The Effect of Amyloid-Beta on the Insulin Signalling Pathway in Neuroblastoma 2a (N2a) Cells: The Characterization of Insulin Resistance in Alzheimer's Disease

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Abstract

Alzheimer's disease (AD) is a progressive neurodegenerative disorder characterized by accumulation and deposition of extracellular beta-amyloid peptides (Aβ) and intra-neuronal hyperphosphorylated tau in the brain. The insulin signalling pathway begins upstream at the insulin receptor (IR), where the intracellular insulin receptor substrate 1 (IRS1) is phosphorylated, thus propagating the signal downstream to the PI3K/Akt signalling pathway, which affects both the glycogen synthase kinase 3 beta (GSK3β), which is a tau kinase, and mTOR, which is a critical part of the mTORC1 and mTORC2 complexes that not only mediate a wide range of cell functions, but also feed back upstream to regulate Akt. Increasing evidence builds a strong case for the role of soluble Aβ oligomers (AβOs) in the impairment of insulin signalling in AD. Our *in vitro* studies with neuroblastoma 2a (N2a) cells stably transfected with human APP695 gene (N2a-APP), which secrete excess Aβ, show that the phosphorylation and expression of several but not all critical signalling proteins along the insulin signalling pathway are dysregulated in the cells in comparison to the parental N2a cells. N2a-APP cells were also found to be phenotypically insulin resistant. Subsequently, N2a-APP cells were treated with the Aβ binding peptide (ABP), which binds Aβ oligomers. The ABP treatment was observed to enhance insulin signalling response compared to untreated controls. The results suggest that Aβ may be responsible for inducing the insulin resistant phenotype in N2a-APP cells, and that the removal of Aβ oligomers is a potential treatment consideration for dysfunctional insulin signalling involved in Alzheimer's disease.

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ABP	Amyloid beta binding peptide
AD	Alzheimer's disease
Akt	(see PKB)
APP	Amyloid Precursor Protein
Aβ	Amyloid beta
AβOs	Amyloid beta oligomers
BBB	Blood-brain barrier
CNS	Central nervous system
Deptor	DEP domain-containing mTOR interacting protein
DMEM	Dulbecco's modified Eagle's medium
DMSO	Dimethyl sulfoxide
DTT	Dithiothreitol
EDTA	Ethylenediaminetetraacetic acid
ECL	Enhanced chemiluminescence
FBS	Fetal bovine serum
GAP	GTPase activating protein
GSK3α/β	Glycogen synthase kinase 3 alpha/beta
GTPase	Guanosine triphosphate-ase
hAPP	Human APP
IR	Insulin receptor
IRS1	Insulin receptor substrate 1
JNK	c-Jun N-terminal kinase
LDP	Long-term depression
LTP	Long-term potentiation

PDK1	3-phosphoinositide-dependent protein kinase 1
PI3K	Phosphoinositide-3-kinase
PIP2	Phosphatidylinositol (3,4)-bisphosphate
PIP3	Phosphatidylinositol (3,4,5)-tris-phosphate
PKB	Protein kinase B
PMSF	Phenylmethanesulfonylfluoride
PRAS40	Proline rich Akt substrate 40 kDa
PVDF	Polyvinylidene fluoride
sAPPβ	Soluble APPβ
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SPs	Senile plaques
mLST8	Mammalian lethal with Sec13 protein 8
mSIN1	Mammalian stress-activated protein kinase-interacting protein
mTOR	Mammalian target of rapamycin
mTORC1/2	mTOR complex 1/2
N2a	Mouse neuroblastoma 2a
N2a-APP	N2a stably transfected with hAPP695
NFTs	Neurofibrillary tangles
NMDAR	N-methyl-D-aspartate receptor
NP-40	Octylphenoxypolyethoxyethanol
P75NTR	Low-Affinity Nerve Growth Factor Receptor
PBS	Phosphate buffer solution
PHF	Paired helical filaments
Rheb	Ras homologue enriched in brain
T2D	Type 2 diabetes

TNFα	Tumor necrosis factor alpha
TSC1/2	Tuberous sclerosis 1/2

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Introduction

Since the first characterization of Alzheimer's disease (AD) by Alois Alzheimer in 1906, the neurodegenerative disease has been known as a devastating, fatal disease that progressively robs victims of their memory and autonomy. Its causative origins still elude researchers, and what knowledge scientists have gleaned through extensive research exists as several different theories of pathogenesis that are linked tenuously at best. These past 30 years of AD research, though they have not yet yielded a cure, has uncovered and emphasized the multi-factorial nature of AD pathogenesis. Not only do genetic variation, age, lifestyle, and most likely other unknown factors interact with each other to determine whether an individual will develop physiological characteristics of AD, increasing evidence suggests that the interaction of factors may further determine whether those physiological characteristics manifest as clinical symptoms.

The early clinical symptoms of AD present as difficulty remembering names and events; as the disease progresses, communication and judgment are impaired and ultimately, the patient is rendered unable to speak, swallow, and walk (Querfurth and Laferla 2010). The most common causes of death at the end stages are pneumonia and dehydration, but in some cases there is no overt cause of death other than the bodily and functional deterioration caused by the disease itself (Ganguli, Shen et al. 2005; Molsa, Marttila et al. 1986). The progression from diagnosis to death lasts for an average of 9 years (Davis and Samuels, 1998). On a neurological level, the disease condition manifests as a loss of synapses and neurons in the cerebral cortex, leading to progressive atrophy of the temporal lobe, parietal lobe, frontal cortex and cingulate gyrus (Wenk 2003).

The economic and social burden of Alzheimer's disease on society is considerable. In 2015, 5.3 million individuals over the age of 65 in the United States suffered from AD, which is projected to increase to 7.1 million by 2025 (Alzheimer's Association 2015). In Canada, the statistics show that in the same age category, there are 747,000 individuals suffering from AD, which, under the current rate of population increase, will rise to 1.4 million (Alzheimer's Society Canada 2012). As medical care advances, the number of people living into their 80s and 90s will increase, therefore the burden of AD care will also increase. Although 11% of people over the age of 65 have AD in the US, the percentage increases to 32% in people over the age of 85 (Alzheimer's Association 2015). The economic impact of the disease not only constitutes the cost of treating and accommodating patients, but also includes the unpaid hours that family members shoulder in caring for their kin. In Canada, the time given by family caregivers in 2011 represented $11 billion in lost income (Alzheimer's Society Canada, 2012). Unless treatments can be improved if not replaced by a cure, the economic cost of AD will continue to rise as the elderly population increases. Therefore, in a society with an increasing number and proportion of elderly citizens, it becomes increasingly vital to advance our knowledge of this ultimately fatal disease that currently has no cure.

The vast majority of AD cases are sporadic and multifactorial, while familial cases only account for 2% of cases (Maiese, Chong et al. 2009). Without the rationalization of inheritance as with familial cases, the etiology of sporadic AD is a difficult conundrum of multiple possible contributing factors that interact with each other to bring about the disease state. The great challenge of AD research is coming to a consensus about which of the many molecular pathologies of AD can be targeted to prevent or cure the disease without harmful side effects.

What distinguishes Alzheimer's disease as a unique form of dementia is two neuropathological markers, senile plaques (SP) and neurofibrillary tangles (NFTs), both defined physiological markers observable by microscopy in the post-mortem brain tissue, that accompany the behavioural and functional dysfunctions that must primarily be used as the diagnostic criteria while the patient is alive. It is these two modes of pathogenesis that give AD its status as a neurodegenerative disease, since the development of these neuronal aberrations leads to cell death.

1.1 APP and Aβ oligomers in AD

Perhaps the single most characteristic pathological indicator of AD is senile plaques, which are accumulations of extracellular protein deposits. SPs are made up of self-aggregating amyloid beta (Aβ) peptide fragments. These fragments originate from the membrane spanning amyloid precursor protein (APP), which is located in the plasma membrane, trans-Golgi network, endoplasmic reticulum, and the endosomal, lysosomal, and mitochondrial membranes (Sakono and Zako 2009). APP undergoes proteolytic cleavage via secretase enzymes, primarily α-and γ-secretase pathway and β-and γ-secretase pathway. The full precursor protein is found in both human neural and non-neural tissues, is 110 – 135 kDa in size, and also highly conserved in rat, cow, and monkey tissues (Selkoe, Podlisny et al. 1988). When APP is cleaved via the β-and γ-secretase pathway, the size of the peptide fragments varies from 39-43 amino acids. The most prevalent of the fragments, Aβ40, is relatively soluble, contains 40 amino acids and makes up about 90% of the total Aβ peptides produced, yet it is the less abundant Aβ42 fragments that are hydrophobic and the most prone to self-aggregation and the most commonly found in senile plaques (Kametani, 2008; Jarrett and Lansbury, 1993). The formation of

senile plaques occurs when these soluble fragment monomers, dimers, and oligomers aggregate to form insoluble fibrils with β-sheet structure, which ultimately form plaques (Klein, 2002). Post-mortem analysis of AD brains has shown that the proportion of Aβ42 to Aβ40 is significantly increased over control non-demented brains (Kuo, Emmerling et al. 1996; Funato, Yoshimura et al. 1998). These Aβ fragments result from the successive cleavage of APP by β-secretase, which releases sAPPβ and the APP N-terminal ectodomain into the extracellular environment, and then by γ-secretase, producing Aβ40 and Aβ42 (Nunan and Small 2000; Vassar, Bennett et al. 1999).

Although it is the increased amount and proportion of Aβ42 that bring about SPs and cell death, studies have shown that Aβ in lower amounts may have roles in normal physiological function. The presence of Aβ is not pathological; it is the excess of Aβ that must be addressed as such. At normal physiological levels in the picomolar range, Aβ is suggested to have a key role in synaptic plasticity, improvement of cognition, and long-term potentiation in the hippocampus; it is when physiological levels are elevated to nanomolar and micromolar concentrations that detrimental effects are seen (Shankar, Bloodgood et al. 2007; Puzzo, Privitera et al. 2008). APP itself is also associated with neuronal growth, synaptogenesis and cell adhesion, while it is also essential in memory formation. (Luo, Sunderland et al. 1996; Moya, Benowitz et al. 1994; Mileusnic, Lancashire et al. 2000).

While it has been long accepted that the accumulation of Aβ and the deposition of senile plaques have major roles in the loss of synapses and neurons, much stronger correlations have emerged between the levels of soluble Aβ oligomers (AβOs), synaptic dysfunction and loss, and the actual severity of cognitive impairment. One of the most confounding shortcomings in the current understanding

of AD is in reconciling AD pathophysiology with the manifestation of cognitive impairment. There is no standard threshold SP load at which individuals begin displaying cognitive symptoms of AD; while it is true that all AD cases with significant impairment have elevated SPs in their brain, in the general continuum of disease progression, particularly in the early stages, there is a weak correlation between SP load and disease severity (Berg, McKeel et al, 1993; Terry, Maslia et al. 1999). This link was further proven tenuous in early human trials, in which vaccines targeting amyloid plaques did not have discernable effects on the progression of dementia symptoms in cognitive decline (Holmes, Boche et al. 2008). In clinical trials with APP transgenic mice, the reduction of plaque burden via Aβ42 immunization did show preservation of cognitive function, but the following human trials were stopped because 6% of patients developed encephalitis, and the immunotherapy did not halt cognitive decline even though plaque counts were decreased (Gilman, Koller et al. 2005). Many recent human clinical trials with Aβ-targeting antibodies consistently show clearing of Aβ from the brain, yet do not improve cognition (Farlow, Arnold et al. 2012; Ostrowitzki, Deptula et al. 2012).

With inconsistencies between plaque pathology and cognitive impairment commonplace in the body of AD research, focus is now shifting towards other pathological agents that affect neurological function. In actuality, there is a stronger correlation between the level of soluble AβOs and the severity of synaptic loss and cognitive decline (Caughey and Lansbury 2003; Haass and Selkoe 2007; Klein, Krafft et al. 2001; Ferreira, Vieira et al. 2007). The correlation of insoluble Aβ, as found in fibrils and plaques, and disease severity is relatively weak (McLean 1999). The key to elucidating a direct mechanism between neurophysiological markers and cognitive indicators may be pursuing further study of the role of AβOs in AD

pathogenesis, particularly how soluble AβOs interfere with functional signalling pathways in neurons. The focus has steadily been shifting away from the plaques consisting of insoluble fibrillar aggregates of AB, which do not affect cellular signalling functions.

The size of the prefibrillar AβOs found in the AD brain vary from 10 kDa to 100 kDa (Kuo, Emmerling et al. 1996). The structural diversity and ubiquity of AβOs in the diseased portions of the AD brain are demonstrated by the presence of AβO dimers and trimers in larger soluble oligomers as well as in amyloid plaque extracts (Walsh, Tseng et al. 2000. Bernstein, Dupuis et al. 2009. Maclean 1999). In various cultured cells that express APP, including human neuroblastomas and Chinese hamster ovary cells, ABOs have been detected to be secreted into the media (Morishima-Kawashima and Ihara 1998; Podlisny, Ostaszewski et al. 1995). In vitro cell toxicity studies involving these AβO dimers and trimers show that these low-MW ABOs are respectively threefold and 13-fold more toxic than the monomeric form (Ono 2009). The cell toxicity is suggested to be linked to the deleterious effect of soluble AβO on various neuronal receptors; it is shown that AβOs can bind to extracellular receptors and in excess amounts can detrimentally affect the associated signalling pathways (Wang, Lee et al. 2000; Demuro, Mina et al. 2005). Studies continue to reveal targets for receptor mediated toxicity, such as the activation of the apoptotic signal upon AβO binding to the nerve growth factor receptor (Yamamoto, Matsubara et al. 2007). Cell death and various dysfunctions have been reported in studies that show AβO binding to the NMDA receptor, Frizzled receptor, and the p75NTR receptor (De Felice, Velasco et al. 2007; Shankar, Bloodgood et al. 2007; Magdesian, Carvalho et al. 2008; Coulson 2006). Of great interest in the context of AD pathogenesis is that AβOs act as antagonists to insulin

at the neuronal insulin receptor, thus reducing the insulin signalling response (Bomfim, Forny-Germano et al. 2012; Heras-Sandoval and Ferrera 2012).

