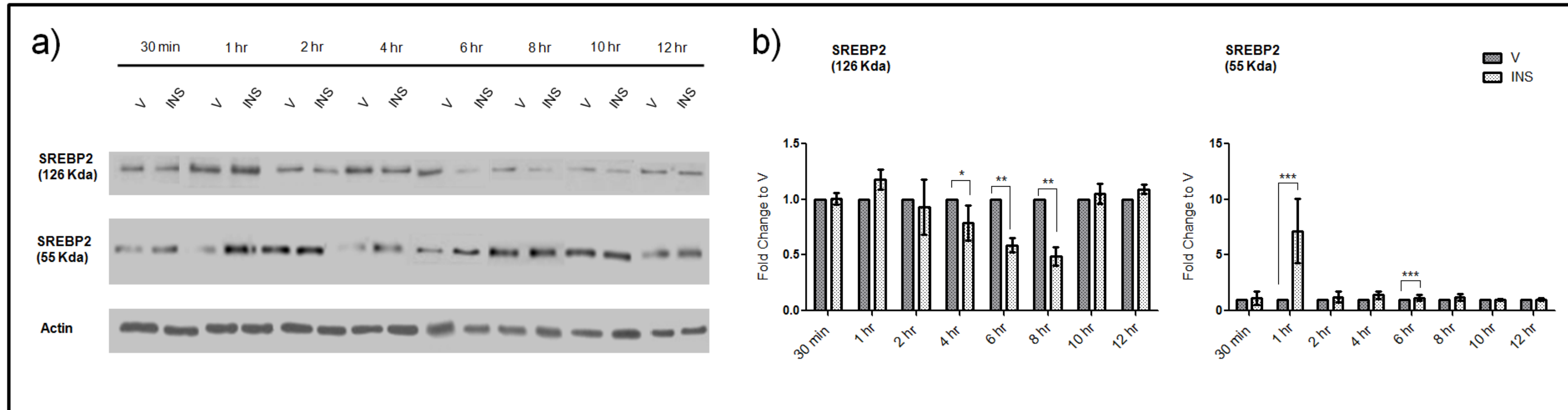

Figure 8: SREBP2 protein levels changes in SHY treated with 1.72 μM insulin

a) SHY cells were treated with 1.72 μM insulin for 30 min, 1, 2, 4, 6, 8, 10, and 12 hours. The 126 kDa and 55 kDa subunits of SREBP2 protein levels were analyzed by western blotting, normalized to β-actin, and presented relative to vehicle (Two-tailed t-test, $p<0.05$, N=3). **b)** There was an observed increase in the126 kDa subunit at 1 hour but it was not significant and it was followed by significant decreases in protein levels starting at 4 to 8 hours. The 55 kDa subunit of SREBP2 was increased significantly at 1 hour only. This indicated that SREBP2 protein levels were changing in response to insulin treatment. The down-regulation of the 126 kDa of SREBP2 may indicate negative feedback control in SHY cells.

3.1.4 QPCR analysis of cholesterol synthesis genes in SHY cells treated with 1.72μM insulin

Since the enzymes involved in the cholesterol synthesis pathway were up-regulated at the protein level, the expression of the genes of some of these enzymes was investigated to determine insulin effects. Cells were treated with 1.72 μM insulin concentration in various time points 1, 2, 4, 6, 8, 10 and 12 hours, and the levels of HMGCR, DHCR24 and SREBP2 gene expression were evaluated by QPCR. SREBP2 gene expression levels were significantly down regulated starting at 2 up to 10 hours (Two-tailed t-test, $p<0.05$, $N=3$) (Figure 9a), which reflected the down-regulation of the protein observed in Figure 8a. This indicated that SREBP2 gene expression levels changed in response to insulin treatment and the decrease may be a negative feedback mechanism in SHY cells. It could also indicate that SHY cells may respond to the high level of insulin by up-regulating SREBP2 very early in the treatment around 15 – 30 minutes time frame, which can drive the up-regulation observed in the two cholesterol synthesis genes HMGCR and DHCR24 later on. HMGCR was up-regulated starting at 6 up to 12 hours (Figure 9b). Similarly, DHCR24 was up-regulated starting at 4 up to 12 hours (Figure 9c). These results were reflective of the protein level increases observed of HMGCR and DHCR24 proteins, which started increasing significantly 2 hours after their gene up-regulation (Figure 7).

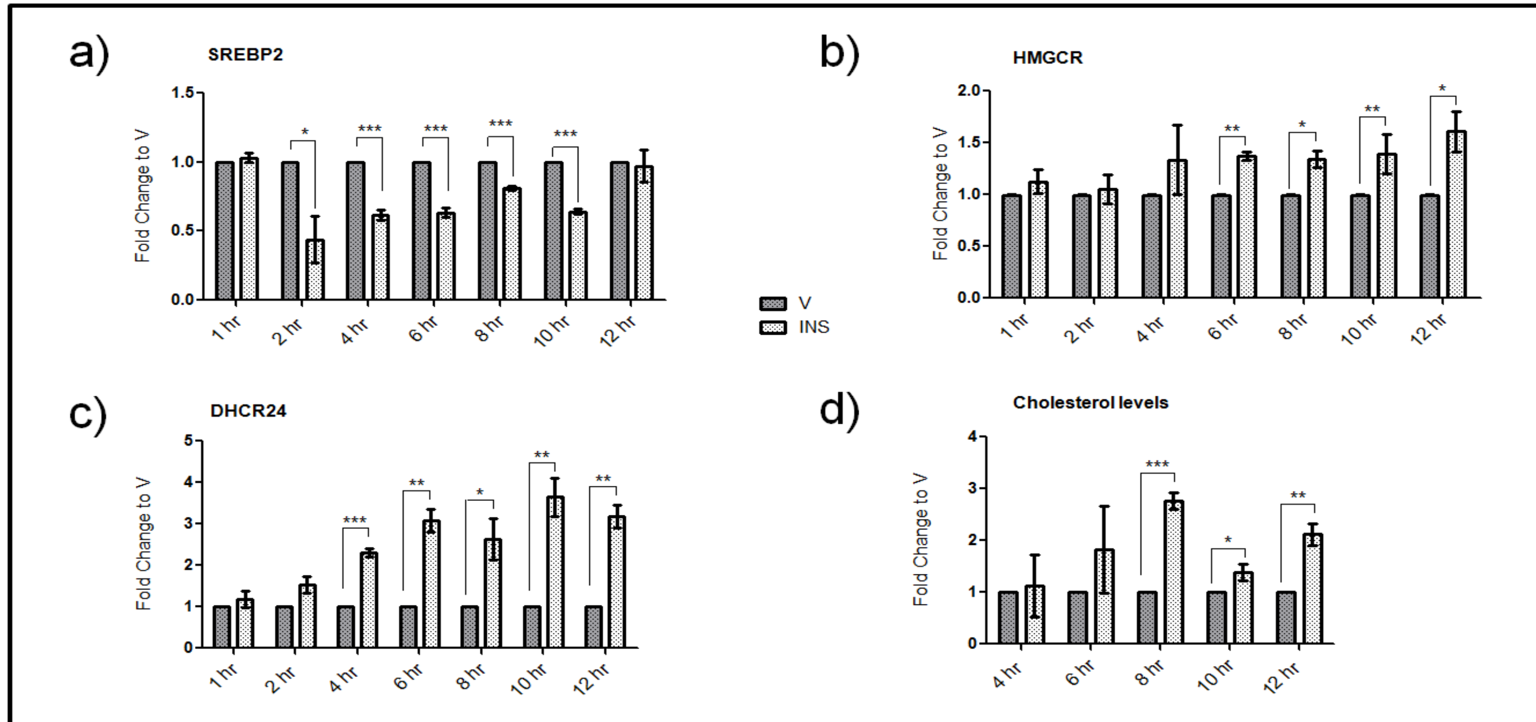

Figure 9: 1.72 μM insulin treatment of SHY cells up-regulates gene expression levels of HMGCR, and DHCR24 and increases total cholesterol levels

a) SHY cells were treated with 1.72 μM insulin for 1, 2, 4, 6, 8, 10, and 12 hours. Gene expression levels were measured by QPCR, normalized to β-actin, and presented relative to vehicle (Two-tailed t-test, $p<0.05$, N=3). SREBP2 was significantly down-regulated starting at 2 hour and up to 10 hours indicating negative feedback mechanism in SHY. **b)** HMGCR gene levels increased significantly starting at 6 to 10 hours. **c)** Levels of DHCR24 gene were increased significantly starting at 4 to 12 hours. **d)** SHY cells were treated with 1.72 μM insulin for 4, 6, 8, 10, and 12 hours. Cholesterol content was analyzed by Amplex Red cholesterol assay, normalized to protein content of cells, and presented relative to vehicle (Two-tailed t-test, $p<0.05$, N=3). Total cholesterol levels increased significantly starting at 8 to 12 hours.

3.1.5 Cholesterol levels increase in SHY cells treated with 1.72μM insulin

After showing the insulin-induced increases in both gene expressions, and protein levels of the cholesterol synthesis enzyme (SREBP2, HMGCR, and DHCR24), the actual cholesterol levels in the cultured cells were evaluated. SHY cells were treated with 1.72 μM insulin for 4, 6, 8, 10, and 12 hours and the total cell content of cholesterol was evaluated by Amplex Red cholesterol assay. Total cholesterol levels significantly increased starting at 8 to 12 hours (Figure 9d) (Two-tailed t-test, $p<0.05$, $N=3$). These results confirm that insulin regulated cholesterol synthesis in human neuronal SHY cells by acting on SREBP2, which leads to up-regulation of cholesterol synthesis enzymes and increased total cholesterol in the cell.

3.1.6 Insulin signaling occurs in N2a and N2a-APP cells at 1.72μM as measured by western blotting

Next, the effects of insulin treatment on the mouse neuroblastoma 2a cell lines (N2a and N2a-APP) were evaluated to determine if similar results would be obtained as compared to SHY. Also, I wanted to determine whether there were any differences between the parental N2a cells and the N2a cells stably expressing APP (N2a-APP). Importantly the N2a-APP cells produce and secrete copious amounts of Aβ into the media. N2a and N2a-APP cells were treated with insulin at 1.72 μM from 30 minutes to 12 hours. Levels of pAkt Ser473 and pGSK-3β were probed by western to confirm insulin signaling and they were normalized to β-actin (Figure 10a & 11a). Analysis by two-tailed t-test showed a significant treatment effect on pAkt and pGSK that showed increased

phosphorylation relative to vehicle control that lasted up to 8 hours for pAKT and 12 hours for pGSK for both cell lines (Two-tailed t-test, $p<0.05$, N=3) (Figure 10b & c, 11b & c) . There was no significant difference between total Akt and GSK to vehicle and there was no differences observed between the two cell lines in response to this concentration of insulin.

