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Dysregulation of Insulin Signaling in Human AD Brain and Alleviation of Aβ-Induced Insulin Resistance by Amyloid-β Binding Peptide (ABP) in Neural Cells

Yuka Sai[1], Balu Chakravarthy[2], Debbie Callaghan[2], Qiao Li[1#] and Wandong Zhang[1,2*]

[1]*Department of Cellular and Molecular Medicine, University of Ottawa, Canada*
[2]*Department of Human Health Therapeutics, Human Health Therapeutics Research Centre, Canda*

Abstract

Alzheimer's disease (AD) is a progressive neurodegenerative disorder characterized by the accumulation of extracellular amyloid-β peptides (Aβ) and intra-neuronal neuro-fibribillary tangles in the brain. Increasing evidence builds a strong case for the role of soluble Aβ oligomers (AβOs) in the impairment of insulin signaling in AD. Insulin signaling pathway begins upstream at the insulin receptor by phosphorylating IRS1 and propagating the signal downstream to the PI3K/Akt which down-regulates GSK3β activity for tau phosphorylation and activates mTORC1 that mediates a wide range of cellular functions. Our study found that human AD brains had high levels of Aβ peptides with cerebral amyloid angiopathy (AD/CAA) and showed low activities of insulin signaling-responsive transcription factors as compared to age-matched non-demented controls (ND). Our further studies with neuroblastoma 2a (N2a) cells stably transfected with a human AβPP695 gene (N2a-AβPP), which secrete excessive Aβ, show that the basal levels of the expression and phosphorylation of several but not all critical signaling proteins along insulin signaling pathway are dysregulated as compared to the parental N2a cells. N2a-AβPP cells were phenotypically insulin resistant in response to insulin stimulation. Pre-treatment of N2a-AβPP cells with the Aβ-binding peptide (ABP), which binds and removes Aβ oligomers, significantly enhanced insulin signaling response in cells compared to controls. Taken together, our data suggest that human AD/CAA brains had dysregulation of insulin signaling and that Aβ oligomers may be responsible for inducing the insulin-resistant phenotype in N2a-AβPP cells and the removal of Aβ oligomers by ABP improved insulin signaling and relieved insulin resistant phenotype.

Keywords: Alzheimer's disease • Amyloid-β peptide • Insulin signaling dysregulation • Insulin resistance • Intracellular signaling proteins • Amyloid-β binding peptide

Introduction

The characteristic pathological markers of Alzheimer's disease (AD) are extracellular senile plaques composed of β-amyloid peptides (Aβ) and intraneuronal neurofibribillary tanglesin the brain [1-3]. While it is known that extracellular 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 [1-6]. Studies show that AD brain displays dysregulation of insulin signaling and insulin resistance which may contribute to brain malfunction [6-10]. Recent work suggests that human herpesvirus and/or other pathogens disrupt the molecular, genetic and clinical networks in Alzheimer's brain [11-13]. Aβ oligomers were shown to bind herpesvirus surface glycoproteins and accelerate β-amyloid deposition leading to entrapment of herpesviruses in a mouse AD model and human neural cell culture model [14]. Over-production and impaired clearance of Aβ peptides and oligomers in response to those stresses may play a critical role in the subsequent development and progression of Alzheimer's disease. It is thus important to pursue further study to understand the role of AβOs in AD, particularly how soluble AβOs interfere with functional signaling pathways in neural cell survival and death.

Of great interest in the context of AD development and progression is that AβOs or/and Aβ peptides may act as antagonists to insulin at the neuronal insulin receptor (INSR), thus reducing the insulin signalling response [6,7,10]. What may precipitate from the binding of AβOs to the insulin receptor is insulin resistance, which happens to be a consistently identified pathology in AD. Insulin resistance may also serve as a link between Aβ and other pathologies [15,16]. Evidence suggests that patients with type 2 diabetes have an increased risk of developing AD and that defective insulin signaling and decreased responsiveness to insulin signaling are present in the AD brain, even in cases where the patient has no prior indications of type 2 diabetes [8-10,17,18]. It is known that insulin production is triggered by glucose. This process may be altered in AD patients with type 2 diabetes or AD patients with insulin resistance. When insulin binds to the insulin binding domain of the extracellular α subunit dimer of the insulin receptor, disinhibiting the tyrosine-kinase activity of the β subunits, thus leading to the phosphorylation of the intracellular insulin receptor substrate 1 (IRS1) that is docked to the INSR and initiating insulin signaling [19] (Figure S1). Phosphorylated IRS1 directly stimulates phosphoinositide-3-kinase (PI3K), which advances the signal downstream through a series of conversions and activations, notably phosphorylating Akt, which mediates many cellular functions like metabolism, survival, and apoptosis [10,20-22]. Downstream of PI3K/Akt, glycogen synthase kinase 3 beta (GSK3β) is of particular interest because of its ability to phosphorylate tau. Tau is a neuronal protein that involves microtubule stability and axonal transport. In AD, hyperphosphorylated tau aggregates into neurofibrillary tangles, causing neuronal dysfunction and death [23]. GSK3β is activated when its tyrosine-216 is phosphorylated; GSK3β is deactivated when phosphorylated at its serine 9 residue by the PI3K/Akt pathway [20,24]. The importance of understanding the role of insulin signaling in AD is further emphasized by its central role in the mTOR (mammalian target of rapamycin) signaling