1.2 Insulin signalling pathway and insulin resistance in AD

What may precipitate from the binding of AβOs to the insulin receptor is insulin resistance, which happens to be a characteristic pathology in AD. Insulin plays an important role in regulating synaptic function and plasticity (Chiu, Chen et al. 2008; Wan, Xiong et al. 1997). The CNS depends on insulin signalling to maintain neuronal survival, normal proliferation, differentiation, and gene expression (Kappeler, De Magalhaes Filho et al. 2008; Adams and Sweatt 2002). The complex signalling cascade that begins at the insulin receptor (IR) mediates this wide range of cell functions. Peripheral insulin resistance primarily affects glucose uptake into the cells, and while the same is also true in the CNS, insulin resistance may also affect the regulation of cognitive function (Freychet 2000). Not only do individuals with type II diabetes (T2D) have increased risk of developing dementia, in the CNS, AD itself is gaining recognition as its own form of diabetes (Ott, Stolk et al. 1999; De la Monte 2008). Indeed, evidence suggests that defective insulin signalling and decreased responsiveness are present in the AD brain, even in cases where the patient has no prior indications of T2D (Steen, Terry et al. 2005; Talbot, Wang et al. 2012; Griffin, Moloney et al. 2010). Within the CNS, the distribution of insulin receptors have important implications for the function of insulin signalling other than glucose uptake, and more importantly, for how impaired signalling affects major cognitive functions. Most notably, the hippocampus and cerebral cortex have high densities of IRs (Adem, Jossan et al. 1989; Hopkins, Williams et al. 1997), and these regions also encompass areas that affect cognitive activities like planning and the

consolidation of short and long-term memory (Bliss and Collingridge 1993). Insulin itself is transported into the brain from the periphery via specific insulin transporters at the blood-brain barrier (Banks, Jaspan et al. 1997; Pardridge, Eisenberg et al. 1985), but is also synthesized de novo by neurons in the hippocampus and pre-frontal cortex (Hoyer 2003). The physiological levels of insulin in the brain is about 10-100 fold the peripheral plasma concentration of periphery circulation (Schecter, Whitmire et al. 1992), which ranges in the blood from about 18-90 pmol/L at fasting concentration to 100-800 pmol/L during digestion and 1-2 hours following meals (Kahn, McCulloch et al. 1997; Hellman, Gylfe et al. 2007). Therefore, insulin response experiments in animal brain tissue and cells typically set the physiological dosage at around 1 nM of insulin, up to 10 nm (Talbot, Wang et al. 2012; Le Roith, Hendricks et al. 1983; Denley, Breirley et al. 2006).

The insulin receptor itself is a heterodimeric tetramer transmembrane protein consisting of two disulphide linked subunits that bind ligand, and two transmembrane β subunits that also form a dimer (Hardie and Hanks, 1995). Insulin binds to the insulin binding domain on the extracellular α subunit dimer, which causes the α subunit to disinhibit the tyrosine-kinase activity of the β subunits, leading to the phosphorylation of the intracellular insulin receptor substrate 1 (IRS1) that is docked to the IR (Sun, Rothenburg et al, 1991) (Figure 1). Once phosphorylated, the IRS1 directly stimulates phosphoinositide-3-kinase (PI3K), which then catalyzes the conversion of the membrane phospholipids phosphatidylinositol (3,4)-bisphosphate (PIP_2) to phosphatidylinositol (3,4,5)-tris-phosphate (PIP_3) (Hemmings and Restuccia 2012). Once PIP3 is activated, 3-phosphoinositide-dependent protein kinase 1 (PDK1) phosphorylates Akt, which mediates many cellular functions like metabolism, survival, and apoptosis (Song, Ouyang et al. 2005; Faissner, Heck et al. 2006;

Dudek, Datta et al. 1997). This phosphorylation of IRS1 and subsequent activation of downstream signalling is one function of insulin in the CNS other than the translocation of glucose transporters to the cell membrane. Further suggestions of the specialized role of insulin in the CNS is put forth in studies of rat brains, which have high density of IRs in the hippocampus and regions of the cerebral cortex where learning and memory functions occur (Hill, Lesniak et al. 1986; Werther, Hogg et al. 1987).

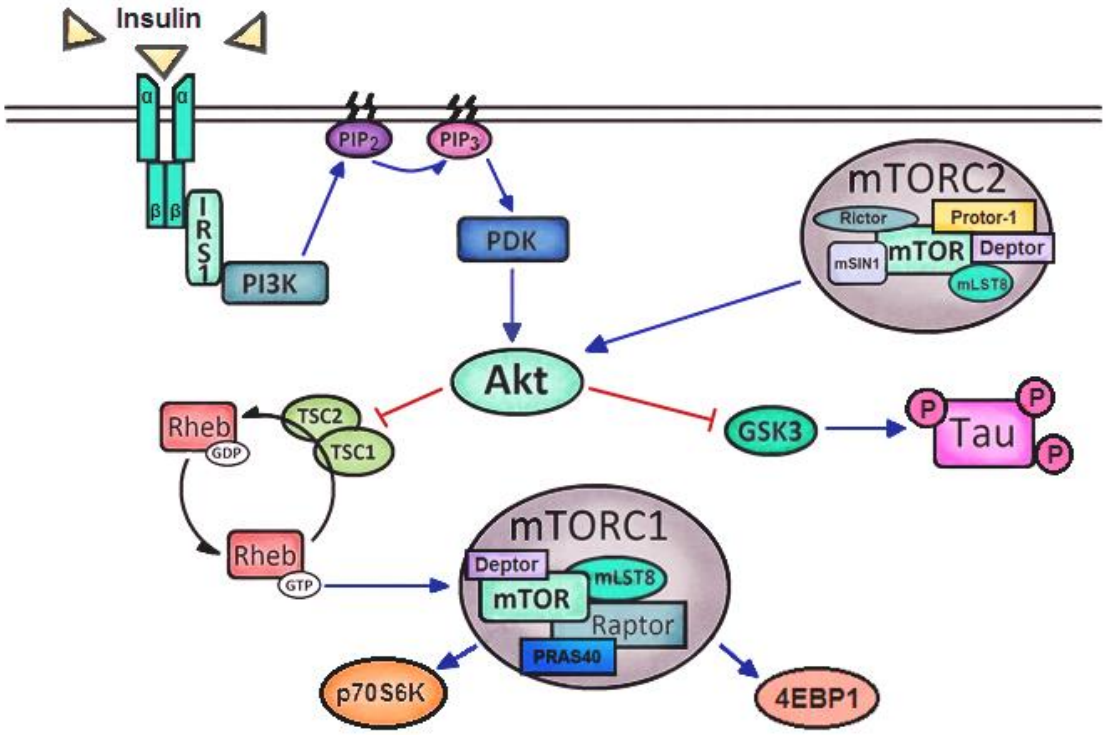

Figure 1. Schematic representation of the association between insulin, PI3K/Akt, and mTOR signaling pathways.

How insulin resistance forms in AD is still unclear, with mixed findings pertaining to the exact cause and molecular mechanisms. Basal levels and activation states of the insulin signalling proteins, as detected in AD brains, vary between studies. In general, consistency is strongest in terms of reduced sensitivity

of IR and increased serine phosphorylation of the downstream IRS-1/2 in the AD brain (Watson and Craft, 2003; Rivera, Goldin et al. 2005; Steen, Terry et al. 2005). Insulin resistance in the AD brain is also apparent in the increased phosphorylation of IRS1 on its serine 616, 636/639 and 312 residues, which are classical indications of insulin resistance in T2D (Bomfim, Forny-Germano et al. 2012; Talbot, Wang et al. 2012; Moloney, Griffin et al. 2010). As mentioned, the dysregulation of insulin signalling is closely linked to the pathogenic effect of AβOs. One significant effect of AβOs is that it induces the internalization of neuronal insulin receptors, yet in the AD brain, the density of IRs is significantly higher than in the healthy brain (Zhao, De Felice et al. 2008; Forlich, Blum et al. 1998). This could be indicative of an insulin resistant state in which compensatory mechanisms are being triggered in an attempt to relieve the downregulation of insulin receptor binding. Downstream of IRS1, the activation of Akt by its phosphorylation at serine 473 seems to occur in response to soluble AβO even in the absence of insulin (Zhao, De Felice et al, 2008).

The clearance of extracellular AβOs seems to be mediated by IRs themselves, because the blocking of IR activation greatly elevates extracellular AβO, while treatment with insulin and anti-diabetic IR-sensitizing agents like metformin leads to reduction in AβO pathology in cell and animal models of AD (Zhao, Lacor et al. 2009; Beeri, Schmeidler et al. 2009; Gupta, Bisht et al. 2011). Insulin signalling itself, in absence of other disease factors, also decreases with age, therefore becoming another factor that likely contributes to the onset of sporadic AD in the elderly, particularly since normal insulin signalling is a protective mechanism against elevated extracellular AβO levels (Fernandes, Saad et al. 2001).

Downstream of PI3K/Akt, glycogen synthase kinase 3 (GSK3) is of particular interest because of its ability to phosphorylate tau. Tau is an axonal protein that regulates microtubule stability and axonal transport. In AD, tau is hyperphosphorylated, which causes the proteins to first aggregate into paired helical filaments (PHF), and then neurofibrillary tangles (Grundke-Iqbal, Iqbal et al. 1986). NFTs, although not a marker exclusive to AD, are characteristic lesions formed from the aggregation of hyperphosphorylated tau protein. In normal, cytosolic tau, there are usually two sites that are phosphorylated, while the tau found in PHFs is phosphorylated at 7 to 8 sites at least, including serine 199, 202, 235, 262, 396, threonine 212, 217, and 231 (Hanger, Anderton et al. 2009; Hanger, Betts et al. 1998; Mandelkow and Mandelkow, 2012). Autopsied samples from AD brains show 3- to 4-fold times more phosphorylation of tau compared to the normal brain (Köpke, Tung et al. 1993). There are about 79 potential phosphorylation sites of serine and threonine on the longest tau isoform (Gong, Liu et al. 2005; Hanger, Byers et al. 2007).

GSK3 is a ubiquitious kinase that is abundantly expressed at nerve terminals in the central nervous system, existing as two isoforms, GSK3α and GSK3β (Leroy and Brion 1999; Doble and Woodgett 2003). Specifically, GSK3β is important in AD pathogenesis because of its ability to phosphorylate tau at most of its phosphorylation sites (Hanger, Anderton et al. 2009). Because it is a tau kinase, the expression of GSK3β must be tightly regulated to prevent the excessive phosphorylation of tau that leads to NFTs. The hyperphosphorylated tau form cannot bind and stabilize microtubules, instead forming insoluble aggregations and causing the destabilized microtubules to degrade (Lee, Perry et al. 2005). GSK3β is activated by negative regulation; it is deactivated when phosphorylated at its serine 9 residue

(Eldar-Finkelman 2002). The PI3K/Akt pathway, which is activated by insulin, serves to suppress its activity by phosphorylating GSK3β (Dudek, Datta et al. 1997). GSK3β could therefore be a significant link between the two main pathophysiologies of AD; overproduction of Aβ leads to the formation of AβOs which desensitize insulin receptors, thus inactivating the PI3K/Akt pathway that phosphorylates - and therefore deactivates - GSK3β, and in doing so leads to the formation of NFTs through the increased phosphorylation of tau.

1.3 The mTOR signalling pathway

The importance of understanding the role of Akt in AD pathogenesis is emphasized by its central role in the mTOR signalling pathway. The mTOR protein is a serine/threonine kinase that is involved in the control of gene transcription, protein synthesis, cytoskeleton structure, metabolism, development, and cell survival and senescence (Maiese 2013). Two protein complexes, mTORC1 and mTORC2, both containing mTOR, are the two main junctions in the mTOR signalling pathway (Figure 1). The differences between the mTORC1 and mTORC2 complexes are two-fold: composition and position. The proteins that are unique to the mTORC1 complex are Raptor, and proline rich Akt substrate 40 kDa (PRAS40), while the proteins unique to the mTORC2 complex are Rictor, mammalian stress-activated protein kinase interacting protein (mSIN1), and Protor-1 (Kim, Sarbassov et al. 2002; Frias, Thoreen et al. 2006; Sarbassov, Ali et al. 2004). Both complexes contain mammalian lethal with Sec13 protein 8 (mLST8) and DEP domain-containing mTOR interacting protein (Deptor). The Raptor protein, which is activated when phosphorylated by various effectors such as the Ras homologue enriched in brain (Rheb), is essential for the binding together of mTORC1 constituents (Wang, Lawrence et al. 2009).

Raptor is prevented from binding by competition with PRAS40, which must be phosphorylated by Akt to be inhibited, therefore promoting mTORC1 activity (Wang, Zhang et al. 2012). Deptor regulates both mTORC1 and mTORC1 activity by inhibition, as knockout cells have elevated level of downstream mTORC1 and mTORC2 activity (Peterson, Laplante et al. 2009). The mTOR within mTORC1 depends upon mLST8 to phosphorylate its downstream targets p70S6K and 4EBP1, while mTORC2 relies on mSIN1 and Rictor to activate and phosphorylate Akt at serine 473 (Chong, Shang et al. 2012). The influence of Akt upon mTOR signalling and vice versa is clear and vital; while Akt is phosphorylated by the mTORC2 complex, mTORC1 relies on Akt phosphorylation of the inhibitory tuberous sclerosis 1/tuberous sclerosis 2 (TSC1/TSC2) complex. Upstream, mTORC1 is regulated by a heterodimer comprised of TSC1/TSC2 acting as a GTPase activating protein (GAP) that controls the activity of mTORC1 activating protein Rheb (Yadav, Burgos et al. 2013). The heterodimer inhibits mTORC1 by inactivating Rheb from its GTP to GDP-bound state. When Akt phosphorylates TSC2, the TSC1/TSC2 complex destabilizes, which interferes with its inhibiting effect upon Rheb, thus allowing its activation to in turn activate mTORC1 (Tee, Manning et al. 2003; Inoki, Li et al. 2003). Insulin signalling is therefore critical to mTOR signalling because Akt is partly regulated by the insulin-PI3K-Akt pathway; in turn, mTOR signalling is also critical in the regulation of Akt activation.

The current range of knowledge on the role of mTOR in AD pathogenesis is wide, but not deep. Much of the body of knowledge centers around the two-complex system of the mTOR signalling pathway, which is both a continuation and an intersection of the PI3K/Akt pathway. Upregulation of mTOR is positively correlated with upregulation of Aβ; studies have found that mTOR upregulation may both

enhance the generation of Aβ and inhibit the clearance of existing Aβ. Inhibition of mTORC1 activation downregulates its downstream effectors that inhibit autophagy, thus inducing the clearance of Aβ and delaying, yet not ameliorating, the progression of AD (Ravikumar, Duden et al. 2002; Spilman, Podlutskaya et al. 2010). The tau pathogenic pathway is also affected with aberrations in mTOR signalling, as the activation of mTOR promotes tau pathology by upregulating the phosphorylation of tau, while the inhibition of mTOR attenuates it (Caccamo, Magri et al. 2013; Spilman, Podlutskaya et al. 2010). Examination of the IRS/PI3K/Akt pathway activity and its surrounding and intersecting pathways in the AD brain reveal disturbances at all levels. Akt activation has been observed to be increased in the hippocampus and cortex in the AD brain, as well as its targets mTOR and GSK3β (Griffin, Moloney et al. 2005; Moloney, Griffin et al. 2010; Pei and Hugon, 2008).