3.1.7 SREBP2 is up-regulated in N2a and N2a-APP cells in response to 1.72µM insulin as measured by western blotting

After the confirmation of insulin signaling in the mouse cell lines, SREBP2 protein levels were probed for by western blotting. N2a and N2a-APP cells were treated with insulin at 1.72 µM from 30 minutes to 12 hours, and the two subunits of SREBP2 (126 kDa, and 55 kDa) were evaluated (Figure 12a &13a). The 55 kDa subunit of SREBP2 protein level was increased significantly at 1 and 2 hours, and decreased significantly at 6 hours in the N2a parental cells (Two-tailed t-test, $p<0.05$, N=3) (Figure 12b). There was a significant increase in the 126 kDa subunit protein level at 4, 6, and 8 hours (Figure 12b) indicating SREBP2 response to insulin in N2a cells. In N2a-APP, the 55 kDa subunit protein level of SREBP2 increased significantly at 1, 2, 4 and 6 hours and decreased significantly at 12 hours (Figure 13b) while the 126 kDa subunit increased significantly starting at 2 hours to 10 hours (Figure 13b). SREBP2 protein levels increased in N2a and N2a-APP cells and there was no down-regulation of the 126 kDa as was observed with SHY cells at this concentration of insulin indicating regulatory differences between the mouse and human cell lines.

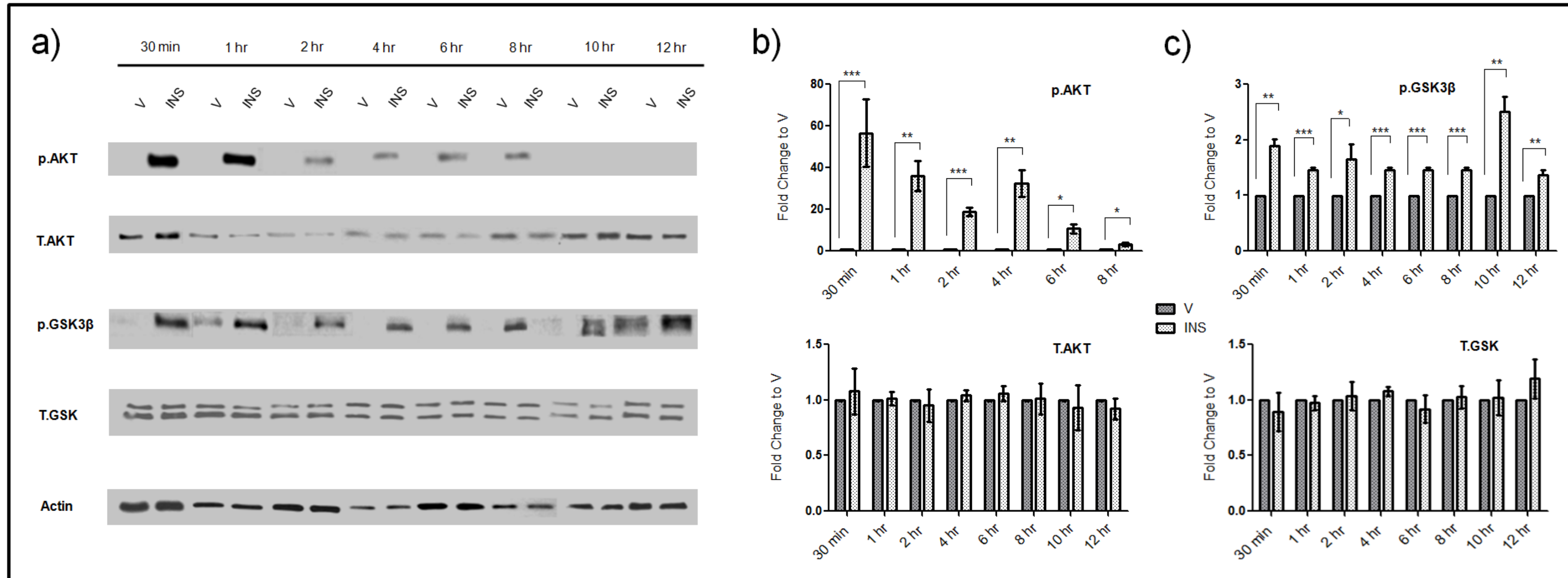

Figure 10: Insulin signaling occurs in N2a cells treated with 1.72 µM insulin

a) N2a cells were treated with 1.72 µM insulin for 30 min, 1, 2, 4, 6, 8, 10, and 12 hours, pAkt Ser473, pGSK3β, and total proteins were determined by western blotting, normalized to β-actin, and presented relative to vehicle (Two-tailed t-test, $p<0.05$, N=3). **b)** There was a significant increase in pAkt at 30 minutes and up to 8 hours and no significant change in total Akt was observed indicating insulin signaling started well before 30 minutes time frame and can last up to 8 hours. **c)** A significant increase in pGSK3β was observed starting at 30 minutes to 12 hours with no significant increase in total GSK. N2a cells responded to insulin similar to SHY cells but insulin signaling in these cells lasted for a longer time period than in SHY and GSK remained inhibited throughout the time course.

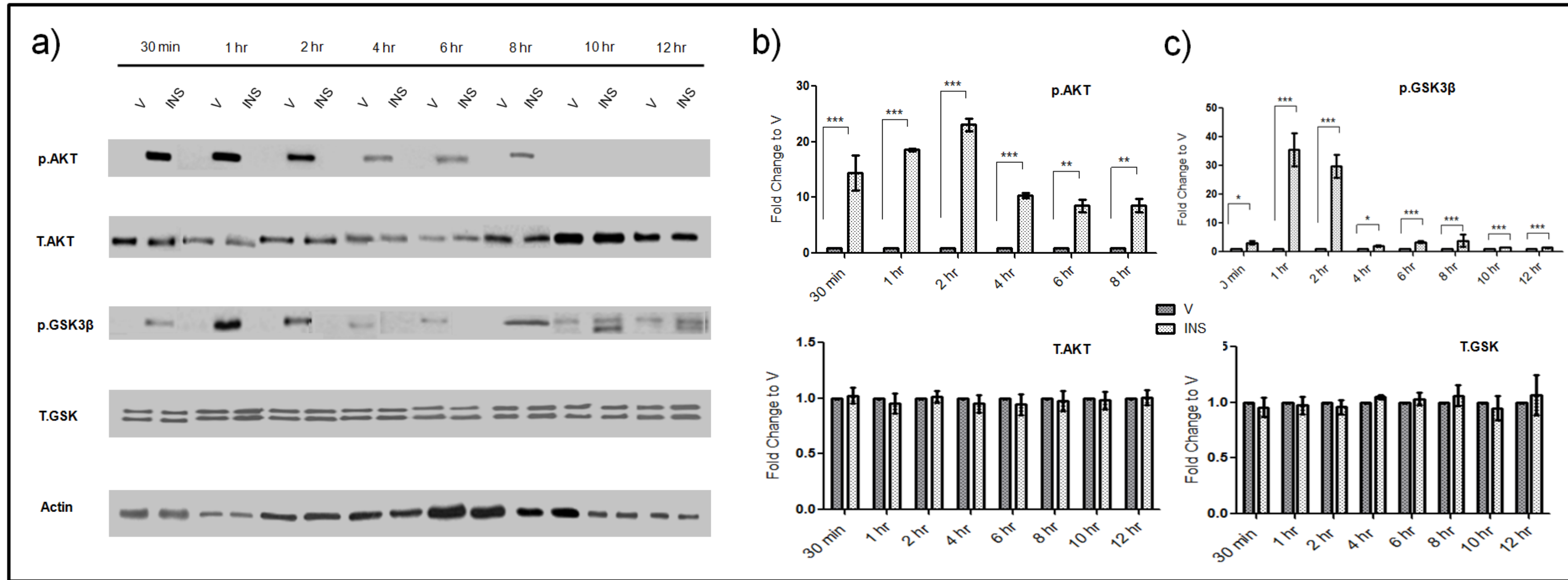

Figure 11: Insulin signaling occurs in N2a-APP cells treated with 1.72 μM insulin

a) N2a-APP cells were treated with 1.72 μM insulin for 30 min, 1, 2, 4, 6, 8, 10, and 12 hours, pAkt Ser473, pGSK3β and total proteins were determined by western, normalized to β-actin, and presented relative to vehicle (Two-tailed t-test, $p<0.05$, N=3). **b)** There was a significant increase in pAkt at 30 minutes and up to 8 hours and no significant change in total Akt was observed indicating insulin signaling started well before 30 minutes time frame and can last up to 8 hours. **c)** A significant increase in pGSK3β was observed starting at 30 minutes and up to 12 hours with no significant increase in total GSK. N2a-APP cells, like their counterpart N2a cells, responded to insulin similar to SHY but insulin signaling in these cells lasted for a longer time period than in SHY and GSK remained inhibited throughout the time course.

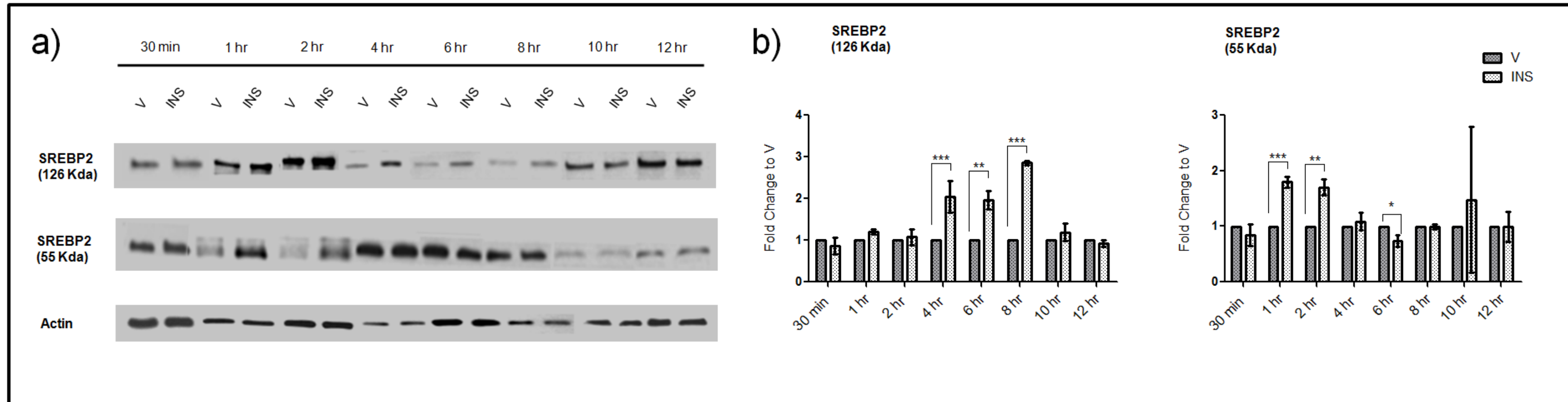

Figure 12: SREBP2 protein levels increase in N2a cells treated with 1.72 μM insulin

a) N2a cells were treated with 1.72 μM insulin for 30 min, 1, 2, 4, 6, 8, 10, and 12 hours. The 126 kDa and 55 kDa subunit levels of SREBP2 protein were analyzed by western blotting, normalized to β-actin, and presented relative to vehicle (Two-tailed t-test, $p<0.05$, N=3). **b)** There was a significant increase in protein levels starting at 4 hours and up to 8 hours in the 126 kDa subunit. The 55 kDa subunit of SREBP2 was increased significantly at 1 and 2 hours and decreased significantly by 6 hours. This indicated that SREBP2 protein levels were changing in response to insulin treatment in N2a mouse cell line.