***Address for Correspondence:** Wandong Zhang, Department of Cellular and Molecular Medicine, University of Ottawa, Canada, Email: Wandong.Zhang@nrc-cnrc.gc.ca*

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pathway. Two protein complexes, mTORC1 and mTORC2, both containing mTOR, are the two main junctions in the mTOR signaling pathway (Figure S1). The influence of Akt upon mTOR signaling and vice versa is clear and vital; while Akt is phosphorylated by the mTORC2 complex, mTORC1 relies on the phosphorylation and subsequent inactivation by Akt of the inhibitory tuberous sclerosis proteins 1 and 2 (TSC1/TSC2) complex [25,26].

Based ± on the high relevance of insulin signaling in AD pathology and the scientific leads on the involvement of soluble AβOs in signaling dysregulation in AD, we studied the insulin signaling responsive transcription factors in human AD brains and further investigated the effects of AβO on the insulin signaling pathway through how insulin signaling is affected in the context of a neural cell model in which the only factor is the overexpression of stably-transfected human AβPP695 gene, and therefore the presence of excess extracellular Aβ. The role of Aβ was further assessed by using the ability of amyloid-β binding peptide (ABP) to relieve Aβ-induced dysregulation of insulin signaling and insulin resistance. ABP has been shown to bind Aß1-42 oligomers (AβOs) *in vitro* and suppress Aß1-42-induced cytotoxicity in human neuroblastoma cells [27,28]. Most importantly, ABP binds Aβ deposits in AD transgenic mouse brain as well as in the brains from AD patients *in vitro*, and when directly injected into live AD transgenic mice brain [28]. Recent *in vivo* studies have shown that ABP can reduce brain Aβ levels and increase cerebrospinal fluid Aβ42/Aβ40 ratio in animal AD models [29]. In this study, we show that there was dysregulation of insulin signaling or low responsive to insulin signaling in human AD brains as compared to control ND brains and that N2a-AβPP cells also display insulin resistance phenotype or dysregulation of insulin signaling as compared to parental N2a cells. Removal of Aβ oligomers/peptides by ABP significantly alleviated insulin resistance and improved insulin signaling in the N2a-AβPP cells.

Materials and Methods

Human brain tissue samples

The use of human brain tissues in this study was approved by the Research Ethics Board of the National Research Council of Canada (NRC-REB). The brain tissue samples of Alzheimer disease with Cerebral Amyloid Angiopathy (AD/CAA) and age-matched non-demented controls (ND) were obtained from the Brain and Body Donation Program at the Banner Sun Health Research Institute (Sun City, Arizona, USA). The Consent form for Participation in the Program was approved by the Banner Sun Health Institutional Review Board (IRB). Brain samples (occipital lobes) of 13 AD/CAA patients and 13 ND controls from both males and females were used in this study. The patients were examined and diagnosed by neurologists, and post-mortem brain samples were examined and diagnosed by neuropathologists. The diagnosis of Cerebral Amyloid Angiopathy (CAA) pathology was made according to the presence of Aβ deposition in leptomeningeal or superficial cortical blood vessels as described [30].

ELISA assay

Amyloid β (Aβ) 1–40 and 1-42 ELISA kits (Signet Lab, Catalog Numbers: SIG-38954 and SIG-38956; Deham, MA) were used to determine the levels of Aβ1–40 and Aβ1–42 peptides in human 13 ND and 13 AD brain tissues or in the supernatant media from cultured N2a-AβPP cells and parental N2a cells according to manufacturer's instructions. The cells were grown to 80-90% confluence, supernatant medium was collected at different time points and replaced with fresh medium. The collected media were frozen at -80˚C until use. The media were thawed on ice and spun at 10,000 rpm for 10 min at 4°C to remove cell debris. Supernatant media were diluted 10 times with 1X dilution buffer and 100-µL from each sample was used. The ELISA was carried out following manufacturer's instructions. The levels of Aβ1–40 and Aβ1–42 peptides in the samples were calculated according to the standard curve prepared on the same ELISA plates.

Tran signal TM protein/DNA array

Nuclear extracts were prepared from 6 ND and 6 AD/CAA brain tissue samples, respectively. The arrays were performed to detect the activities of the 52 transcription factors (TF) in the nuclear extracts of the brain tissues following the manufacturer's instructions (Panomics Inc., Fremont,CA) and as described previously [30-34]. The levels of activated TFs on the two blots were analyzed by using a densitometer with Kodak 1D 3.6 Version program.