The study of Alzheimer's disease in vitro is often performed in transfected cell lines that overexpress the amyloid precursor protein (APP) at the cell membrane. APP is the large precursor protein that is integral to the cell membrane; when cleaved by various secretase enzymes, some of the fragments secreted extracellularly are the beta-amyloid (Aβ) peptides that aggregate to form toxic oligomers and plaques, ultimately leading to neuronal dysfunction and death (Turner, O'Connor et al. 2005). While APP expression is a major factor in AD pathogenesis, it does have a role in normal cell functions that has not yet been well-characterized. For one, APP seems to be involved in neurite outgrowth and synaptogenesis (Kirazov, Kirazov et al. 2001). A commonly used cell line for studying Aβ toxicity is a mouse N2a neuroblastoma cell line that has been stably transfected with a human gene encoding APP695, which expresses significantly higher levels of APP (Xiong, Callaghan et al. 2008). The general morphology of N2a compared to APP cells is

shown in the staining of the membrane associated protein IRS; N2a cells seem to be generally larger (Figure 1). The APP-transfected cells generate Aβ40 and Aβ42, the former Aβ fragment being the predominant form of Aβ, and the latter being the form that aggregates most readily into amyloid oligomers (Roher, Lowenson et al. 1993; Masters, Simms et al. 1985). The parental cell line, the neuroblastoma 2a (N2a), was developed from a spontaneous tumour of a strain A albino mouse and is a favoured research model for its fast growth, suitability for transfection, and versatility for various neurotoxicity studies, like prion propagation (Solassol, Crozet et al. 2003).

The current standard of treatment for AD patients aims to address the symptoms and progression of the disease. On a molecular level, specific drugs that target demonstrated signalling dysfunctions have been tested and are continually being developed and subjected to clinical trials. However, the high rate of clinical trial failure is owed to the fact that inhibition or activation of various proteins known to affect AD pathogenesis also carries a high risk of unwanted side effects from interference with their normal functional roles. Treatments targeting β- and y-secretase to reduce the production of Aβ42 have been tested, but trials were ultimately aborted because of cell death and undesirable side effects stemming from the fact that not only do these enzymes also cleave proteins other than APP, they also cleave APP into fragments other than Aβ which have important functions (Doody, Raman et al. 2013). Drugs that target Aβ itself, as described previously, do not commonly show positive effects on cognitive function. An exception may be solanezumab, which binds Aβ monomers and is shown to have modest slowing of cognitive decline in mild AD patients, yet the benefit is still small compared to that of existing cholinesterase-inhibitors that treat memory and learning deficits (van Marum

2015; Sadowski and Galvin 2012). Phase 3 clinical trials for solanezumab are ongoing. However, more recently, antibodies and other binding agents are in development to specifically target soluble AβOs,

Targeting GSK3β in theory seems attractive, since inhibiting its kinase activity would decrease the phosphorylation of tau and therefore decrease the formation of NFTs. However, as with any of the endogenous signalling proteins, knocking out or inhibiting GSK3β in an effort to treat AD pathology is more simply said that done. GSK3β, when functioning normally, plays an important role in the regulation of synaptic plasticity, gene expression, and cell survival (Grimes and Jope 2001). Much of the focus on AD treatment lies in addressing tau pathology directly by using vaccines against hyperphosphorylated tau. Animal studies show some promising results, mainly in the reduction of hyperphosphorylated tau and NFT pathology, as well as some recovery of cognitive and motor function (Theunis, Crespo-Biel et al. 2013; D'Abramo, Acker et al. 2013; Boutajangout, Quartermain et al. 2010). Human trials are in the early stages, and the lack of adverse inflammatory response in mice suggests a good safety profile for humans (Theunis, Crespo-Biel et al. 2013). To combat insulin resistance itself, clinical trials applying intranasal insulin treatments in AD patients present a strong case for the association of systemic insulin resistance and cognitive decline. Intranasal doses of insulin were administered to cognitively impaired patients in a small clinical study, and those patients displayed significant improvements in recall exercises (Reger, Watson et al. 2006). Further investigations on the mechanisms of insulin resistance in AD are needed to improve the therapy or to develop new therapies targeting insulin resistance or the insulin signalling pathway.

The importance of understanding the way that AβOs affect insulin signalling in the brain is emphasized in the growing shift in research focus and development of treatments towards addressing soluble AβOs. The success of future clinical trials with drugs that target AβOs depends on a comprehensive body of research that details how effectors in the insulin signalling pathway are dysregulated to bring about a state of insulin resistance, and whether dysregulated signalling can be rescued by clearing AβO from the brain.

1.4 Aims and hypothesis of this study

The major aim of this study is to characterize the effect of Aβ on the insulin signalling pathway. By comparing the parental and APP-transfected N2a cells, proteins in the insulin signalling pathway can be examined for any effect in response when amyloid beta is the only differing characteristic between cell lines. It is known that amyloid beta can cause insulin resistance, but there is limited information as to how each specific step in the insulin signalling pathway is affected in the context of a model in which the only pathogenic factor is the presence of excess extracellular Aβ. The mTOR protein in particular, as a key component of the mTORC1 and mTORC2 complexes, is a potentially critical regulator of signalling pathway. Also considering the ubiquity of the Akt protein in connection with the mTOR signalling pathway, another major aim of this study is to determine how mTOR and Akt interact. The experiments to be performed will shed light on the central hypothesis: Aβ induces an insulin resistant state that dysregulates the insulin signalling pathway at various points and clearance of Aβ may relieve the insulin resistant state in the cell model. Because of the position of the mTORC complexes in the signalling pathway, mTOR

activation or inhibition may also reveal pathway mechanisms that regulate insulin signalling.

Materials and Methods

2.1 Chemical Reagents

Geneticin, DMSO, trypsin-EDTA, streptomycin sulfate, were purchased from Thermo Fisher Scientific (Grand Island, NY, USA). Dulbecco's modified Eagle medium was purchased from Wisent Inc. (Montreal, ON, Canada). Fetal bovine serum (FBS), penicillin G potassium salt, and Beta-amyloid 1-16 6E10 antibody was purchased from Sigma-Aldrich Canada Co. (Oakville, ON, Canada). Bradford Assay Reagent, protein standard ladder, and PVDF membranes were purchased from Bio-Rad (Mississauga, ON, Canada). Western-Lightning Plus-ECL was purchased from Perkin Elmer Inc. (Waltham, MA, USA). Autoradiography film was purchased from Diamed Lab Supplies Inc. (Mississauga, ON, Canada). Total Akt, phospo Akt s(Ser473), total GSK3β, phospho GSK3β (Ser9), phospho IRS-1 (Ser612), total IRS1, total mTOR, phospho mTOR (Ser2448), and phospho p70S6K (Thr389) antibodies were purchased from New England Biolabs (Whitby, ON, Canada). Phospho tau (ser199/202) antibody was purchased from EMD Millipore Corporation (Etobicoke, ON, Canada). NP-40 was purchased from Bio Basic Canada Inc. (Markham, ON, Canada). Beta-tubulin antibody was made in-house in hybridoma cells, and was a generous gift from Dr. Alexandre Blais at University of Ottawa. Amyloid beta binding peptide (ABP), solubilized in sterile H2O, was a generous gift from Dr. Balu Chakravarthy at the National Research Council Canada.

2.2 Cell Culture

Immortalized mouse neuroblastoma 2a (N2a) and N2a cells stably transfected with a human gene encoding APP695 (N2a-APP) were grown in DMEM with 5% FBS and 0.2% penicillin-streptomycin. The cells were incubated in a 5% CO_2 incubator at 37°C incubator. From passage 9 to 29, the cells were passaged three times a week. After washing the cells with 1xPBS to remove all media, 1ml of 1x trypsin was added to lift the cells from the plate surface, and then growth media were added to the trypsinized cells to neutralize the protease effect of trypsin. The N2a-APP cells were cultured with the antibiotic geneticin at 200 µg/L to select for cells carrying the APP gene.

2.3 Insulin time course treatment

The two cell lines were cultured to 80% cell confluence in separate 100mm dishes. The growth media were removed and the cells were washed with 1xPBS twice before the treatment media were added. Cells were treated with solubilized human recombinant insulin in serum-free media at concentrations of 0.1 nM, 0.5 nM, 5 nM, and 100 nM for 15, 30 min, 1, 2, 4, and 8 hours.

2.4 Amyloid Beta binding peptide (ABP) treatment

The two cell lines were cultured to 70% confluence in separate 100 mm dishes. The growth media were removed and the cells were washed with 1xPBS twice before the pre-treatment was started. Cells were treated with 0.44 µM ABP, which is 1000x the concentration of extracellular Aβ, which is 2000 pg/mL as determined by the lab of Dr. Wandong Zhang, in serum-free media for 24 and 48

hours; the media were replaced with fresh treatment media at 24 hours in plates treated for 48 hours. After the ABP treatment period, the ABP media were removed, and the cells were treated with 0.5 or 5 nM insulin in serum-free media for 30 minutes, 1 hour, and 2 hours.

2.5 Protein isolation and western blotting

After treatment, the media were removed from the plates and the cells were washed 3 times with PBS, 1 ml of PBS was added, then the cells were gently scraped from the dish with a rubber policeman and collected into new tubes on ice. The cells were then centrifuged for 5 minutes at 4000 rpm. The supernatant was discarded and the pellet kept for protein isolation. The cells in tubes were then lysed with whole cell extraction buffer (10% glycerol, 1% NP-40, 1 mM PMSF, 1 mM DTT, 50 mM Tris-HCl, 5 mM EDTA, 400 mM NaCl). The cell lysates were rotated for 30 minutes at 4°C, then centrifuged at 14000 rpm for 10 minutes. The supernatant was transferred to new tubes, the pellet discarded, and total protein levels were determined by Bradford assay. 1 µl of each sample was individually mixed into 1 ml of Bradford dye, then all samples were vortexed at the same time at high speed for 5 seconds, then 200 µl of each mixture was immediately loaded into the wells of an Immulon 1B non-UV microplate. The absorbance analysis was carried out with the Thermo Lab Systems Microplate Spectrophotometer using Multiskan Spectrum software. The test wavelength was 595 nm, and the plate was shaken for 10 seconds before the absorbance reading was taken. 40 mg of each protein sample was mixed with loading buffer (40% SDS, 25% glycerol, 5% β-mercaptoethanol, 5% bromophenol blue, 0.5 M Tris-HCl) onto a SDS-PAGE gel of 6% resolving gel and 4% stacking gel, and run for 1.5 hours at 100 V. The proteins were transferred to

PVDF membrane overnight at 35V in a walk-in 4°C fridge. The membranes were blocked at room temperature for 1 hour in blocking buffer [5% skim milk powder in 1X PBS with 0.2% Tween 20 (PBS-T)]. The blots were incubated while rotating with the primary antibody at 1:1000 dilution overnight at 4°C, then washed with 1X PBS-T, and incubated in the appropriate secondary antibody at 1:5000 dilution for 1 hour. After washing with PBS-T, the blot was treated with ECL Plus solution for 1 minute and then developed on autoradiography film. Densitometry analysis was performed using Scion Image software. Treatment condition data were presented relative to controls as fold-change of treated samples to the control samples. For additional probing, the blots were stripped with stripping buffer (0.71% β-mercaptoethanol, 20% SDS, and 12.4% 0.5 Tris-HCl in distilled H2O) at 50°C for 30 minutes, then blocked and incubated with primary and secondary antibody as described above.

2.6 Phase-contrast microscopy

N2a and N2a-APP cells were grown to 70% confluence in growth media on coverslips in 6-well tissue culture plates. The media were aspirated and the cells were washed with 1xPBS 2 times for 5 minutes each. The cells were then fixed with ice cold methanol, shaking gently for 10 minutes at room temperature, then washed again in 1xPBS 2 times for 5 minutes each. The coverslips were removed from the wells, excess PBS was dried off and mounted onto microscopy slides with 20ul of 70% glycerol. The edges of the coverslips were sealed with nail polish, then the slides were visualized with Axio Imager M2 under phase-contrast settings, with 40x oil objective.

2.7 Immunocytochemistry

N2a and N2a-APP cells were plated as described above. After fixation with ice cold methanol and washing with 1xPBS, the cells on the coverslips were permeabilized by applying 1 ml of PBS/0.5% Triton x-100 to each well, shaking gently for 15 minutes at room temperature. The permeabilizing solution was aspirated. The primary antibody was diluted in PBS/0.1% Triton x-100, then for each coverslip, a 60ul drop was placed onto the parafilm-covered lid of the 6-well plate. The coverslips were lifted from the wells and then each overturned onto the drop containing the primary antibody and incubated overnight in 4°C, or at room temperature for 2 hours. After incubation, the cover slips were placed face-up back into the wells, then washed with PBS/0.1% Triton x-100, 3 times for 5 minutes. The appropriate secondary antibody was diluted into PBS/0.1% Triton x-100, then again the coverslips were incubated face down in 60ul drops of the antibody solution, for 1 hour at room temperature in the dark. The coverslips were washed in PBS/0.1% Triton x-100, then incubated in 500 ul Hoescht stain (0.5ug/ml), to stain the nucleus, for 1 minute while gently shaking. The coverslips were immediately washed with 1xPBS twice for 5 minutes, then mounted onto microscope slides with 20 µl of 70% glycerol. The edges of the coverslips were sealed with nail polish. The cells were visualized with an Axio Imager M2 microscope under fluorescence illumination settings with 63x oil objective.

2.8 Statistical Analysis

Statistical analyses were performed in GraphPad Prism 6 using ANOVAs to assess significance of differences where multiple comparison are made, followed by

the Bonferroni post-hoc test. The two-tailed *t*-test was also used to determine statistical significance.

Results:

3.1 N2a and N2a-APP cells have similar growth rate and morphology.

Immunocytochemical and phase contrast microscopy visualizations of the N2a and N2a-APP cells showed that the general morphology of the two cell lines do not differ. Undifferentiated, both cell lines were observed to have round morphology with short extending neurofilaments (Figure 2). The size of both cells lines ranged from about 10 to 18 um.