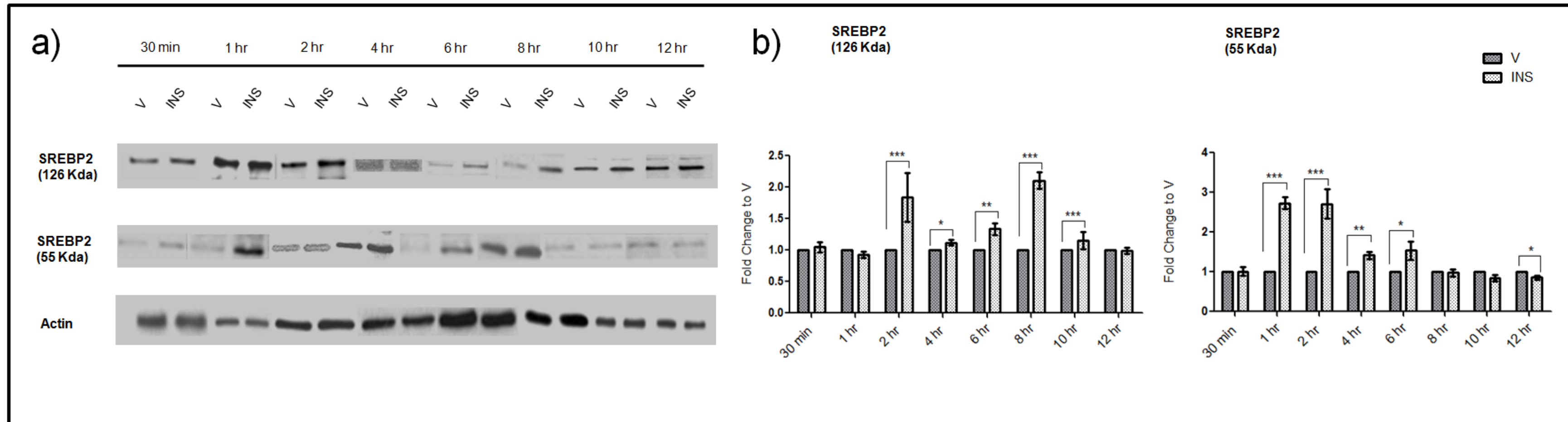

Figure 13: SREBP2 protein levels increase in N2a-APP cells treated with 1.72 µM insulin

a) N2a-APP cells were treated with 1.72 µM insulin for 30 min, 1, 2, 4, 6, 8, 10, and 12 hours. The 126 kDa and 55 kDa subunit levels of SREBP2 protein were analyzed by western, normalized to β-actin, and presented relative to vehicle (Two-tailed t-test, $p<0.05$, N=3). **b)** There was a significant increase in protein levels starting at 2 hours and up to 10 hours in the 126 kDa subunit. The 55 kDa subunit of SREBP2 was increased significantly at 1 hour and up to 6 hours and decreased significantly by 12 hours. This indicated that SREBP2 protein levels were changing in response to insulin treatment in N2a-APP mouse cell line.

3.1.8 *QPCR analysis of cholesterol synthesis genes in N2a and N2a-APP cells treated with 1.72μM insulin*

Since SREBP2 protein levels were increased in the mouse cell lines, the expression of the enzymes involved in the cholesterol synthesis pathway were hypothesized to be up-regulated in response to the regulatory protein. The gene expression levels of some of these enzymes were investigated by QPCR. N2a and N2a-APP cells were treated with 1.72 μM insulin for 1, 2, 4, 6, 8, 10 and 12 hours, and the levels of HMGCR, DHCR24 and SREBP2 gene expressions were evaluated by QPCR. SREBP2 gene expression levels were significantly up-regulated at 1 up to 6 hours in N2a (Two-tailed t-test, $p<0.05$, $N=3$) (Figure 14a), which reflected the up-regulation observed of the protein in Figure 12a. HMGCR gene expression level was up-regulated starting at 6 up to 12 hours (Figure 14b), and similarly, DHCR24 gene expression level was up-regulated starting at 4 up to 12 hours (Figure 14c). For N2a-APP cells, SREBP2 gene expression levels increased significantly starting at 1 up to 10 hours (Figure 15a), HMGCR gene expression level increased significantly starting at 8 up to 12 hours (Figure 15b) and levels of DHCR24 gene expression level increased significantly starting at 4 hours and up to 12 hours (Figure 15c). This indicated that SREBP2 gene expression levels changed in response to insulin treatment in both mouse cell lines causing the up-regulation of HMGCR and DHCR24 enzyme levels.

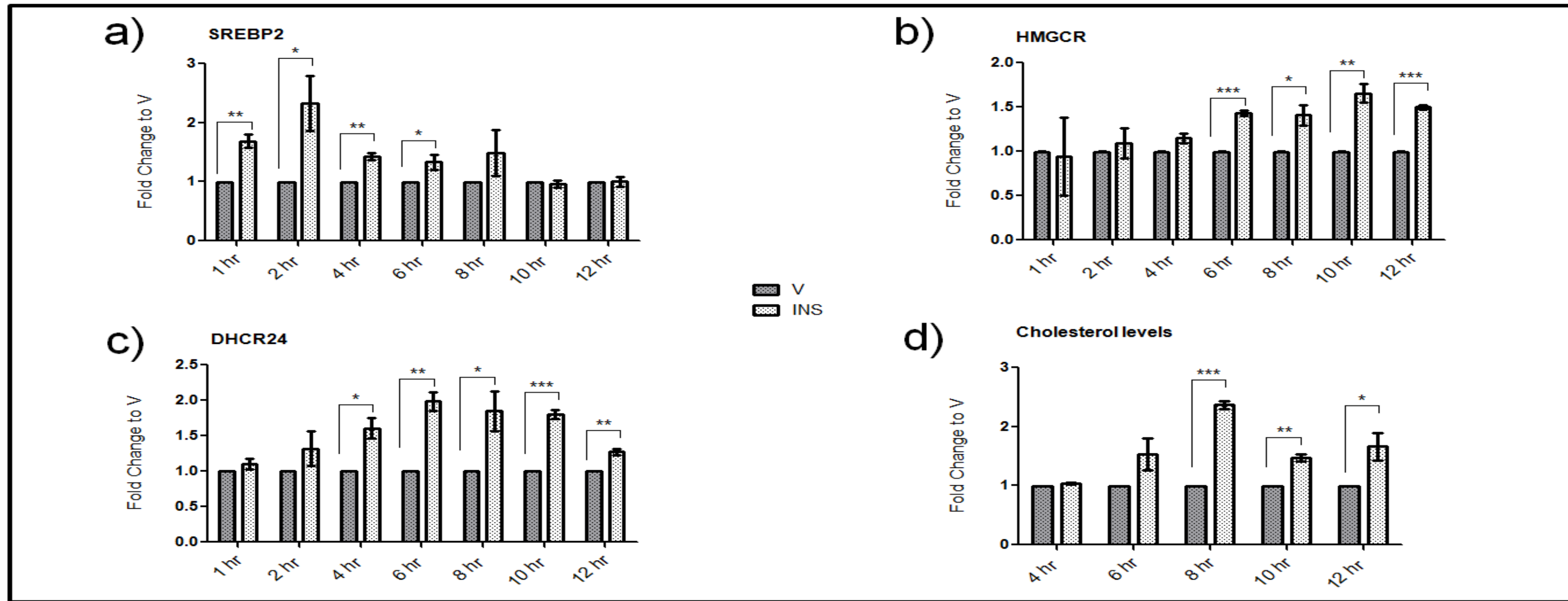

Figure 14: Treatment with 1.72 μM insulin up-regulates gene expression levels of SREBP2, HMGCR, and DHCR24 and increases total cholesterol levels in N2a cells

a) N2a cells were treated with 1.72 μM insulin for 1, 2, 4, 6, 8, 10, and 12 hours and cholesterol synthesis gene expression levels were determined by QPCR, normalized to β-actin, and presented relative to vehicle (Two-tailed t-test, $p<0.05$, N=3). SREBP2 gene expression level was significantly up-regulated starting at 1 to 6 hours indicating activation of the cholesterol synthesis pathway. **b)** HMGCR gene expression levels increased significantly starting at 6 to 12 hours. **c)** Levels of DHCR24 gene expression were increased significantly starting at 4 to 12 hours. **d)** N2a cells were treated with 1.72 μM insulin for 4, 6, 8, 10, and 12 hours and cholesterol content was determined by Amplex Red cholesterol assay. Levels were normalized to protein content of cells and presented relative to vehicle (Two-tailed t-test, $p<0.05$, N=3). Total cholesterol levels increased significantly starting at 8 to 12 hours.

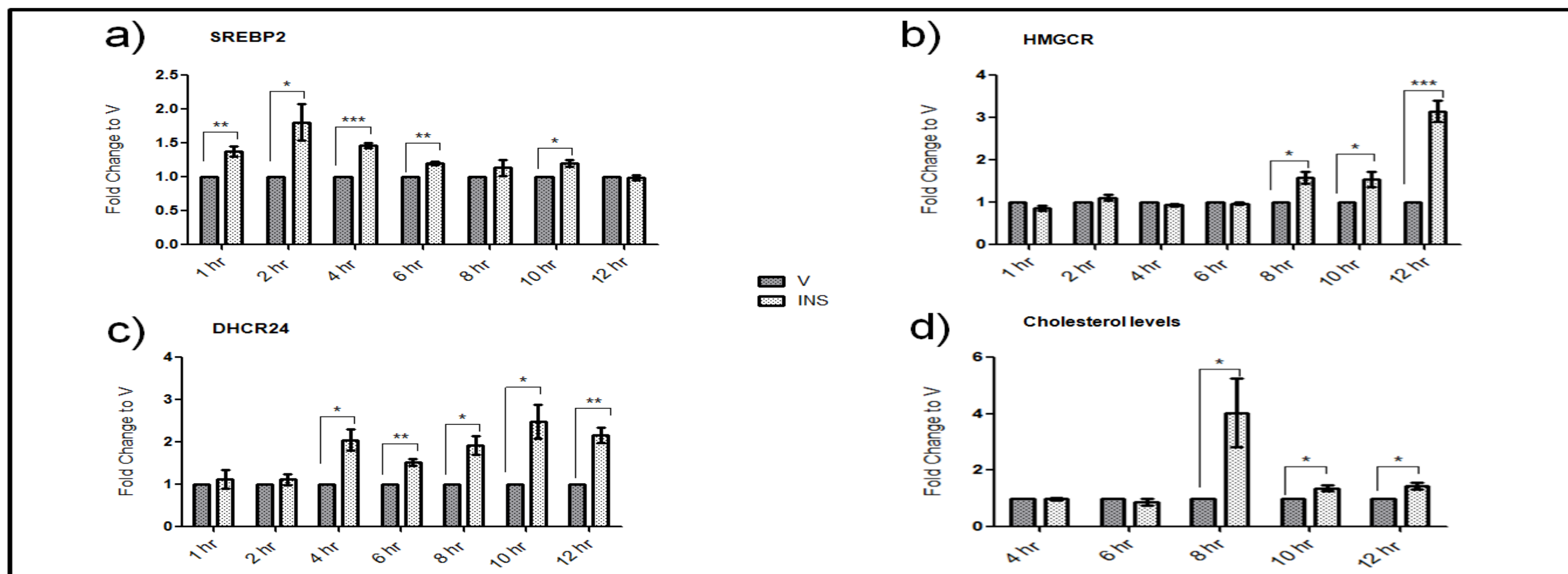

Figure 15: Treatment with 1.72 µM insulin up-regulates gene expression levels of SREBP2, HMGCR, and DHCR24 and increases total cholesterol levels in N2a-APP cells

a) N2a-APP cells were treated with 1.72 µM insulin for 1, 2, 4, 6, 8, 10, and 12 hours and cholesterol synthesis gene expression levels were determined by QPCR. Levels were normalized to β-actin, and presented relative to vehicle (Two-tailed t-test, $p<0.05$, N=3). SREBP2 gene expression level was significantly up-regulated starting at 1 to 10 hours indicating activation of the cholesterol synthesis pathway. **b)** HMGCR gene expression levels increased significantly starting at 8 to 12 hours. **c)** Levels of DHCR24 gene expression were increased significantly starting at 4 to 12 hours. **d)** N2a-APP cells were treated with 1.72 µM insulin for 4, 6, 8, 10, and 12 hours and cholesterol content was analyzed by Amplex Red cholesterol assay. Levels were normalized to protein content of cells, and presented relative to vehicle (Two-tailed t-test, $p<0.05$, N=3). Total cholesterol levels increased significantly starting at 8 to 12 hours .

3.1.9 Cholesterol levels increase in N2a and N2a-APP cells treated with 1.72μM insulin

After showing that insulin increased both gene expression and protein levels of some key cholesterol synthesis enzyme, the cholesterol levels in the treated cells were evaluated. N2a and N2a-APP cells were treated with 1.72 μM insulin for 4, 6, 8, 10, and 12 hours and the total cell content of cholesterol was evaluated by Amplex Red cholesterol assay. Total cholesterol levels significantly increased starting at 8 hours and up to 12 hours in both cell lines (Figure 14d & 15d) (Two-tailed t-test, $p<0.05$, N=3). These results confirm that insulin treatment increased the level of cholesterol in these cells.

3.2 Aβ causes insulin resistance in neural cells

3.2.1 Aβ inhibits insulin signaling in N2a-APP cells treated with insulin at physiological levels

N2a and N2a-APP cell lines were treated with insulin for 1 hour using physiological level concentrations 0.1 nM, 0.5 nM, 1 nM and higher concentrations of 5 nM and 1.72 μM. A comparison of insulin signaling between the parental cell line N2a and N2a-APP was carried out by visualizing pAkt Ser473 and pGSK3β in western blot (Figure 16a). N2a cells displayed significant increases in pAkt at all insulin concentrations, while N2a-APP had significant increases in pAkt at 1 nM and higher concentrations of insulin (Figure 16b). pGSK3β was significantly increased in N2a cells at all insulin concentrations and it was significantly increased in N2a-APP cells starting at 0.5 nM up to 1.72 μM

(Figure 16c). These results indicated that insulin signaling may be compromised in the N2a-APP cells due to the presence of Aβ peptides or APP. An evaluation of signal intensities between N2a and N2a-APP cells was carried out on the same x-ray film under the same exposure to show significantly reduced pAkt signals at lower concentrations of insulin and at 5 nM (Figure 16d). The signaling intensity of pGSK was also reduced in N2a-APP cells at 1 and 5 nM insulin concentrations compared to parental N2a cells (Figure 16e). Overall, there seemed to be an inhibition of insulin signaling or interference in the cells over-expressing APP and producing Aβ.

3.2.2 Aβ inhibits insulin-dependent up-regulation of cholesterol synthesis enzymes in N2a-APP cells treated with insulin at physiological levels

To support the notion that N2a-APP cells were not responding to physiological levels of insulin, a treatment from 1 to 10 hours at concentrations of insulin 0.1 nM, 0.5 nM, and 5 nM were carried out in N2a and N2a-APP cell lines. SREBP2, HMGCR and DHCR24 gene expression levels were evaluated by QPCR in both cell lines. SREBP2 gene expression levels were significantly up-regulated starting at 1 up to 6 hours in N2a cells and significantly down-regulated at 10 hours at concentrations 0.1 and 0.5 nM of insulin (Two-tailed t-test, $p<0.05$, N=3) (Figure 17a, & d). There was no significant change in SREBP2 observed at these low insulin concentrations in N2a-APP cells (Figure 17a & d), which reflected the poor response observed in insulin signaling in figure 16. At 5 nM insulin concentration, SREBP2 gene expression level was up-regulated at 1, 2 and 4 hours in N2a cells and at 1, 4 and 6 hours in N2a-APP cells (Figure 17g).

Similarly, HMGCR gene expression level was up-regulated in N2a cells at all insulin concentrations starting at 6 and up to 10 hours (Figure 17b, e, &h), but, it was only up-regulated at 5 nM insulin concentrations in N2a-APP cells at 4, 8 and 10 hours (Figure 17b, e & h). DHCR24 gene expression level followed a similar trend where it was up-regulated significantly in N2a cells at all insulin concentrations from 6 up to 10 hour; while it was only up-regulated at 5 nM in N2a-APP cells at 6 and 8 hours (Figure 17c, f & i). These results supported the observation of insulin dysregulation and resistance in N2a-APP cells at physiological levels as compared to parental N2a cells in Figure 16.

3.2.3 Aβ causes phosphorylation of pIRS Ser612 in N2a-APP cells at baseline levels

Further investigations were carried out to determine how insulin signaling was blocked in N2a-APP cells at physiological levels of stimulation by looking at the phosphorylation state of different IRS subunits. N2a and N2a-APP cells were plated in serum-deprived media for 24 hours and proteins were harvested for western blots. There was no difference between pAkt, pGSK, and pIRS Y895, which indicated that no insulin signaling was occurring in either cell line (Figure 18a). There was a significant phosphorylation of IRS at Ser612 in N2a-APP cells compared to N2a cells (Two-tailed t-test, $p<0.05$, N=3) (Figure 18b). Phosphorylation of IRS at this subunit has been associated with insulin resistance in the literature.

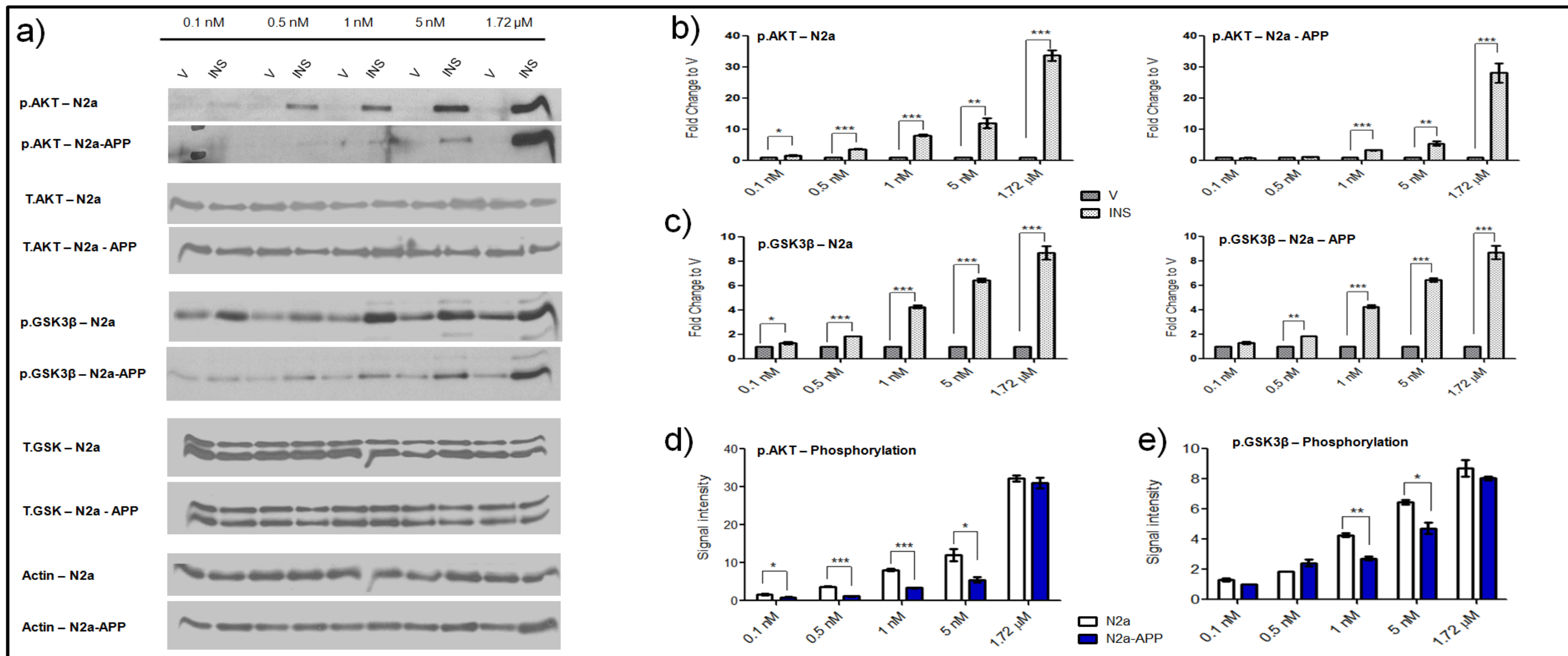

Figure 16: N2a-APP cells do not display insulin signaling when stimulated at physiological levels

a) N2a and N2a-APP cells were treated for 1 hour with insulin at concentrations 0.1 nM, 0.5 nM, 1 nM, 5 nM, and 1.72 μM and pAkt, pGSK-3β, total Akt, and total GSK protein levels were analyzed by western blotting, normalized to β-actin, and presented relative to vehicle (Two-tailed t-test, $p<0.05$, N=3). **b)** There was a significant increase in pAkt Ser473 in response to all concentrations of insulin used in N2a cells while a significant increase in N2a-APP occurred at 1 nM and higher concentrations only. **c)** pGSK3β was significantly increased at all insulin concentrations in N2a cells and at 0.5 nM and higher for N2a-APP cells. **d)** A comparison of signal intensities of pAkt was carried out between N2a and N2a-APP cells using the same x-ray film under the same exposure conditions. There was a significantly reduced signal starting at 0.1 nM up to 5 nM. **e)** Similarly, a signal intensity comparison of pGKS3β revealed significantly reduced signal at 1 and 5 nM in N2a-APP compared to N2a cells. These results indicate compromised insulin signaling in N2a-APP cells due to over expression of APP.