3.2 N2a-APP cells have basal dysregulation of insulin signalling proteins.

To evaluate the effect of Aβ on the insulin signalling pathway, N2a and N2a-APP cells were treated with insulin and the activation of various pathway proteins was determined by western blotting. This cell model is centered around the effect of amyloid beta on signal regulation; indeed the only difference between the parental N2a cells and the transfected N2a-APP cells is the overexpression of amyloid precursor protein (APP) at the membrane; these transfected cells release Aβ extracellularly. A western blot was performed to detect p-IRS1 (ser 612), IRS1, p-Akt (ser 473), Akt, p-GSK3β (ser 9), GSK3β, p-tau (ser 199/202), p-mTOR (ser 2448), and mTOR which were then normalized to β-tubulin (Figure 3a). The results showed that there is a significant decrease in IRS1 phosphorylation in N2a-APP cells at baseline, that is, untreated with insulin (Figure 3b). The opposite trend is true of the expression of IRS1 itself, which seems to be overexpressed in N2a-APP cells in comparison to the parental N2a cells. At baseline, N2a-APP cells also significantly

express higher levels of p-Akt, Akt, and p-tau (Figure 3b). These results suggest that insulin signalling may be dysregulated and mTOR pathway may be suppressed in N2a-APP cells as a result of APP overexpression and the production of Aβ peptides in comparison with N2a cells.

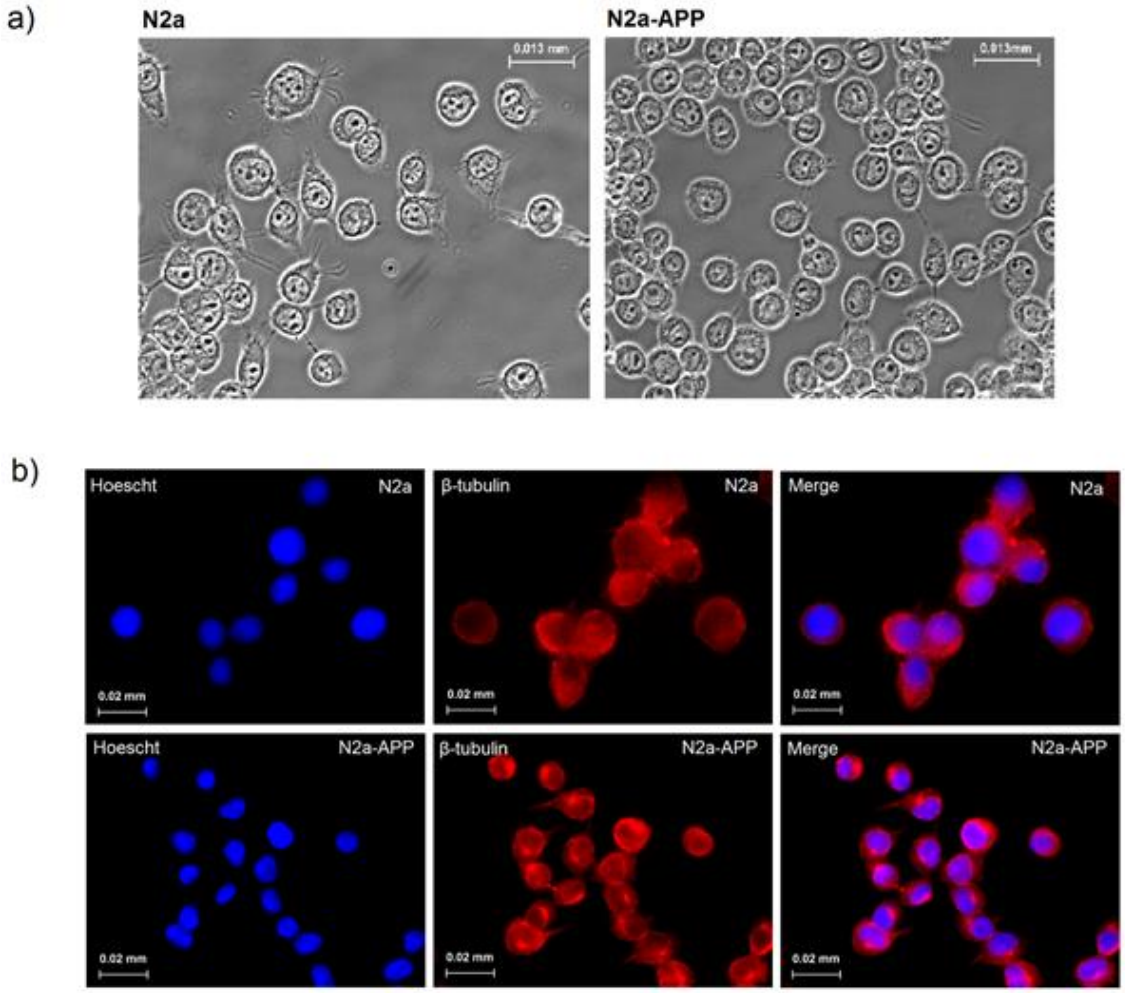

Figure 2a: Examination of N2a and N2a-APP cells by phase-contrast microscopy. b: Immunofluorescence visualization of N2a and N2a-APP cells using beta-tubulin mouse mAb (red). Nuclei were stained with Hoechst fluorescent DNA dye (blue).

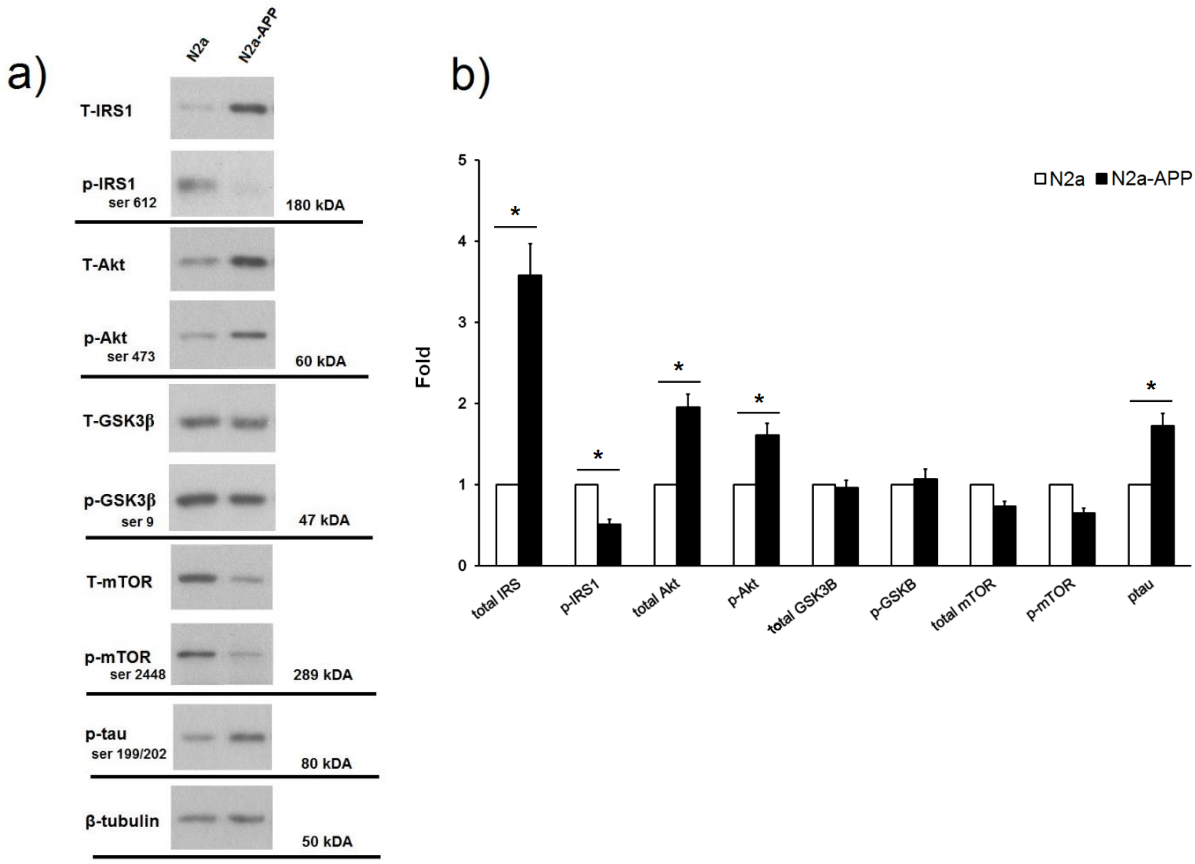

Figure 3: Basal levels of insulin signaling proteins in N2a and N2a-APP cells.

a: Mouse neuroblastoma 2a (N2a) cells, and N2a-APP cells stably transfected with the gene encoding hAPP695, were plated in serum-free media for 24 hours, then collected without any applied treatment to determine the cell lines' basal protein levels. **b**: N2a-APP cells have significantly lower basal levels of p-IRS, while having significantly higher levels of total IRS1, total Akt, p-Akt, and p-tau compared to basal levels in parental N2a cells (One-way ANOVA, Bonferroni post hoc test, *$p < 0.05$, $N \geq 37$). Levels of p-GSK3β, total GSK3β, p-mTOR, and total mTOR were not significantly changed between N2a and N2a-APP cells.

3.3 N2a-APP cells show insulin resistance phenotype.

To assess how the N2a-APP cells may respond differently to insulin than N2a cells, the two cell lines were treated with 0.1 nM, 0.5 nM, 5 nM, and 100 nM concentrations of insulin – a range that encompasses both physiological and superphysiological levels – over a time course of 15 minutes, 30 min, 1h, 2h, 4h, and 8 hours (Figure 4). The aim was to compare any fluctuations in the level of proteins in the insulin signaling pathway, and therefore any difference in insulin response between the two cell lines. The total level of IRS1 in response to insulin is significantly increased in N2a-APP cells. With the level of IRS1 at each treatment condition measured as fold change of IRS1 in relation to each cells' respective untreated controls, N2a-APP cells have higher positive change in IRS1 compared to N2a cells at each treatment condition, shown to be increased significantly at 30 minutes, 1 hour and 2 hours of 0.5 nM and 5 nM (Figure 4c). Given this pronounced increase of IRS1 in N2a-APP cells, when p-IRS1 is further normalized after the loading control to the increased IRS1, the final fold change from control of IRS1 phosphorylation is decreased in N2a-APP cells compared to N2a cells, shown to be significant statistically, particularly at 30 minutes, 1 hour, and 2 hours of 0.5 nM and 5 nM of insulin treatment. Together, these results show that the effect of APP or/and Aβ peptide on N2a cells is twofold: one, that Aβ induces an insulin resistant state in which the phosphorylation response of IRS1 is significantly diminished, and two, that the Aβ-mediated induction of resistance at IRS1 may also trigger the cell to overexpress IRS1 in an attempt to compensate for the lack of phosphorylation response. Since IRS is located at the upper stream of insulin signalling,

dysregulation of IRS expression or/and phosphorylation disturbs the transmission of insulin signalling downstream.

Further down the pathway, mTOR phosphorylation also displays a degree of insulin resistance in N2a-APP cells, in that at the lower insulin treatment concentrations of 0.5 nM and 5 nM, the level of p-mTOR is significantly lower in N2a-APP cells than in N2a cells (Figure 7c). However, unlike IRS1, response can be recovered with the application of higher treatment concentrations of insulin, as a significant increase of p-mTOR was observed with 5 nM and 100 nM of insulin treatment over the entire 15 minute to 8 hour time course (Figure 7b). N2a and N2a-APP otherwise respond similarly to insulin at corresponding treatment conditions, in terms of total expression and phosphorylation of Akt and GSK3β (Figure 5 and 6).

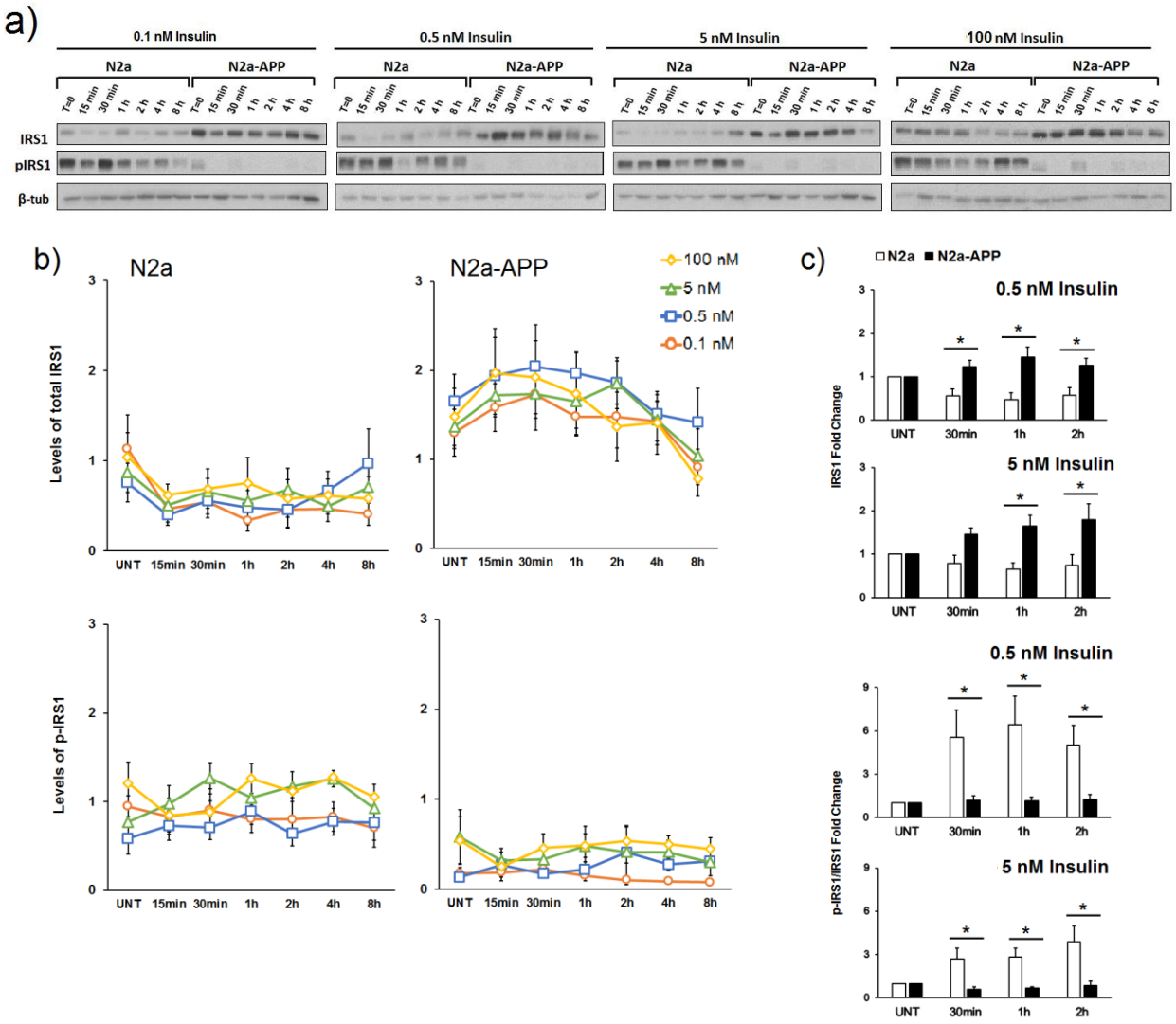

Figure 4: Effect of insulin on phosphorylation and expression of IRS1.

a: N2a-APP cells display decreased IRS1 phosphorylation and increased total IRS1 level compared to parental N2a cells. N2a and N2a-APP cells were treated with 0.1, 0.5, 5, and 100 nM insulin for 30 minutes, 1, 2, 4, and 8 hours. Western blot was performed to detect p-IRS1 (ser 612) and total IRS1, which were normalized to β-tubulin and both cell lines were presented relative to their respective untreated controls; p-IRS1 was also normalized to total IRS1. ANOVA was used to account for multiple comparisons across concentrations and time points (Two-way ANOVA, Bonferroni post hoc test, *$p < 0.05$, N=9). **b**: There is no significant concentration-dependent difference in the fold change of IRS1 or p-IRS1 in response to insulin treatment. **c**: N2a-APP cells have significantly higher fold change in total IRS1 levels compared to parental N2a cells, particularly at 30 minutes, 1 hour, and 2 hours of 0.5 nM and 5 nM of insulin treatment. When p-IRS1 is further normalized to the increased IRS1, the fold change of IRS1 phosphorylation is significantly decreased

in N2a-APP cells compared to N2a cells, specifically at 30 minutes, 1 hour, and 2 hours of 0.5 nM and 5 nM of insulin treatment.