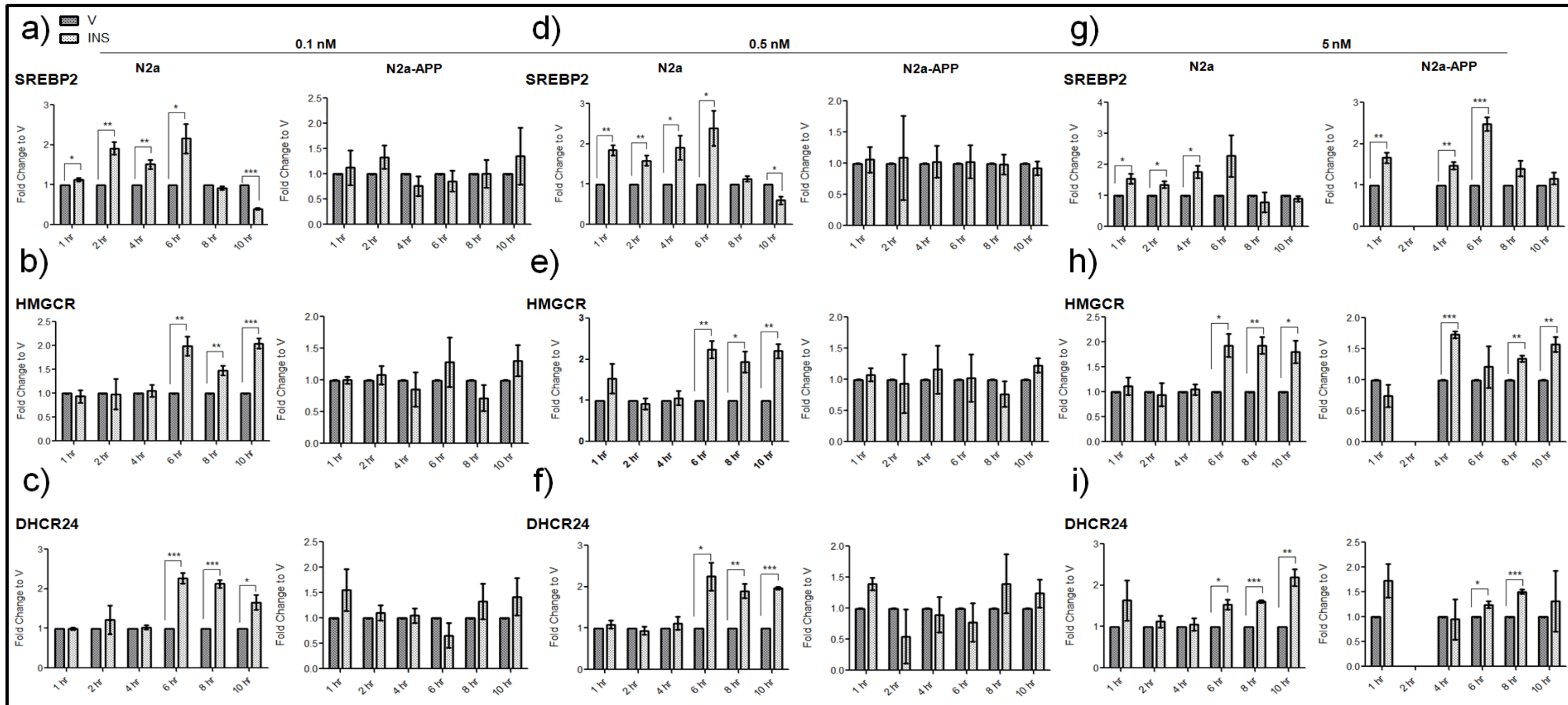

Figure 17: Cholesterol gene expression levels in N2a-APP cells do not change as in N2a cells at physiological insulin concentrations, but they increase at higher concentrations

N2a and N2a-APP cells were treated with 0.1, 0.5 and 5 nM insulin for 1, 2, 4, 6, 8, and 10 hours. Cholesterol synthesis gene expression levels were determined by QPCR, normalized to β-actin, and presented relative to vehicle (Two-tailed t-test $p<0.05$, N=3). **a), d), g)** SREBP2 was significantly up-regulated at all insulin concentrations starting at 1 up to 6 hours in N2a, and at 5 nM in N2a-APP. There was no change in N2a-APP cells at 0.1 and 0.5 nM indicating insulin resistance. **b), e), h)** HMGCR gene expression level was significantly up-regulated in N2a from 6 to 10 hours at all insulin concentrations, and at 5 nM in N2a-APP, but no change was observed in N2a-APP at 0.1 and 0.5 nM. **c), f), i)** DHCR24 gene expression level was significantly increased at 6 to 10 hours at all insulin concentrations in N2a cells, but only at 5nM in N2a-APP cells.

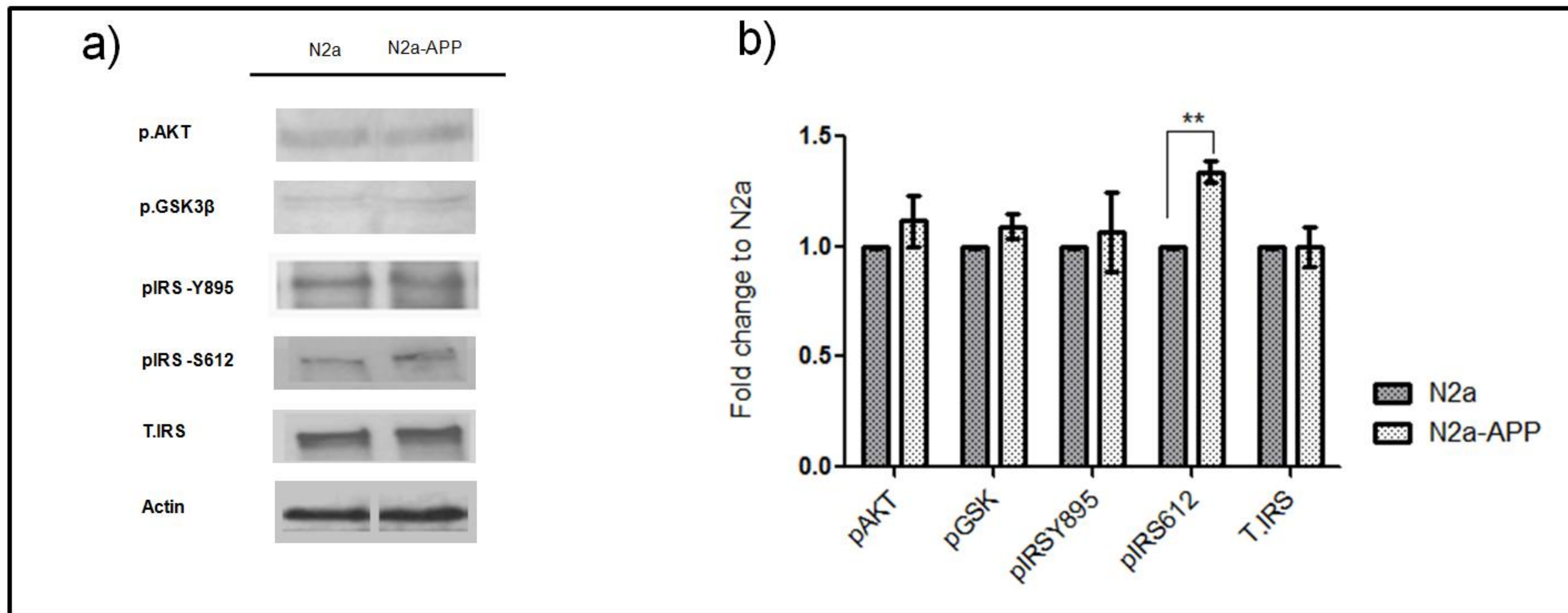

Figure 18: N2a-APP cells exhibit phosphorylated IRS on Ser612 compared to N2a cells at baseline

a) N2a and N2a-APP cells were plated in serum-free media for 24 hours and western blot was carried out for pAkt, pGSK3β, pIRS-Y895, pIRS-S612 and Total IRS. **b)** There were no significant differences between the cell lines with respect to pAkt, pGSK, pIRSY895 and T.IRS, but there was a significant increase of phosphorylation of IRS S612in N2a-APP cells. Levels were normalized to β-actin and presented relative to N2a cells (Two-tailed t-test, $p<0.05$, N=3).

3.2.4 Aβ causes phosphorylation of pIRS Ser612 activates inflammatory response, and affects cholesterol enzyme levels in SHY cells

To determine the effect of Aβ peptides on human neural cells, SHY was treated with 5 μM of Aβ1-42 and Scr Aβ1-42 for 1, 2, 4, 6 and 10 hours and western blot was performed on phosphorylation states of IRS Ser612 and c-Jun Ser73, and levels of HMGCR, and DHCR24 (Figure 19a). pIRS Ser612 was significantly up-regulated at 2, 4, 6, and 10 hours in comparison to the vehicle control (one-way Anova, $p<0.05$, N = 3) (Figure 19b). However, a significant up-regulation of pIRS Ser612 was also observed for the scrambled peptide compared to vehicle at 2 and 4 hours indicating that SHY cells were reacting to both Aβ and its scrambled peptide at earlier time points. At 6 and 10 hours there was a significant difference between Aβ and both of the controls indicating phosphorylation of IRS Ser612 in response to Aβ1-42 during that time frame. This indicated a potential insulin resistance effect in SHY cells due to Aβ.

The levels of phosphorylated c-Jun Ser73 was up-regulated significantly at 6, 8 and 10 hours in comparison to both controls (Figure 19c), which indicated inflammatory response in SHY cells to Aβ1-42 and activation of JNK. The significant increase in c-Jun at 2 hours in response to scrambled Aβ indicated that the cells were reacting to the foreign peptide, but this response normalized at later time points.

Levels of DHCR24 proteins were significantly decreased in SHY after treatment with Aβ starting at 2 and up to 8 hours in comparison to both controls and at 10 hours in comparison to vehicle control (Figure 19d). On the other hand,

levels of HMGCR protein increased significantly in response to Aβ at 6 hours in comparison to both controls (Figure 19e). These results indicated that Aβ1-42 interferes with cholesterol homeostasis in human neural SHY cells.

3.3 Insulin causes Aβ1-40 and Aβ1-42 secretion from intracellular to extracellular space in N2a-APP cells

After observing the effects of Aβ1-42 on insulin signaling, the effect of insulin treatment on Aβ metabolism was investigated. N2a-APP cells were treated with 1.72 μM insulin in a time course from 1 to 12 hours and with 5 nM insulin in a time course from 2 to 8 hours. Aβ1-40 and Aβ1-42 ELISA assays were carried out to measure the Aβ levels in the media following treatment compared to vehicle. A significant increase in Aβ1-40 was observed in the media of N2a-APP cells starting at 2 and up to 4 hours after treatment with 1.72μM insulin. Similarly, Aβ1-42 levels increased in the media at 4 to 8 hours (Figure 20a). Treating N2a-APP with 5 nM of insulin yielded similar results where Aβ1-40 and Aβ1-42 levels were significantly increased in the media from 2 to 8 hours (Figure 20b). These results indicated that insulin treatment mediated the secretion of Aβ from the intracellular space of cells to the extracellular space.

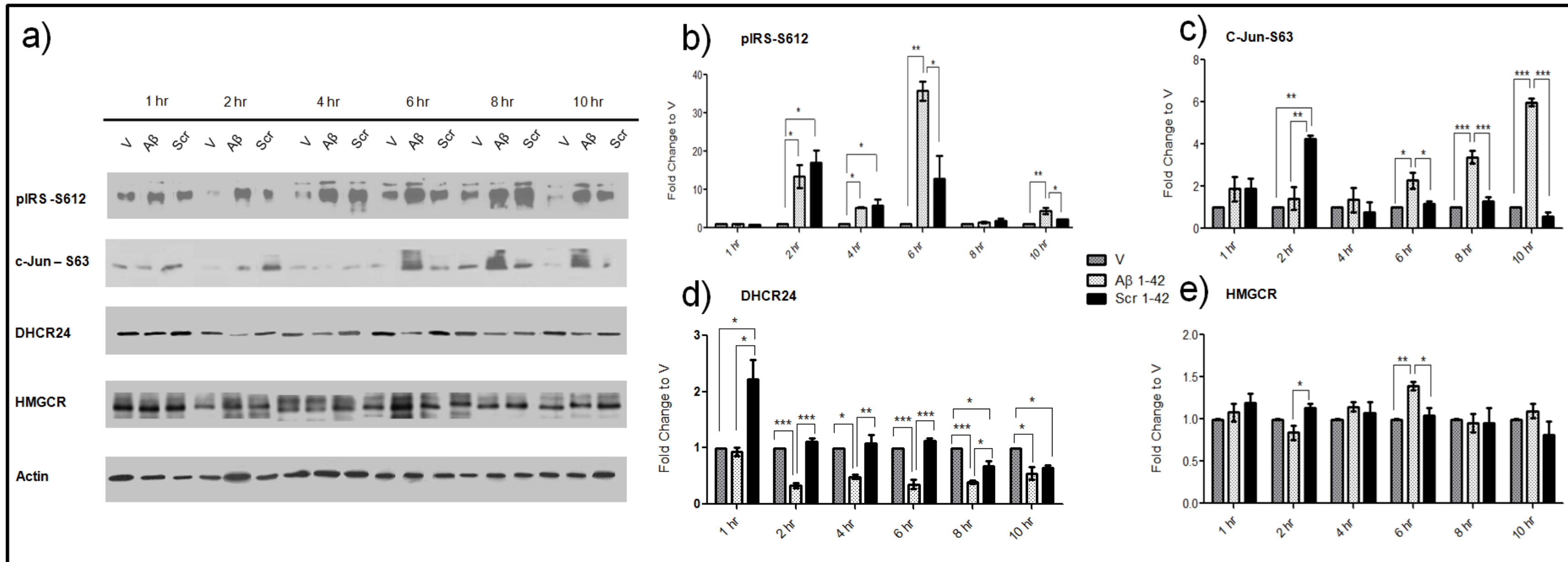

Figure 19: Aβ1-42 treated SHY cells displayed signs of insulin resistance, inflammation and cholesterol homeostasis dysregulation

a) SHY was treated with Aβ1-42 (Aβ) and its scrambled peptide (Scr) for 1, 2, 4, 6, 8 and 10 hours, pIRS Ser612, pc-Jun Ser63 and protein levels of DHCR24 and HMGCR were determined by western, normalized to β-actin, and presented relative to vehicle (one-way Anova, $p<0.05$, N = 3). **b)** Significant increase of pIRS Ser612 in SHY was observed at 2 and 4 hours in response to both Aβ1-42 and scrambled peptide and at 6 and 10 hours in response to Aβ1-42 only. **c)** Phosphorylation of c-Jun Ser63 was significantly increased starting at 6 to 10 hours in response to Aβ1-42 treatment indicating inflammatory response in SHY cells. **d)** Levels of DHCR24 proteins significantly decreased in response to the Aβ1-42 starting at 2 to 10 hours. **e)** Levels of HMGCR protein significantly increased at 6 hours in response to Aβ1-42 treatment.

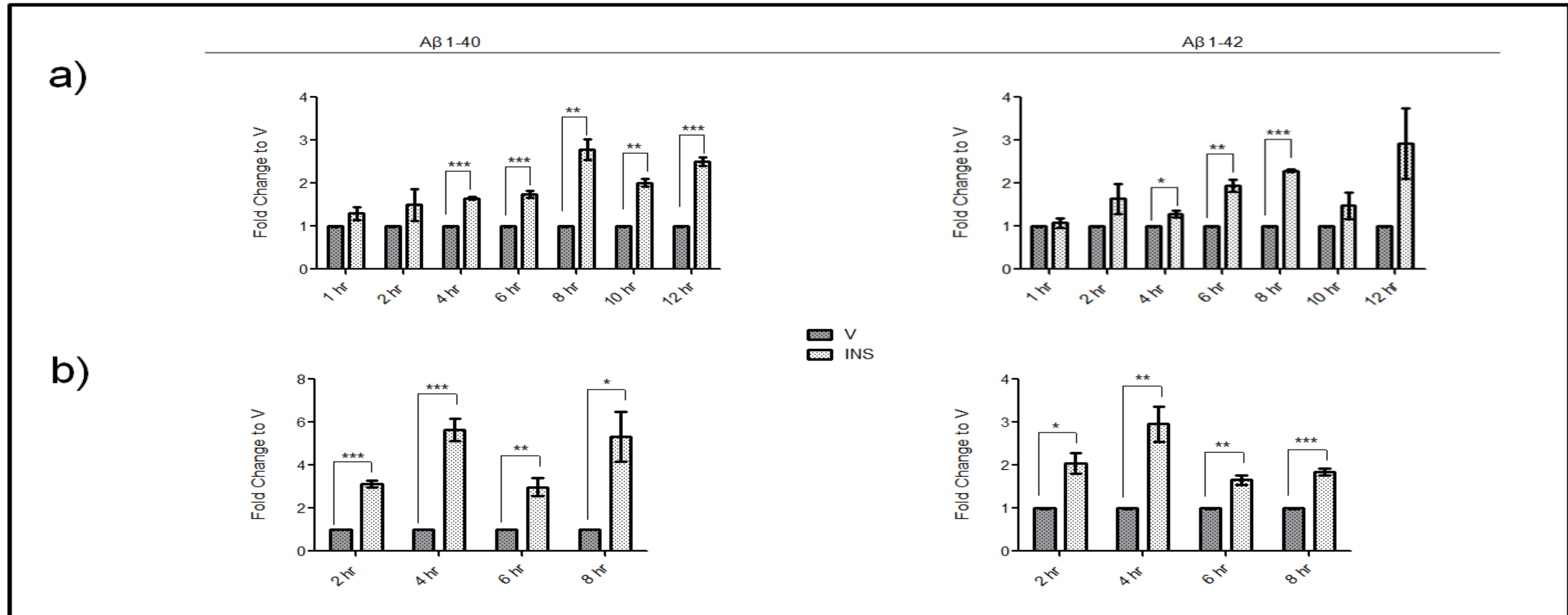

Figure 20: Insulin treatment causes Aβ1-40 and Aβ1-42 secretion in N2a-APP cells

a) N2a-APP cells were treated with 1.72 μM insulin from 1 to 12 hours and Aβ1-40 and Aβ1-42 ELISA assays were carried out on media of treated cells. Levels were normalized to cellular protein content, and presented relative to vehicle (Two-tailed t-test, $p<0.05$, N=3). There was a significant increase of Aβ1-40 in the media starting at 4 to 12 hours, and a significant increase of Aβ1-42 in the media from 4 to 8 hours in response to 1.72μM insulin treatment. **b)** N2a-APP cells were treated with 5 nM insulin from 2 to 8 hours. Aβ1-40 and Aβ1-42 ELISA assays were performed on media of treated cells. Levels were normalized to cellular protein content and presented relative to vehicle. Significant increase of Aβ1-40 and Aβ1-42 in the media from 2 to 8 hours after 5 nM insulin treatment was observed (Two-tailed t-test, $p<0.05$, N=3).

Discussion

Recently, insulin resistance and deficiency have been considered as an important feature in the progression of AD. Multiple studies looked at clinical correlations between failure of signals in insulin pathways and their association with brain disorders including stroke, AD, and Parkinson disease (Schubert, Gautam et al. 2004). Impairments of insulin signaling were not only described in human AD, but also in AD animal models (Takeda, Sato et al. 2010; Bomfim, Forny-Germano et al. 2012) and in rodents receiving intracerebral streptozotocin injections (Salkovic-Petrisic and Hoyer 2007). In addition, lower levels of insulin and IRs in AD brains further implicated insulin resistance in AD neuropathology (Steen, Terry et al. 2005; Craft 2012). Another recognized feature of the disease is the dysregulation of cholesterol and lipid homeostasis in AD brains. Several studies linked cholesterol, lipid, and lipoprotein dysregulation to insulin resistant state (Avramoglu, Basciano et al. 2006). One study confirmed the link between cognitive deficits in diabetic rodents and the impaired regulation of cholesterol synthesis in the brain to insulin resistance (Suzuki, Lee et al. 2010) However, identifying the molecular signaling pathways in the brain experiencing failure continues to be a challenge and no previous study looked at both insulin resistance and cholesterol homeostasis disruption within an AD model context.

Two mouse neuroblastoma 2a cell lines N2a and N2a transfected with human APP, and one human neurobalstoma cell line SHY were used in this study to investigate how Aβ and insulin interact with each other and affect insulin signaling and cholesterol homeostasis. Insulin effects on cholesterol signaling in

human and rodent neural cells were investigated as well as some signaling pathways in insulin resistance, specifically phosphorylation of IRS on Serine 612. Finally, insulin effects on Aβ trafficking was also examined.

4.1 Insulin regulates the synthesis of cholesterol in neural cells through SREBP2

Suzuki et al (2010) showed that in insulin-deficient diabetic mice, there was a reduction in the expression of SREBP2, the major transcriptional regulator of cholesterol metabolism, and in its downstream genes in the hypothalamus leading to reduction in cholesterol content of the synapses. This was corrected by insulin that was shown to regulate these genes in neurons and glial cells of the mice (Suzuki, Lee et al. 2010). Treatment of SHY cells with insulin in this study also showed that insulin is a regulator of cholesterol synthesis in human neural cells SHY and in mice neural cells N2a. Insulin signaling was gauged by looking at IRS downstream targets, specifically phosphorylation of Akt and GSK3β (Figure 2). SHY and N2a cells were treated by both physiological insulin levels (0.1 – 5 nM) and supraphysiological insulin levels (1.72 μM). Both cell types responded to all of insulin doses starting at physiological level doses 0.1 – 1 nM and higher doses 5 nM – 1.72 μM (Figure 4 & 16). The time course treatment from 30 minutes to 12 hours allowed for visualizing the effects of insulin from an early window and measure when the effects tapered off in each cell type. Also, the time course allowed for measuring any differences between the responses of human cells versus mouse cells to insulin signaling. It was determined that SHY's response to insulin was faster and shorter in duration compared to the response of N2a cells. This was due to the observation that

phosphorylation of Akt and GSK returned to baseline at 6 hours in SHY (Figure 5), while the phosphorylation was sustained in N2a cells up to 8 and 12 hours respectively (Figure 10 & 11).