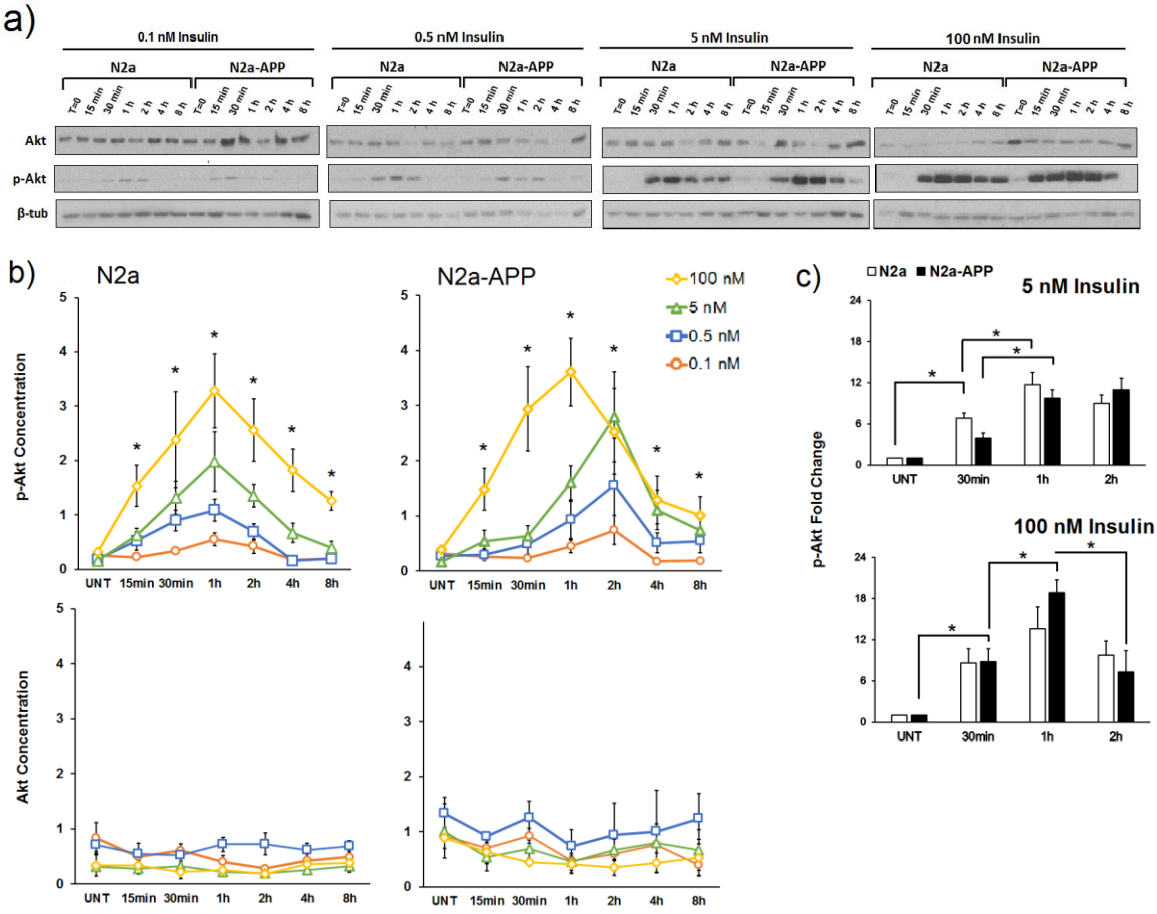

Figure 5: Effect of insulin on phosphorylation and expression of Akt in N2a and N2a-APP cells.

a: Both N2a and N2a-APP cells display significant time-dependent p-Akt phosphorylation at higher insulin concentrations. In the same time course experiment, western blot was performed to detect p-Akt (ser 473) and total Akt, which were normalized to β-tubulin and both cell lines were presented relative to their respective untreated controls. (two-way ANOVA, Bonferroni post hoc test, *$p < 0.05$, N=9). **b:** Although N2a and N2a-APP cells do not significantly differ from each other in fold change response to insulin, each cell line displays a response curve of Akt phosphorylation over the 8 hours of treatment. N2a cells peak in response to all concentrations of insulin at 1 hour of treatment, while N2a-APP cells display a delayed peak at 2 hours of treatment with 0.1 nM, 0.5 nM, and 5 nM of treatment. At the highest treatment concentration of 100 nM, however, the peak Akt phosphorylation remains at 1 hour in N2a-APP cells. 100 nM of insulin treatment in N2a cells elicits a significantly higher Akt phosphorylation response than 5 nM of insulin treatment at all treatment time points. Furthermore, 5 nM of insulin treatment in N2a cells produces significantly higher levels of p-Akt than 0.5 nM of insulin treatment for 2 and 4 hours. Finally, 0.5 nM of insulin in N2a cells induces

significantly more phosphorylation of Akt than 0.1 nM of insulin treatment for 30 minutes and 1 hour. N2a-APP cells display a significant increase of p-Akt at 100 nM of insulin treatment compared to 5 nM for 30 minutes and 1 hour. **c:** The fold change of p-Akt from untreated control in N2a cells at 30 minutes is significantly increased with 5 nM of insulin treatment, and with 100 nM insulin in N2a-APP cells at 30 minutes. Subsequently, with 1 hour of 5 nM insulin treatment, the level of p-Akt is significantly increased in both N2a and N2a-APP cells from the previous 30 minute time point. Significant increase is also seen in N2a-APP cells at 1 hour of 100 nM insulin treatment compared to 30 minutes. At 100 nm of insulin, at 2 hours of insulin treatment, the p-Akt fold change response in N2a-APP cells is significantly decreased from the 1 hour peak. Total Akt levels over the time course did not show a response curve pattern nor differentiating response levels between the two cell lines.

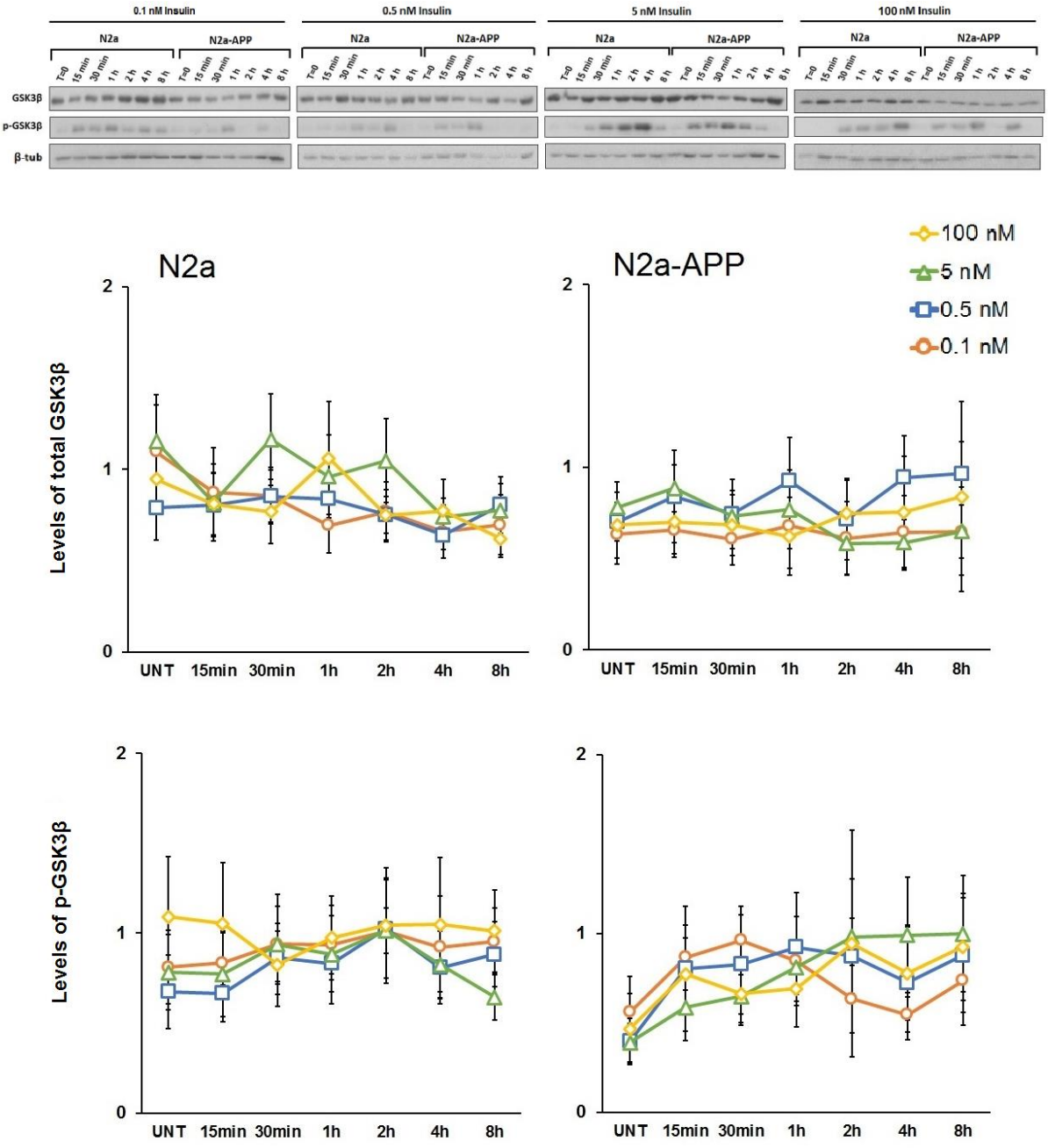

Figure 6: Effect of insulin on phosphorylation and expression of GSK3β in N2a and N2a-APP cells.

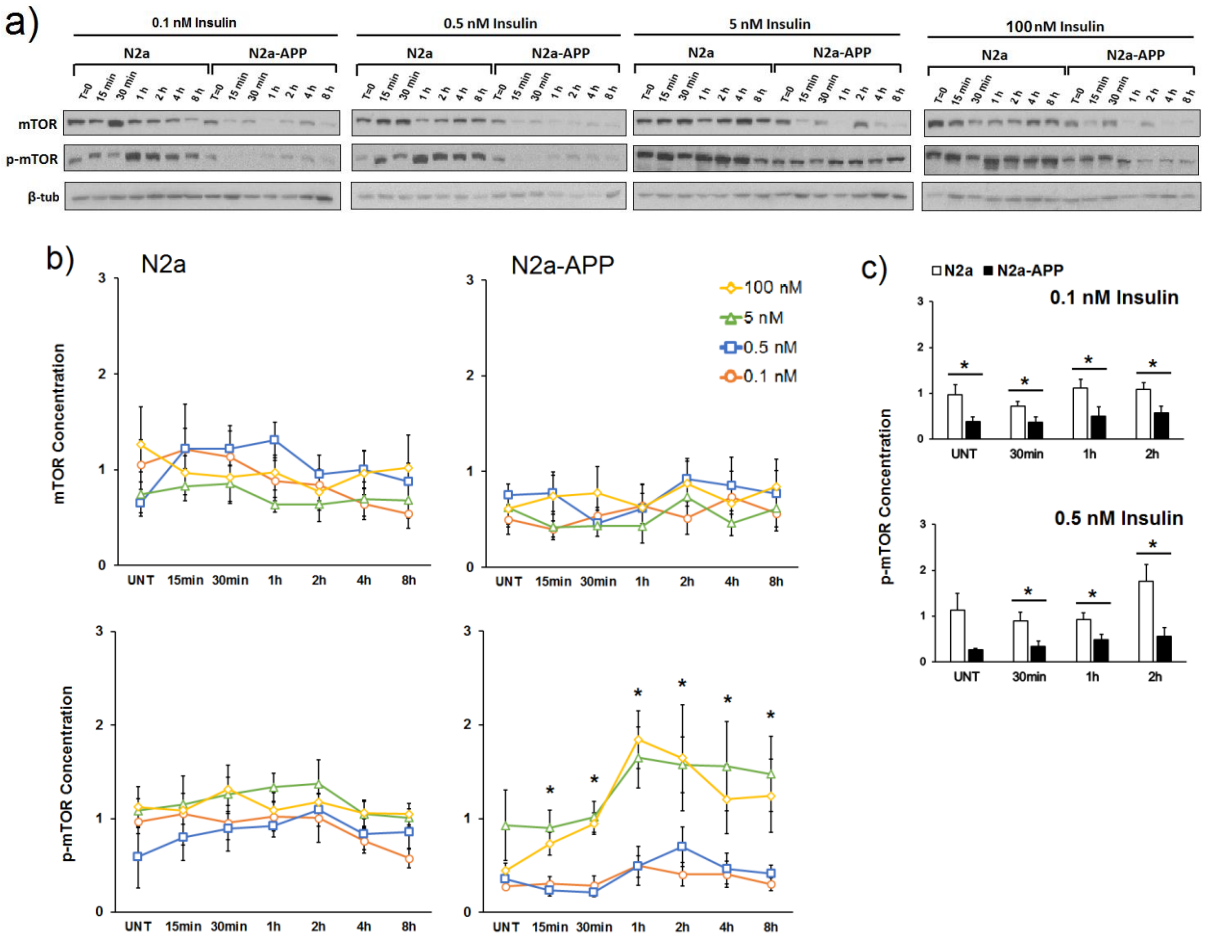

Figure 7: Effect of insulin on phosphorylation and expression of mTOR.

a: Phosphorylation of mTOR in N2a-APP cells is resistant at lower insulin treatment concentrations, but can be recovered with higher concentrations of insulin treatment. **b:** N2a-APP cells have significantly higher mTOR phosphorylation levels at 5 nM and 100 nM of insulin treatment compared to 0.1 nM and 0.5 nM treatments, across all time points, from 15 minutes to 8 hours. **c:** N2a-APP cells have significantly less phosphorylation of mTOR compared to N2a cells at 0.1 nM and 0.5 nM of insulin treatment, particularly at baseline, 30 minutes, 1 hour, and 2 hours of treatment.

3.4 Phosphorylation of Akt in both N2a and N2a-APP cells shows concentration-and time-dependent sensitivity to insulin.

Distinct concentration and time dependent patterns of sensitivity to insulin of both cell lines are apparent only in the phosphorylation of Akt. With 5 nM and 100 nM of insulin treatment, the time-dependent phosphorylation of Akt is apparent as a response curve of fold change, in that both N2a and N2a-APP cells have significantly increased phosphorylation at 15 minutes of treatment compared to the control, then subsequently higher fold increase of Akt phosphorylation at 30 minutes of treatment compared to 15 minutes (Figure 5c). With 5 nM of insulin treatment, N2a cells' phosphorylation response to insulin peaks at 1 hour, at which p-Akt is significantly increased from 30 minutes. With 5 nM of insulin, N2a-APP cells seem to peak at around 2 hours of treatment (Figure 5b). With the highest insulin treatment concentration of 100 nM, both N2a and N2a-APP cells seem to peak at 1 hour of treatment; in N2a-APP cells, p-Akt is significantly increased at 1 hour compared to 30 minutes, but is significantly decreased at 2 hours compared to the peak at 1 hour (Figure 5c). Although this response curve is visible at the lower treatment concentrations of 0.1 nM and 0.5 nM insulin, the concentration of p-Akt is too low to show any significant fold changes between time points.

Phosphorylation of Akt in response to insulin is also concentration dependent; starting at a very low absolute concentration of p-Akt is apparent at 0.1 nM of insulin treatment, the fold change of p-Akt from control increases with higher insulin treatments (Figure 5b). In general, N2a and N2a-APP cells share a similar pattern of progressive concentration dependent increase in Akt phosphorylation in response to insulin. The peak of the response curve for N2a cells is at 1 hour of treatment with all four insulin concentrations of 0.1, 0.5, 5 and 100 nM. The response of N2a-APP

cells, however, shows a comparatively delayed peak at 2 hours of treatment with 0.1 nM, 0.5 nM, and 5 nM of treatment. At the highest treatment concentration of 100 nM, the peak of Akt phosphorylation response remains at 1 hour in N2a-APP cells. 100 nM of insulin treatment in N2a cells elicits a significantly higher Akt phosphorylation response than 5 nM of insulin treatment for 15 minutes, 1h, 2h, 4h, and 8 hours. Likewise, 5 nM of insulin treatment of N2a cells for 2 and 4 hours produces significantly higher levels of p-Akt than 0.5 nM of insulin treatment. Finally, 0.5 nM of insulin in N2a cells induces significantly more phosphorylation of Akt than 0.1 nM of insulin treatment for 30 minutes and 1 hour. N2a-APP cells, while in trend shows the same pattern of concentration-dependent increase as N2a cells, only displays a significant increase of p-Akt at 100 nM of insulin treatment compared to 5 nM for 30 minutes and 1 hour. The above results suggest that the response of Akt phosphorylation to insulin is delayed and the magnitude of the response is minimized in N2a-APP cells as compared to N2a cells.

3.5 Amyloid-beta binding peptide (ABP) pre-treatment in N2a-APP cells enhances insulin signalling response

While the effect of Aβ on insulin signalling dysregulation has been established, it is important to determine whether clearing or inhibiting Aβ in the N2a-APP cells' extracellular environment can relieve the stress on the signalling pathway. To this end, N2a and N2a-APP cells were treated with 0.5 nM and 5 nM insulin for 30 minutes, 1 hour, and 2 hours. Before the insulin treatment, N2a-APP cells were pre-treated with Aβ binding peptide (ABP) at a concentration of 1000 times that of extracellular Aβ, which was previously found by Dr. Wandong Zhang's lab to be 2000 pg/mL. Cell protein was extracted and probed via western blot for p-IRS1 (ser 612), IRS1, p-Akt (ser 473), Akt, p-GSK3β (ser 9), GSK3β, p-tau (ser 199/202), and p-mTOR (ser 2448), mTOR, and p-p70S6 Kinase (thr 389). Results were normalized to β-tubulin, and the combined treatment condition of ABP and insulin was presented relative to corresponding treatment concentration of insulin only.

At the beginning of the insulin signalling pathway, there is an immediate significant increase of p-IRS1 in N2a-APP cells treated with ABP followed by 0.5 nM insulin for 30 minutes as compared to N2a-APP cells treated only with insulin (Figure 8c). In N2a-APP cells, p-IRS1 is also significantly increased in response with 5 nm insulin treatment for 1 hour when preceded with 24 or 48 hours of ABP pre-treatment as compared to N2a-APP cells treated only with insulin (Figure 8c). With regards to total IRS1 levels, significant response increase in N2a-APP cells is also observed at 30 minutes of 0.5 nm insulin after 24 hours of ABP pre-treatment compared to insulin only; the significant increase in IRS1 seems to be sustained with 24 hour pre-treatment and subsequent 0.5 nM or 5 nM insulin treatment, then also after 48 hours of pre-treatment and following 2 hours of 0.5 nM or 5nM treatment (Figure 8b).

These results suggest that ABP may be binding up the extracellular Aβ that would otherwise competitively interact with the insulin receptor, thus allowing for increased receptor binding of insulin and increased phosphorylation activity upon the IRS1 protein directly associated with the receptor. As for the increase in IRS1, it was previously concluded that the upregulation of IRS1 in APP cells may be in compensation of the decreased phosphorylation of IRS1, yet if some phosphorylation is being recovered with ABP treatment, it would also be expected that IRS1 expression would decrease since the compensation is not needed. However, ABP pre-treatment seems to additionally increase IRS1 response significantly. The reason for this may be that the cells are still in a state of trying to compensate for the lack of p-IRS1, in that expression of IRS1 may take longer to adjust accordingly to improved IRS1 phosphorylation.

As for p-Akt downstream of IRS, pre-treatment with of N2a-APP cells with ABP for 24 hours does seem to significantly increase levels of p-Akt in response to insulin than the cells treated with 5 nm insulin for 30 minutes (Figure 9). Similarly, with 48h ABP pre-treatment, the same significant increase at 30 minutes of 5nm insulin was seen. ABP may be enhancing insulin signalling for the phosphorylation of Akt in these conditions.

Further downstream in the signalling pathway, phosphorylation of mTOR at ser 2448 seems to be also increased in trend with 24 hours of ABP pre-treatment followed by 5 nm insulin, that is, compared to N2a-APP cells only treated with 5 nm insulin (Figure 10). Directly downstream of mTOR is p70S6 Kinase, which is a direct indicator of the level of mTORC1 activity since its phosphorylation is dependent on the activation of mTORC1. The phosphorylation of p70S6 kinase in response to insulin is diminished in N2a-APP cells compared to N2a cells, suggesting decreased

mTOR activity. However, pre-treatment with ABP significantly increases phosphorylation of p70S6 Kinase in N2a-APP cells in response of insulin compared to non ABP-treated N2a-APP cells, specifically with 24 hours of ABP pre-treatment followed by 0.5 nm or 5 nm insulin (Figure 11). This is consistent with the aforementioned trend of increase in p-mTOR and mTOR.

Interestingly, IRS1, Akt, mTOR, and p70S6 kinase seems to be the only proteins we examined within the pathway that significantly exhibits enhanced phosphorylation after ABP pre-treatment. Analysis of the phosphorylation GSK3β and of tau did not show any significant trends in changing response to insulin in the cells. However, there are about 79 phosphorylation sites of serine and threonine on tau protein, therefore the phosphorylation sites of ser 199/202 targeted in this experiment may not be the ones affected in these treatments.

The timing of each of these proteins' phosphorylation increase is important to consider. At 30 minutes of insulin treatment, only IRS1, Akt and p70S6K seem to show significant increases in phosphorylation, while the rest of the pathway proteins are significantly phosphorylated or expressed later at 1 or 2 hours. This may suggest that functioning of the insulin signalling pathway, and its adaptations to exogenous effectors, do not constitute a global shifting in phosphorylation or expression in one direction or the other along the pathway, nor do these shifts occur at the same time. Some proteins may need more time to resume their normal levels or function, or their recovery may not even be reflected by changes in phosphorylation or expression, but instead as increases in activity at a particular time point.

3.6 Co-treatment of phosphatidic acid and insulin does not affect insulin response in N2a-APP cells

An additional experiment was performed that investigated the effect of regulating mTOR activity on the insulin signaling pathway: a co-treatment of N2a and N2a-APP cells with phosphatidic acid (PA) and insulin. Since PA is an mTOR agonist, it tests how mTOR, as well as its upstream and downstream signaling proteins, functions within the parental and N2a-APP cell models when its activity is upregulated. The data showed that there was no significant change in phosphorylation or expression of the proteins in the insulin signalling pathway in response to insulin when cells were co-treated with PA.

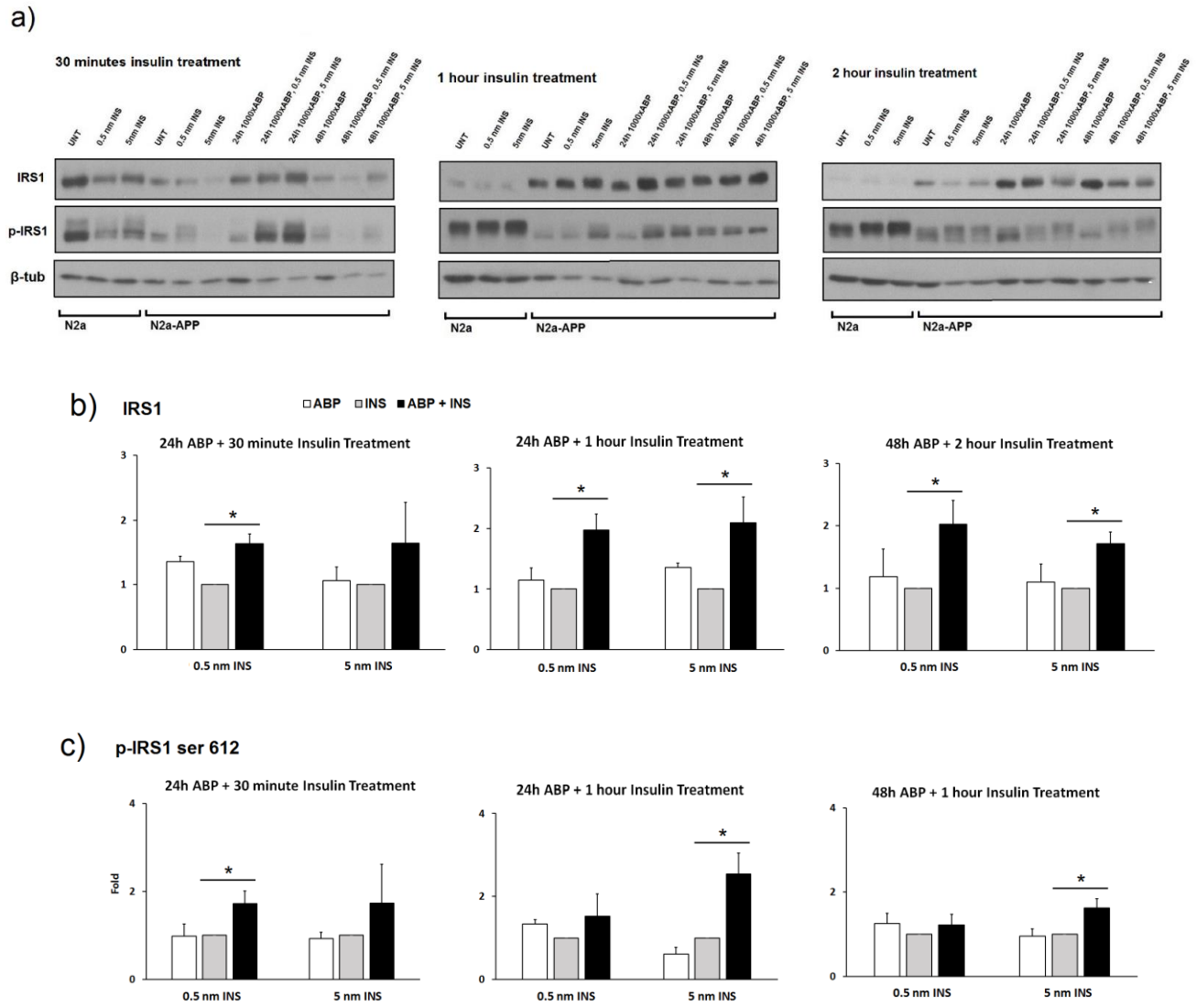

Figure 8: Effect of amyloid binding peptide (ABP) on the phosphorylation and expression of IRS1 in response to insulin.

a: N2a and N2a-APP cells were treated with 0.5 nM and 5 nM of insulin for 30 minutes, 1 and 2 hours. N2a-APP cells were also pre-treated with Aβ binding peptide (ABP) obtained from Dr. Balu Chakravarthy at the NRC, at 1000x the extracellular concentration of amyloid beta (2000 pg/mL as determined by Dr. Wandong Zhang's lab) for 24 or 48 hours. The media were replenished with ABP at 24 hours for plates that were treated for a total of 48 hours. Cell protein was extracted and probed via western blot for p-IRS1 (ser 612), IRS1, p-Akt (ser 473), Akt, p-GSK3β (ser 9), GSK3β, p-tau (ser 199/202), and p-mTOR (ser 2448), mTOR, and p-p70S6 Kinase (thr 389), then normalized with β-tubulin. The ABP + insulin conditions were presented relative to the insulin only conditions. The *t*-test was used to assess statistical significance of the ABP + insulin-treated samples' response compared to only that of the insulin-treated samples. (Two-tailed, equal or unequal

variance *t*-test, *p < 0.05, N=4). **b:** At 30 minutes and 1 hour of 0.5 insulin treatment after 24 hours of ABP pre-treatment, N2a-APP cells express significantly higher levels of IRS1. IRS1 response is also significantly elevated with 1 hour of 5 nM insulin after 24h pre-treatment. Also at two hours of either 0.5 or 5 nm insulin after 48 hours of ABP, IRS1 is significantly higher in N2a-APP cells compared to insulin treatment without ABP. **c:** p-IRS1 (s612) protein is significantly increased in N2a-APP cells trend as compared to N2a cells, in response to 30 minutes of 0.5 nM insulin treatment after 24 hours of ABP pre-treatment. At the one hour 5nm insulin treatment condition after 24 and 48 hours of ABP pre-treatment, p-IRS1 response is significantly higher in N2a-APP cells compared to their insulin-only condition.