The levels of enzymes that are involved in the cholesterol synthesis pathways increased in SHY cells in response to insulin treatment starting at 4 hours, and continued to be elevated up to 12 hours (Figure 7). The enzymes selected were from various points along the synthesis pathways and they include: 1) HMGCR, the rate limiting enzyme of cholesterol biosynthesis that is subjected to complex regulatory controls; 2) SQLE, the enzyme that catalyzes the conversion of squalene to lanosterol; and 3) DHCR7 and DHCR24 which catalyze the last steps of cholesterol synthesis (Figure 3). QPCR analysis of HMGCR and DHCR24 gene expression levels also showed an up-regulation in SHY cells in response to insulin treatment (Figure 9b & c), which preceded the increase in the proteins by two hours. Similarly, up-regulation of HMGCR and DHCR24 gene expression levels was observed for N2a and N2a-APP cells in response to 1.72 μM of insulin at similar time points to SHY cells (Figure 14-15b & c). These results indicated insulin can activate cholesterol synthesis pathway in these cell lines. Cholesterol levels were tested in all the cell lines and it was indeed elevated starting at 8 hours time point and continued to be elevated up to 12 hours (Figure 9-14 &15d). It is important to note that this elevation of total cholesterol levels in cultured cells might not be mimicked *in vivo* due to the fact that the majority (70%) of cholesterol present in the CNS is in myelin sheaths and it turns over very slowly (Barres 2008). Changes in cholesterol *in vivo* will

probably be observed in the membranes of astrocytes and neurons where this pool turns over more quickly and could be affected by short-term changes in cholesterol homeostasis (Okabe, Kim et al. 1999; Bjorkhem and Meaney 2004).

The cleaved form of SREBP2 protein at 55 kDa was observed to be elevated in SHY at 1 hour (Figure 8), whereas its 126 kDa counterpart that is associated with the ER showed significant decrease in protein levels starting at 4 and up to 8 hours indicating a possible mechanism of negative feedback regulation in SHY cells. Similarly, SREBF2 gene expression level was down-regulated in SHY cells starting at 2 hours (Figure 9a). In contrast, 55 kDa SREBP2 protein level in N2a cells was increased for a longer period of time than SHY and the126 kDa protein level was also elevated starting at 4 and up to 8 hours in both N2a and N2a-APP cells (Figure 12 & 13). Also, SREBP2 gene expression level was up-regulated in N2a and N2a-APP cells reflecting the observations in protein levels (Figure 14-15a). The elevated protein levels of SREBP2 in mouse cells match closely with results obtained from the *in vivo* study by Suzuki et al, 2010, but the decrease of the protein in SHY suggests different signaling events in human cells compared to mice. Literature search on the topic offered no information about potential differences between mouse and human cells pertaining to insulin signaling and cholesterol synthesis. One possible explanation can be that tight regulation of SREBP2 in the brain is required since it has been shown that increased levels of SREBP2 protein can induce BACE1 expression in human cells (Mastrocola, Guglielmotto et al. 2011). A quick effect on SREBP2 protein levels by insulin can serve to activate cholesterol biosynthesis and a quick down-regulation ensures

that prolonged SREBP2 signaling does not occur. This hypothesis is based on the observations that statins activate SREBP2, which induces apoptosis of various cells (Xia, Tan et al. 2001; Gibot, Follet et al. 2009) indicating that aberrant regulation of SREBP2 could lead to SREBP2-dependent pro-apoptotic signaling (Yoshino, Tabunoki et al. 2011). An article by Lovestone, 2010, outlined how transgenic mice only offer a partial model for AD by summarizing the observed differences between studies in the literature. The authors suggested that differences between longevity in humans and mice as well as its relationship to insulin signaling could explain why age is the most important risk factor for AD development (Lovestone and Killick 2010). It is important to study multi-factorial diseases such as AD in an organism model that resembles as much as possible the pathologies seen in human patients, which would allow discerning abnormal molecular failures and identifying potential therapeutic targets. Further research is warranted into potential differences of insulin signaling in Homo sapiens versus other species to improve models of AD. Nevertheless, the observation that insulin treatment regulated cholesterol synthesis in neural cells is an important one. Cholesterol is shown to be essential for synaptogenesis and synapse function and depletion of cholesterol leads to blocking of the biogenesis of synaptic vesicles leading to decreased neurotransmitter release (Thiele, Hannah et al. 2000; Hering, Lin et al. 2003; Rohrbough and Broadie 2005). Insulin resistance and deficiency in AD could cause altered synaptosomal membrane cholesterol, which in turn might contribute to glutamatergic synapse dysfunction and altered glutamate release

(El-Husseini, Schnell et al. 2000; Trudeau, Gagnon et al. 2004). The mechanisms of insulin up-regulation of cholesterol synthesis genes through SREBP2 will be of future work to this manuscript where a potential pathway of analysis is the PI3K/Akt/mTOR pathway. This is based on work done by Porstmann et al, which demonstrated that SREBP activity is regulated by mTORC1 in an Akt-dependent cell growth manner (Porstmann, Santos et al. 2008).

4.2 Aβ causes insulin resistance in neural cells

My work demonstrates that N2a-APP cells have an insulin resistant phenotype. There was a reduced signaling intensity of pAkt and pGSK in N2a-APP cells at low insulin concentrations (0.1 – 0.5 nM) and no signaling of pAkt and pGSK at 0.1 nM compared to N2a cells (Figure 16). This indicates that insulin signaling may be compromised in N2a-APP cells due to the presence of Aβ peptides or/and hAPP. My work shows that this insulin resistance phenotype in N2a-APP cells led to disrupted insulin-dependent cholesterol regulation when treated by low insulin concentrations of 0.1 – 0.5 nM. None of the cholesterol biosynthesis genes responded to insulin stimulation in N2a-APP cells at these concentration levels in comparison to parental N2a cells that showed up-regulation of all genes (Figure 17). Cholesterol genes did get up-regulated at higher concentrations of insulin in N2a-APP cells [5 nM insulin treatment (Figure 17) and 1.72 μM insulin (Figure 15)] including SREBF2, DHCR24 and HMGCR.

Molecularly, Aβ appeared to cause phosphorylation of IRS-1 on serine 612 in N2a-APP cells when compared to N2a cells (Figure 18). IRS-1 contains multiple

tyrosine phosphorylation motifs serving as docking sites for proteins that mediate the metabolic and growth-promoting functions of insulin (Sun, Miralpeix et al. 1992; Myers, Sun et al. 1993; Wang, Myers et al. 1993). It also contains over 30 serine and threonine phosphorylation sites that upon phosphorylation uncouple IRS-1 from the insulin receptor, thereby inhibiting its tyrosine phosphorylation and insulin signaling (Ozes, Akca et al. 2001; Li, Soos et al. 2004). Ser612 can be phosphorylated by protein kinase C (PKC), ERK, and mTOR (De Fea and Roth 1997; Ozes, Akca et al. 2001; Gual, Gremeaux et al. 2003; Boura-Halfon and Zick 2009). In AD post-mortem analysis, mTOR is reported to be excessively activated (An, Cowburn et al. 2003; Griffin, Moloney et al. 2005) and Aβ oligomers were shown to activate ERK2 (Chong, Shin et al. 2006), JNK (Ma, Yang et al. 2009; Bomfim, Forny-Germano et al. 2012) and PKC (Miscia, Ciccocioppo et al. 2009). A recent study showed that Aβ1-42 required PKC to induce neuronal toxicity and death (Manterola, Hernando-Rodriguez et al. 2013). Treatment of SHY with Aβ1-42 caused phosphorylation of IRS-1 at Ser612 at 6 hours which continued to be phosphorylated at 10 hours (Figure 19). These results suggested that pIRS-1 Ser612 could be one of the targets of Aβ induced insulin resistance in neural cells. Moreover, Aβ1-42 caused increased phosphorylation of c-Jun at serine 63 starting at 6 hours indicating activation of JNK and an inflammatory response in SHY cells (Figure 19). It has been demonstrated in many studies that AD brains exhibit a profound loss of synapses, neuronal degeneration, microglial proliferation and activation of many inflammatory processes (Jellinger 2000; Yao, Chinnici et al. 2004; Lull and Block

2010). While the classically defined features of inflammation such as edema and neutrophil invasion is not seen in AD brain, well characterized inflammatory processes are constant elements of the neuropathology associated with brain degeneration in AD (Akiyama, Barger et al. 2000; Jellinger 2000). Inflammation mediators in AD include activated complement proteins, cytokines, chemokines, lipoproteins, growth factors and many other proteins and enzymes (Pratico and Trojanowski 2000). Two specific cytokines, TNFα and interleukin 1 (IL-1β), have been extensively studied and are markers of inflammation in serum and CSF of AD brain (Laurin, David Curb et al. 2009; Schuitemaker, Dik et al. 2009; Thambisetty, Simmons et al. 2010). Over-expression of IL-1β and TNFα has a role in amyloid plaque formation and levels of TNFα in CSF of individuals with MCI predicted the likelihood of developing AD (Tarkowski, Andreasen et al. 2003; Clark and Vissel 2013). It has been well recognized that inflammatory cytokines particularly IL-1β and TNFα can induce insulin resistance (Zúñiga, Shen et al. 2010) while IL-4, an anti-inflammatory cytokine, protects against it (Chang, Ho et al. 2012). This is where the toxic effects of Aβ, inflammatory responses, dysregulation of cholesterol homeostasis can converge onto insulin resistance. Incidentally, activation of inflammatory responses in SHY after treatment with Aβ demonstrated by activated JNK was in the same time frame of IRS-1(Ser612) phosphorylation (Figure 19). Surprisingly, N2a-APP showed no phosphorylation of c-Jun ser63 and there was no activation of JNK in comparison to N2a cells (data not shown). N2a-APP continuously generates hAPP and Aβ in the medium yet they survive in cultured dishes, thrive and can be continually passaged. This

indicates that there has been an adaptation mechanism in these cells so they do not go under apoptosis due to Aβ toxicity. This shows that N2a-APP cells do not demonstrate all the pathological hallmarks that are seen in response to Aβ and results from studies involving N2a-APP should be interpreted carefully.

4.3 Aβ dysregulates the cholesterol synthesis pathway

Another effect of treating SHY with Aβ1-42 was the marked decrease in DHCR24 protein levels in the cells starting at early time points and continuing to be decreased to 10 hours, while HMGCR protein levels increased significantly at 6 hours (Figure 19). This clearly shows that Aβ disrupts cholesterol homeostasis in SHY cells at 5 μM concentration and in the utilized media conditions.

DHCR24 enzyme catalyzes the reduction of the Δ^{24} double bond in desmosterol to produce cholesterol in the cholesterol biosynthesis pathway. Another name for DHCR24 is selective AD indicator 1 (seladin 1) and it is a multifunctional protein due to its enzymatic, antioxidant, anti-apoptotic and neuroprotective activities (Lu, Kambe et al. 2008; Drzewinska, Pulaski et al. 2009). Seladin 1 is ubiquitously expressed in endocrine glands and in the brain (Greeve, Hermans-Borgmeyer et al. 2000) . It is found to be down-regulated in AD vulnerable brain regions such as the inferior temporal cortex (Greeve, Hermans-Borgmeyer et al. 2000; Sarajarvi, Haapasalo et al. 2009) and decreased seladin 1 mRNA levels are shown to be inversely correlated with hyper-phsophorylated Tau in AD brain (Iivonen, Hiltunen et al. 2002). Some genetic studies indicate that there is a gender dependent effect of seladin 1 polymorphisms on the susceptibility to AD and can be associated with AD risk in

men (Lamsa, Helisalmi et al. 2007; Feher, Juhasz et al. 2012). Interestingly, seladin 1 is found to be a target gene of LXR, which is reported to decrease in AD brain (Wang, Rogers et al. 2008; Xiong, Callaghan et al. 2008).

DHCR24 enzyme has been shown to regulate lipid raft formation (Wang, Rogers et al. 2008), protect neurons from Aβ mediated toxicity, and regulate Aβ1-42 formation through counteracting the β-secretase cleavage of APP and placement of β-secretase in lipid rafts (Crameri, Biondi et al. 2006; Lamsa, Helisalmi et al. 2007). Recent findings indicate that there is a disorganization of lipid rafts in AD and that reduction of cellular cholesterol favors the co-localization of BACE1 and APP in non-raft membrane domains and hinders generation of the Aβ degrading enzyme, plasmin (Stefani and Liguri 2009). Depletion of DHCR24 enzyme under stress conditions can result in enhanced levels and activity of BACE1 and increased amyloidogenic cleavage of APP (Sarajarvi, Haapasalo et al. 2009). This is due to depletion of Golgi-localized gamma-ear containing ADP-ribosylation factor binding proteins (GGAs), which act as monomeric adaptors involved in cargo selection, clathrin binding and membrane localization (Hirst, Lindsay et al. 2001; Sarajarvi, Haapasalo et al. 2009). Moreover, the DHCR24 dependent cholesterol synthesis reduces membrane aggregate interaction and cell damage associated to amyloid-induced imbalance in cytosolic Ca^{2+} (Cecchi, Rosati et al. 2008)

DHCR24 protein has been shown to have anti-inflammatory activities where it can directly scavenge hydrogen peroxide protecting cells from oxidative stress-induced apoptosis (Lu, Kambe et al. 2008). Also, it inhibits the activation of

caspase-3, a key modulator of apoptosis (Peri and Serio 2008) and can mediate the anti-inflammatory effects of high-density lipoproteins (McGrath, Li et al. 2009). In one study, Simvastatin was shown to modulate DHCR24 protein levels in human neuronal cells by increasing its production in a dose and time dependent manner (Ramos, Sierra et al. 2012). This can enhance neuroprotection from inflammation and can be one of the ways statins appear to reduce progression of AD, rather than exerting effects on cholesterol levels (Fonseca, Resende et al. 2010). Moreover, a study by Lu and colleagues demonstrated that seladin 1 knockout mice had lower cholesterol biosynthesis in their embryonic fibroblasts, which resulted in impaired insulin-dependent phosphorylations of IRS-1, Akt, and Bad and induced apoptosis in the absence of external cholesterol supply (Lu, Kambe et al. 2006).

My results of decreased DHCR24 protein levels in SHY cells in response to Aβ1-42 treatment reflected other work in the literature and the observed decrease of the protein in AD. However, the mechanism by which Aβ1-42 caused the decrease in the protein and whether it is associated with insulin signaling impairments remains to be elucidated.

My work shows that HMGCR levels were up-regulated at 6 hours in SHY after treatment with Aβ1-42 coinciding with inflammatory responses in the cells. There is no literature, to the author’s knowledge, that looks specifically at levels of HMGCR in AD. Some research was done on the mevalonate pathway in which HMGCR is the rate limiting step. The mevalonate pathway does not only mediate cholesterol synthesis but also other lipids such as isopernoids (Brown and

Goldstein 1980). Isoprenoids are complex active lipids which include long chain isoprenoid dolichol, and short chain isoprenoid farnesyl pyrophosphate (FPP) and geranylgeranyl pyrophosphate (GGPP) (Hall 2005). FPP and GGPP lipid levels and their synthase gene expression levels have been reported to be elevated in the frontal cortex of AD patients (Hooff, Peters et al. 2010; Hooff, Wood et al. 2010). FPP and GGPP covalently attach to small guanosine triphosphoate-ases (GTPases), which enables these proteins to be inserted into membranes resulting in activation of pathways involved in inflammation, oxidative stress, and cell proliferation (Cole and Vassar 2006; Gelb, Brunsveld et al. 2006; Linseman and Loucks 2008). GTPase signaling can also control multiple aspects of amyloidogenesis including trafficking of APP, BACE1 and γ-secretase (Di Paolo and Kim 2011). HMGCR inhibitors or statins, have been shown to affect APP distribution and reduce Aβ generation by depleting GGPP independent of cholesterol levels (Ostrowski, Wilkinson et al. 2007; Won, Im et al. 2008; Tamboli, Barth et al. 2010). Statins are also reported to inhibit Aβ stimulated expression of IL-1β, and other inflammatory markers like nitric oxide (Willey and Elkind 2010). These anti-inflammatory actions of statins are attributed to their ability to reduce the levels of isoprenyl intermediates in the mevalonate pathway (Cordle and Landreth 2005; Won, Im et al. 2008). This suggests that the up-regulation seen in HMGCR in SHY could be to increase isoprenoids levels needed for the inflammatory response to Aβ1-42. Specific mechanisms of how HMGCR up-regulation occurs, and whether there is an increase in total

cholesterol or a decrease in response to treatment with Aβ1-42 requires further investigation.

Conflicting reports on cholesterol's role in AD exist in the literature. Some report lowered brain cholesterol/phospholipid ratio levels in aged and AD brains and increases in APP processing and Aβ accumulation (Stefani and Liguri 2009). While other demonstrate that increased cholesterol levels and hypercholesterolemia may increase AD risk (Sharpe, Wong et al. 2012). Clearly, the relationship between cholesterol and AD is controversial and requires further research.

4.4 Insulin may accelerate Aβ clearance and mitigate its effects on neural cells

Treatment of N2a-APP cells with insulin (1.72 μM and 5 nM) resulted in increased levels of Aβ1-40 and Aβ1-42 in the media as early as 2 hours (Figure 20). These results indicate that insulin elevated secretion of Aβ to the extracellular space, which is in line with other studies (SOLANO, SIRONI et al. 2000; Gasparini, Gouras et al. 2001). The insulin-induced release of Aβ into the culture medium is PI3K dependent (Pandini, Pace et al. 2013) and can reduce intracellular accumulation of the peptide. The extrusion of Aβ into the extracellular compartment can lead to its degradation by membrane associated and secreted IDE (Vekrellis, Ye et al. 2000). Several other ways that insulin can reduce Aβ accumulation and aggregation in plaques are reported by Pandini et al, 2013. Along Aβ secretion to the media, insulin favors α-secretase activity with increased neurotrophic action, inhibits the translocation of AICD into the nucleus by dephosphorylation of APP at T668, and phosphorylates GSK3β inhibiting its

enzymatic activity (Pandini, Pace et al. 2013). Finally, inhibiting AICD nuclear activity by insulin has been shown to reduce transcription of genes that encode pro-amyloidogenic enzymes such as GSK3β and BACE1, and increase transcription of anti-amyloidogenic enzymes such as α-secretase and IDE (Farris, Mansourian et al. 2003; Pandini, Pace et al. 2013). However, these effects have not yet been demonstrated *in vivo* and this can be of important future investigation into insulin's role in AD and potential new targets for therapy.

4.5 Future work and concluding remarks

In this study, I have demonstrated that insulin regulates the synthesis of cholesterol in neural cells N2a, N2a-APP and SHY through modulation of SREBP2 enzyme and gene expression levels. I have also shown that Aβ causes insulin resistance in N2a-APP as well as the lack of response of cholesterol biosynthesis enzymes, and decreased phosphorylation of Akt and GSK3β when stimulated by physiological levels of insulin. One of the mechanisms involved in such resistance is the inhibition of IRS-1 signaling by phosphorylating it at Ser612 in N2a-APP and Aβ1-42 treated SHY neural cells. Inflammatory activation in SHY in response to Aβ1-42 treatment is seen through phosphorylation of c-Jun Ser63 indicating JNK activation, down regulation of neuroprotective/anti-inflammatory DHCR24 protein and possible increase of isopernoids due to the temporary activation of mevalonate pathway indicated by increased HMGCR protein levels.

These results suggest that AD is a multi-factorial disease that converges on three main intertwined events: insulin resistance and deficiency, Aβ induced

toxicity and neuro-inflammation, and dysregulation of cholesterol homeostasis. Future therapies should take that into consideration while developing a pharmaceutical intervention that can target multiple facets of the disease. Such therapies may include the conjunctional use of statins, non-steroidal anti-inflammatory drugs (NSAIDs), and insulin remediation drugs.

There are limitations to this study. The use of immortalized neuroblastoma cells and some that have been transfected with hAPP (N2a-APP) may only partially reflect what could happen in normal neuronal cells or *in vivo*. Although treatment conditions are kept identical, there are some variations in response to insulin between mice and human cells, perhaps due to species specificity and some cells did not express some hallmarks of AD such as JNK activation indicative of inflammatory response that was absent in N2a-APP. Finally, this study was focused on *in vitro* responses and mechanisms triggered by insulin and Aβ treatment. Therefore, it may not completely reflect responses that can occur *in vivo* in response to similar stimuli. These discrepancies and factors should be taken into account when interpreting data from different AD cell models.

Future work can be done to expand this study, and explore further mechanisms of actions, the relationship between insulin signaling, Aβ/APP metabolism, neuroninflammation and cholesterol synthesis. First, mechanisms of insulin modulation of SREBP2 protein and gene expression will be explored in both human and mouse neural cells. Inhibitors of insulin signaling and down-stream insulin activated enzymes can be utilized to discern a pathway where

cholesterol biosynthesis depends on. One pathway of interest will be the PI3K/Akt/mTOR pathway. Also, SREBP2 silencing can be used to confirm that regulation of cholesterol by insulin signaling is SREBP dependent in these cells. Other mechanisms of Aβ induced insulin resistance can be explored by looking at IR distribution, other Ser/Ther inhibitory phopshorylation, and glucose sensitivity. Neuro-inflammatory responses can be further analyzed to include other cytokines, isoperoids, and ways to mitigate them. Cholesterol levels and distribution, levels of SREBP2 protein and gene expression, and cholesterol synthesis gene expression levels should be analyzed in response to Aβ treatment. Further analysis of insulin signaling mediated Aβ/APP processing, trafficking and regulation can be carried out by looking at phosphorylation states of APP, levels of Aβ degradation, up-regulation/down-regulation of related proteins, and the level of their activities. Whether or not insulin remediation alone can mitigate neuro-inflammation, Aβ induced insulin resistance and dysregulation of cholesterol synthesis enzymes can also be explored. Finally *in vivo* studies can be carried out utilizing AD mouse models and human AD brain samples to further investigate the effects seen *in vitro*.

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