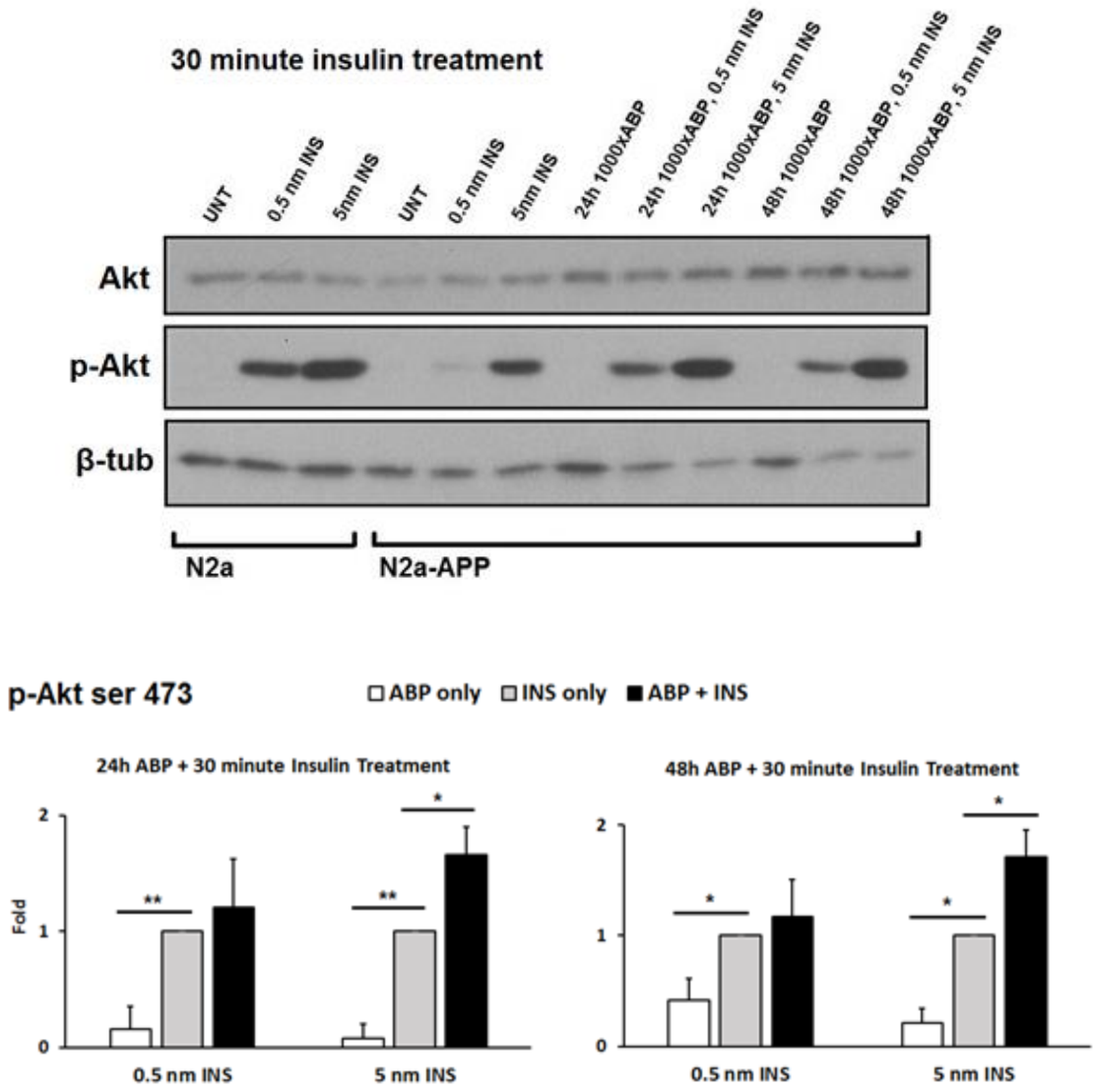

Figure 9: Effect of amyloid binding peptide (ABP) on the phosphorylation and expression of Akt in response to insulin.

There is a significant increase in Akt phosphorylation at ser 473 in N2a-APP cells that have been treated for 30 minutes with either 0.5 or 5 nm insulin after 24 and 48 hours of ABP pre-treatment as compared to N2a-APP cells treated with insulin or ABP only (two-tailed, equal or unequal variance *t*-test, *p < 0.05, ** < 0.001, N=4). In addition, N2a-APP cells treated only with 0.5 or 5 nm insulin shows significantly increased levels of p-Akt compared to N2a-APP cells treated only with ABP, which indicates that insulin alone already has a pronounced effect on Akt phosphorylation, that which is enhanced with ABP pre-treatment.

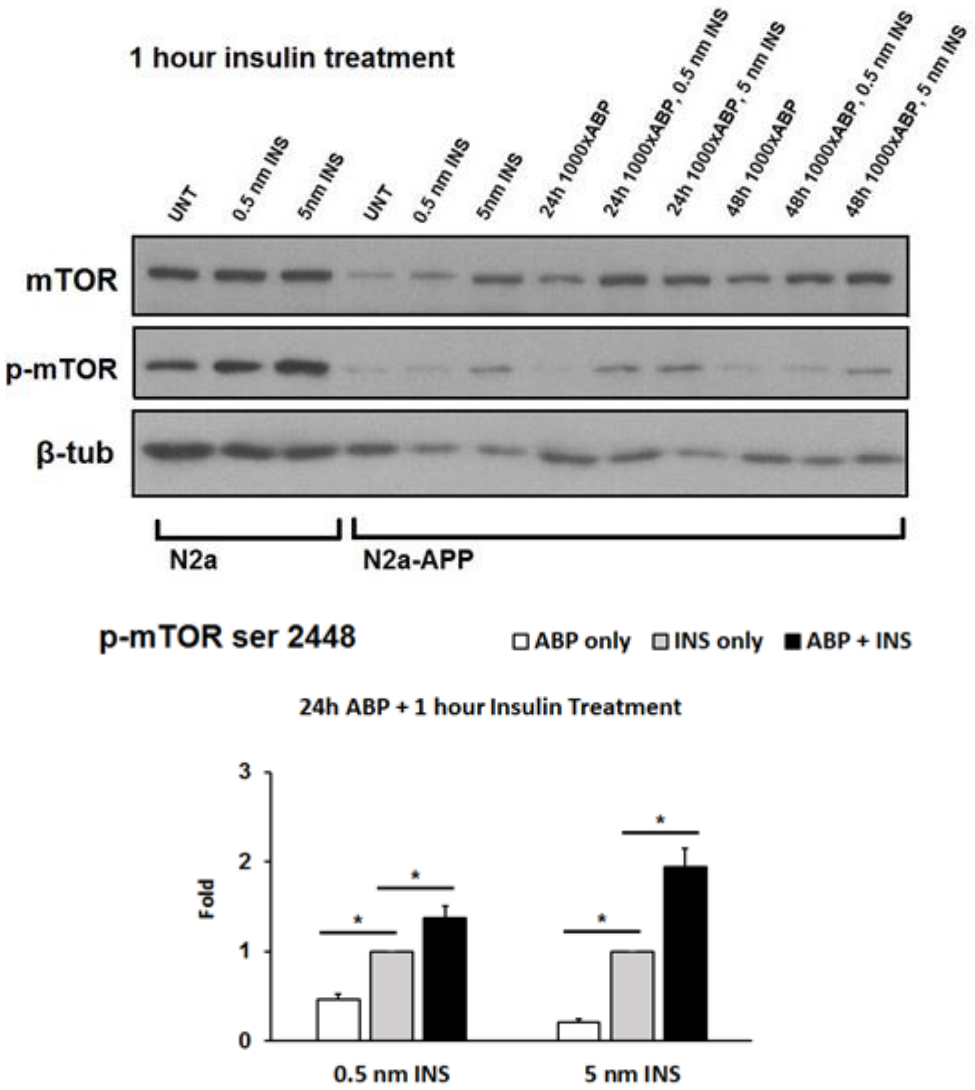

Figure 10: Effect of ABP on the phosphorylation of mTOR in response to insulin.

The phosphorylation of mTOR at ser 2448 to 1 hour of 5 nm insulin treatment after 24 hours ABP pre-treatment, as well as to 1 hour of either 0.5 or 5 nm insulin treatment after 48 hours of ABP is significantly increased in N2a-APP cells as compared to control N2a-APP cells (two-tailed, equal or unequal variance *t*-test, *p < 0.05, N=4). Insulin treatment alone also induces significantly higher mTOR phosphorylation than ABP alone; though insulin already enhances phosphorylation, the combined treatment further increases it.

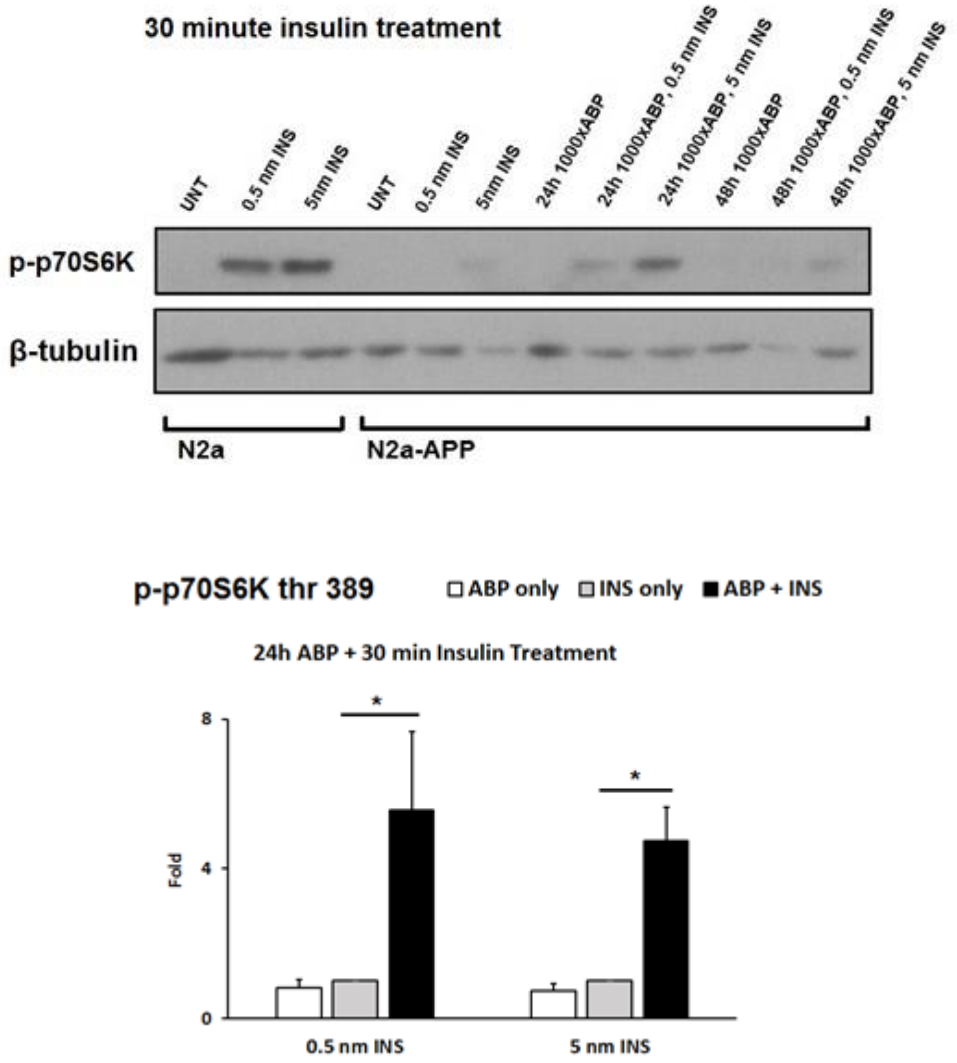

Figure 11: Effect of ABP on the phosphorylation of p70S6K in response to insulin.

Phosphorylation of p70S6K is significantly increased in N2a-APP cells with 30 minutes of 0.5 or 5 nm insulin treatment after 24 hours of ABP pre-treatment as compared to control N2a-APP cells (two-tailed, equal or unequal variance *t*-test, *p < 0.05, N=4).

Discussion

4.1 Aβ oligomers disrupt basal insulin signalling and induce insulin resistance

Insulin resistance in Alzheimer's disease is increasingly becoming an important area of research focus. Understanding how insulin resistance affects the ultimate progression of neurodegeneration in the context of both Aβ and tau pathogenesis is a critical step in linking these previously established pathogenic theories to more recently recognized realities about insulin signalling regulation in Alzheimer's disease. To characterize the effect of Aβ-mediated insulin resistance on these signalling proteins, a cell model was used in which the signaling response is compared between the parental N2a cells and the N2a-APP line. The N2a-APP cell line that has been transfected to express the isoform of APP695, mainly found in the human brain, consistently expresses significantly elevated levels of APP and Aβ compared to the parental N2a cells. Both Aβ40 and Aβ42 were significantly higher in concentration in the culture medium of the N2a-APP cells. No senile plaques are present in the culture medium since SPs develop in the brain tissue of the AD brain. These Aβ peptides may present as monomers or multimers, while Aβ42 may aggregate into the soluble oligomers in the medium. This model is a commonly used *in vitro* model to study the effect of Aβ on cell function and signaling.

My analyses found that N2a-APP cells have dysfunctional insulin responses at several but not all points in the insulin signaling pathway when compared to the parental N2a cells. Even without insulin stimulation, portions of the insulin signalling pathway are dysregulated. Basal levels of Akt and mTOR expression as well as their phosphorylation states are significantly increased and decreased in N2a-APP cells, respectively, which suggests that the presence of excess AβOs, or at least Aβ itself,

affects basal signalling functions (Figure 3). There was significantly reduced p-IRS1 signalling but increased IRS1 expression at basal level and at all treatment concentrations, as well as a trend of p-mTOR signalling reduction at lower physiological concentrations of insulin, but there was no significant difference across the insulin treatment concentrations in the levels of Akt, p-Akt, GSK3β, and p-GSK3β. The clearest indication of insulin resistance in the N2a-APP cells in comparison to the parental N2a cells is the sustained lack of phosphorylation of IRS1 in response to insulin. It is known that AβOs not only compete with insulin to bind to IRs, it also subsequently induces the internalization of IRs (Zhao, De Felice et al. 2008). Since insulin must bind to the IR for the associated IRS1 to be phosphorylated, it would follow that if there are no IRs for the insulin to be bound to, no amount of applied exogenous insulin would elicit a response.

In the AD brain analyzed post-mortem, it is commonly found that IRS1 is decreased and p-IRS1 at serine 312, 616 and 636 are increased, with elevated p-IRS1 at serine 616 in humans.(Steen, Terry et al. 2005. Talbot, Han et al. 2006. Boura-Halfon and Zick 2009). In the N2a and N2a-APP cells collected from the current in-vitro experiments, the opposite is true: IRS1 is increased and p-IRS1 is decreased. The significant downregulation of IRS1 phosphorylation at serine 612 in N2a-APP cells is an indication that the N2a-APP cells are not responding to insulin stimulation as N2a cells do. With regards to IRS1 phosphorylation response, N2a-APP cells appear display insulin resistance, but for the most part, the insulin response of downstream signaling is comparable between N2a and N2a-APP cells. In this regard, N2a-APP cells may be employing compensatory mechanisms that preserve much of the downstream signaling functions from AβO-mediated insulin resistance.

Observed changes may be attributable to the disparity between an in-vitro and in vivo systemic models of AD. In a human body, or an in-vivo animal model, cellular functioning is potentially influenced by the various body systems present within a systemic model, including immune and inflammatory responses. In addition, the post-mortem studies only examined a small piece of brain sample from one patient, which may not represent the global picture of the diseased brain with regards to the different distribution and density of IR and IRS1 in different regions of the brain. Furthermore, AβOs also have the capacity to stimulate TNF-α release by targeting microglia and astroglia (Ledo, Azevedo et al. 2013). Accordingly, TNF-α, a pro-inflammatory cytokine, is found to be elevated in the AD brain in some studies (Tarkowski, Liljeroth et al. 2003. Ye, Price et al. 2003). It is established that TNF-α mediates obesity-induced insulin resistance in mice, and is associated with humans who are obese and peripherally insulin resistant (Uysal, Wiesbrock et al. 1997. Hotamisligil, Arner et al. 1995). One of the effects of elevated TNFα is the activation of the c-Jun N-terminal kinase (JNK), which then phosphorylates IRS1 at serine 312, or serine 307 in mouse IRS1 (Lee, Giraud et al. 2003). The phosphorylation of IRS1 at these specific sites has been associated with the dissociation of IRS1 from IR, and the subsequent degradation of IRS1 and insulin resistance (Zhande, Mitchell et al. 2002, Aguirre, Werner et al. 2002). Since the TNFα is primarily produced by T-cells and macrophages, specifically the microglia in the CNS, a cell culture model is limited by the absence of microglia (Falvo, Tsytsykova et al. 2010). It would follow that the IRS1 levels in the N2a-APP cells would not be as subject to effect of TNFα; the significant upregulation observed in the N2a-APP cells could partly be a result of extracellular AβOs exerting an effect on insulin signaling without the crosstalk of the TNFα, among other systemic influences.

While N2a-APP cells are seemingly mostly protected from insulin resistance-mediated signaling downstream of IRS1, the mystery of how this occurs deepens. It is likely that other signaling pathways are involved which may also affected by AβO. A candidate to consider is the N-methyl-D-aspartate receptor (NMDAR), which is known to be affected by AβOs, although the exact mechanism is not fully understood (De Felice, Velasco et al. 2007. Decker, Jurgensen et al. 2010). It is suggested that AβOs interfere with glutamate reuptake, therefore causing the overactivation of NMDAR (Jacob, Koutsilieri et al. 2007, Talantova, Sanz-Blasco et al. 2013). The involvement of NMDARs in AD is clear; impaired LTP and enhanced LTD in AD is mediated by NMDAR and its downstream effectors (Zhao, Watson et al. 2004. Walsh, Klyubin et al. 2002. Li, Hong et al. 2009). Of particular important is the position of Akt as one of the downstream targets of the NMDAR. The activation of NMDAR through synaptic release of glutamate is linked to downstream activation of Akt (Yano, Tokumitsu et al. 1998. Sutton and Chandler, 2002). The AβO-mediated overactivation of NMDAR, when considered with NMDAR-mediated downstream activity, may explain the apparent preservation of Akt phosphorylation response in the N2a-APP cells. Any downregulation of Akt phosphorylation through AβO binding to the IR may be balanced by the upregulation of Akt phosphorylation through the AβO-mediated overactivation of the NMDAR, ultimately leveling Akt phosphorylation in N2a-APP to that of the parental N2a cells. An indication of insulin resistance is suggested in the delay of the response peak in N2a-APP cells (Figure 5b). The downstream effect of this net preservation of Akt activation is that GSK3β and mTOR activity would also theoretically be preserved.

Indeed, it seems there are no differences between the parental N2a and mutant N2a-APP cells in GSK3β expression or tau phosphorylation response to

insulin. The fact that the active form of GSK3β never wavers in amount is indicative of a robust regulatory system in this cell model that keeps GSK3β levels constant, thus protecting the cell from tau hyperphosphorylation. The cell is then also protected from dysregulation of axon-dendrite polarity (Jiang, Guo et al. 2005), long term potentiation and depression (Bradley, Peineau et al. 2012), which all involve GSK3β in their regulation. These results are in contrast to the common finding that GSK3β is less phosphorylated in the AD brain, and is correlated with tau hyperphosphorylation and overproduction of Aβ, the latter being specifically linked to the aberrant processing of APP with GSK3β upregulation (Hernandez, Gomez de Barreda et al. 2012. Sun, Sato et al. 2002, Phiel, Wilson et al. 2003). The fact that Akt activation in response to insulin was not changed in N2a-APP cells compared to the parental N2a cells would suggest that GSK3β also would not be changed. At basal levels however, Akt and p-Akt are both upregulated in N2a-APP cells while GSK3β and p-GSK3β are not, even though Akt activation is directly correlated with GSK3β inactivation by its phosphorylation (Bijur and Jope, 2003). As GSK3β is not changed in response to insulin treatment, the phosphorylation of tau is also accordingly unchanged in the in-vitro experiments.

In contrast to the unchanging nature of GSK3β, the phosphorylation of mTOR in N2a-APP cells does exhibit a significant lack of response to insulin at lower concentrations of insulin. This would support the hypothesis that the interaction between Akt and the mTOR pathway is a not straightforward progression of response and effect. If the Akt phosphorylation response to insulin is preserved in N2a-APP cells, the downstream phosphorylation of mTOR should also then be preserved, but current observations show that mTOR phosphorylation at the lower

treatment concentrations of insulin are not consistent with the conserved Akt phosphorylation levels (Figure 7b). At 0.1 and 0.5 nM of insulin treatment, N2a-APP cells have significantly less mTOR phosphorylation than N2a cells, while the level of upstream phosphorylation of Akt is not different between the two cell lines. The conclusion then follows that Akt-mediated mTOR phosphorylation is subject to AβO-mediated insulin resistance that requires higher concentrations of insulin treatment to overcome.

4.2 Removal of extracellular Aβ oligomers relieves insulin resistance

A hint of the complexity of the insulin signalling pathway was seen when investigations turned to attempting to modulate the extracellular AβOs by binding them, thus uncovering any changes – or lack thereof – in the pathway's response to insulin. As before, the results of these treatment experiments suggest that when the insulin signaling pathway is acted upon either endogenously by the N2a-APP cells' excess Aβ secretions, or by exogenous treatments, not all signaling proteins in the pathway are affected. In binding the Aβ in the extracellular environment, some enhancement of insulin response was suggested along the pathway, particularly with p-IRS, p-Akt, p-mTOR and p-p70S6 kinase, all of which showed increases in insulin response after ABP pre-treatment. Given that the ABP preferentially binds the oligomeric forms of Aβ42, as confirmed by the Dr. Balu Chakravarty's laboratory who developed the binding peptide, the effects precipitating from the ABP pre-treatment would be a result of lessening the load of AβOs that would bind to the insulin receptors (Chakravarthy, Menard et al. 2013). The first interesting effect is the increase of IRS1 and p-IRS1 to insulin treatment in N2a-APP cells after ABP pre-treatment, though one would expect a downregulation of IRS1 when AβOs are

cleared away. Removal of the main pathogenic agent would presumably bring the N2a-APP cells back towards the normal state of the parental cells, which is indeed observed in the recovery of p-IRS1 phosphorylation at serine 612 (Figure 8c). ABP pre-treatment seems to further increase IRS1 expression in response to insulin in N2a-APP cells, which already express significantly more IRS in response to insulin alone than N2a cells (Figure 7b). It is possible that IRS1 is being further upregulated in immediate response to the clearing of AβOs from the culture media in an attempt to adapt to the sudden increased availability of insulin; although this is simply speculation and should be investigated through further studies involving IR expression and extracellular insulin load.

Downstream of IRS1, the observed increase of p-Akt, p-mTOR, and p-p70S6K is consistent with the expected effect of increased serine phosphorylation of IRS1, which is an indication of recovered insulin response in N2a-APP cells by ABP treatment. These results are promising considerations for developing treatments targeting AβOs, since it is suggested that reducing the extracellular AβOs that would competitively bind to insulin receptors recovers signalling throughout the insulin signalling pathway. Of particular interest is the stimulation of Akt and mTOR phosphorylation, which are downregulated in the pathogenesis of AD. The observation that GSK3β and p-GSK3β remains unchanged in N2a-APP cells suggests that some protective mechanism maintains normal active levels of GSK3β in the face of AβO pathology and the relevant treatments. The relatively lower mTOR signaling at the lower insulin treatment range is able to be somewhat recovered with ABP pre-treatment, and with subsequent 0.5 and also 5 nm insulin treatment (Figure 10). The timing of this recovery of response is important to consider in how mTOR signaling functions in this context. Significant recovery of mTOR phosphorylation

comes after, not before or at the same time, as the ABP-mediated enhancement in Akt phosphorylation at 30 minutes of insulin treatment after the ABP pre-treatment (Figure 9). This implies that Akt is located upstream of the mTOR pathway, and that most likely the effect of the ABP pre-treatment is first exerted upon the mTORC1 pathway, which is downstream of Akt. The increase in p70S6K phosphorylation is also an indication of increased mTORC1 activity. Whether activated mTORC2 also further phosphorylates additional Akt is unclear.

These findings establish that in N2a-APP cells, AβOs disturb insulin signaling and induce insulin resistance by interacting with the insulin receptor. By the observed reduction in IRS1 phosphorylation, which is directly associated with insulin binding to the insulin receptor, the presence of excess Aβ in the N2a-APP cells' culture media affects the PI3K/Akt signaling directly downstream, as well as the closely associated mTOR signaling pathway. Clearance of Aβ or AβO by binding agents such as ABP would then reduce the interactions of Aβ or AβO with insulin receptor and improve insulin signalling. The implications of these results, if the observed effect of ABP treatment in the N2a-APP cells can be adapted for human models of AD, are substantial. If insulin signaling can be rescued or stimulated where resistance is apparent, the pathophysiology of AD that is mediated by insulin resistance in the brain could be attenuated or even halted altogether. Clearing soluble AβOs extracellularly in the brain could not only reduce the formation of insoluble plaques, but also reduce the formation of NFTs; Akt phosphorylation can be enhanced by agents such as ABPs, then GSK3β activation could be decreased, thus reducing the hyperphosphorylation of tau. The cognitive decline that is correlated with neuronal dysfunction and death and cerebral atrophy precipitating from the progressive formation of SPs and NFTs could be relieved or halted,

therefore potentially improving the prognosis of patients diagnosed with AD. Whether treatments like ABPs can be developed as preventative measures remains to be further investigated.

4.3 Future work and concluding remarks

With each experiment, the complexity of the insulin signaling pathway becomes more evident, with intersecting response pathways, feedback or compensation mechanisms at work to preserve cell function. As to why there are contradictions in the phosphorylation and expression levels of various signaling proteins between this in-vitro model and in-vivo, systemic models, the likely explanation would be based on the disparity of complexity. The stresses that are exerted on the human CNS system suffering from AD may promote or exacerbate dysfunctions in signaling that are not otherwise aggravated in a simplified in-vitro model. As for insulin signaling, excess soluble AβO induces a state of insulin resistance, observed as a pronounced decrease in the serine phosphorylation of IRS1 in the N2a-APP cells at basal levels as well as in reduced response to insulin, and also as dysregulation of basal levels and phosphorylation of Akt, mTOR, and tau. Furthermore, it was shown that by binding AβO from the culture media with ABP, thus preventing its competitive binding on insulin receptors, PI3K/Akt/mTOR signaling can be enhanced upon insulin treatment. The effects of AβO may go through insulin signalling or may be bypassing the upstream insulin signaling pathways through other avenues to affect downstream signaling, which is entirely plausible since Akt, GSK3β, and mTOR are also effectors involved in other receptor-mediated pathways. Using this cell model of AD, research focus should be expanded to evaluate the activity of other signaling pathways, such as the aforementioned

NMDA and the Wnt pathways, which are known to be affected in AD. All of these considerations for study should also be extended to in-vivo models, where systemic influences can also be accounted for, particularly the inflammatory response. There is a wealth of knowledge as of yet untapped as to which other additional signaling mechanisms are dysfunctional in AD, and the extent to which these interact and influence pathogenesis with regards to APP processing and tau phosphorylation, ultimately leading to the neuronal death that underlies progressive cerebral atrophy and cognitive decline. In addition, studies with AD animal models of insulin resistance may address the issues that the in vitro models cannot resolve. My findings that ABP can relieve some of insulin resistance in the N2a-APP cells suggest that Aβ or/and AβO is responsible for the dysregulation of insulin signalling in this model and that clearance or binding of Aβ or AβO may be one of potential therapeutic options for further investigations to relieve insulin resistance and improve insulin signalling in Alzheimer's disease.

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