Cell culture

Mouse neuroblastoma 2a (N2a) and N2a cells stably transfected with a human gene encoding AβPP695 (N2a-AβPP) were kindly provided by Dr. Huaxi Xu at the Sanford Burnham Prebys Medical Discovery Institute (CA., USA) and grown in DMEM with 5% FBS and 0.2% penicillin-streptomycin. The cells were incubated in a 5% CO2 incubator at 37°C. The general morphology of N2a and N2a-AβPP cells is shown in the staining of β-tubulin; N2a cells seem to be generally larger (Figure S2). After washing the cells with 1x PBS to remove the medium, 1mL of 1x trypsin was added to lift the cells from the plate surface, and then growth medium was added to the trypsinized cells to neutralize the protease effect of trypsin. The N2a-AβPP cells were cultured with the antibiotic geneticin at 200 µg/L to select for the cells carrying the human AβPP gene.

Insulin time-course treatment

The two cell lines were cultured to 80% cell confluence in separate 100-mm dishes. The growth medium was removed, and the cells were washed with 1x PBS twice before the treatment medium was added. Cells were treated with solubilized human recombinant insulin (Sigma) in serum-free medium at concentrations of 0.1 nM, 0.5 nM, 5 nM, and 100 nM for 0, 15 min, 30 min,1, 2, 4, and 8 hours.

Amyloid-Beta binding peptide (ABP) pre-treatment

The amyloid-β binding peptide (ABP) used in this study was a synthetic product [27-29]. The two cell lines were cultured to 70% confluence in separate 100-mm dishes. The growth medium was removed, and the cells were washed with 1x PBS twice before the pre-treatment was started. Cells were treated with 0.44 µM ABP, which is about 500x the concentration of extracellular Aβ1-40 at ~1700 pg/mL [31] and Aβ1-42 at ~2400 pg/mL, in serum-free medium for 24 and 48 hours; the medium was replaced with fresh treatment medium at 24 hours in plates treated for 48 hours. After the ABP treatment period, the ABP medium was removed, and the cells were treated with 0.5 or 5 nM insulin in serum-free medium for 30 minutes, 1 hour, and 2 hours.

Protein isolation and western blotting

After treatment, the medium was removed from the plates and the cells were washed 3 times with PBS, then the cells were gently scraped from the dish in 1 ml of PBS 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. One (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. Forty (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 an 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)]. Total Akt (CAT#2966S), p-Akt (Ser473) (CAT#4060), total GSK3β (CAT#12456), p-GSK3β (Ser9) (CAT#5558), total IRS1 (CAT#2382), p-IRS1 (Ser612) (CAT#2386), total mTOR (CAT#2983), p-mTOR (Ser2448) (CAT#2971), and p-p70S6K (Thr389) (CAT#9234) antibodies were purchased from New England Biolabs (Whitby, ON, Canada). Phospho-tau (ser199/202) antibody (CAT#AB7694) was purchased from EMD Millipore Corporation (Etobicoke, ON, Canada). Anti-Aβ IgG (6E10) antibody was purchased from Signet Lab (CAT# SIG-39320). The blots were incubated while rotating with the primary antibody at 1:1000 dilution overnight at 4°C, washed with 1X PBS-T, and then incubated in the appropriate secondary antibody at 1:5000 dilution for 1 hour. After washing with 1X 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. In general, the total protein was detected first, and its phosphorylated form of the protein was detected next after stripping the blots.

Phase-contrast microscopy

N2a and N2a-AβPP cells were grown to 70% confluence in growth medium on coverslips in 6-well tissue culture plates. The medium was aspirated, and the cells were washed with 1xPBS 2 times for 5 minutes each. The cells were then fixed with ice cold methanol, shaken 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 the coverslips were mounted onto microscopy slides with 20µl 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.

Immunocytochemical staining

N2a and N2a-AβPP 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 anti-β-tubulin antibody was diluted in PBS/0.1% Triton x-100, then for each coverslip a 60 µL 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. A goat anti-mouse secondary antibody conjugated with Alexa 568 (Invitrogen) 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 µL Hoechst stain (0.5 µg/mL) for 1 minute while gently shaking to stain the nucleus. The coverslips were immediately washed with 1x PBS 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.

Statistical Analysis

All the *in vitro* cell experiments were repeated at least 5 times. Statistical analyses were performed in GraphPad Prism 9 using One-way ANOVA to assess significance of differences where multiple comparisons are made, followed by the Bonferroni post-hoc test. The two-tailed Student t-test was also used to determine statistical significance between two groups. The data were presented as Mean ± SD. Statistical significance was defined at $p<0.05$.

Results

Dysregulation of insulin signaling/low responsiveness to insulin signaling in human AD/CAA brain

The characteristic of the human AD brain examined in this study is the presence of large amounts of Aβ40 and Aβ42 in the brain and cerebral vessels with CAA pathology (AD/CAA), which may contribute to the dysregulation of insulin signaling in the AD brain. ELISA assays showed that the levels of Aβ40 in the 13 non-demented age-matched controls (ND) and 13 AD/CAA brain samples were 6.83 ± 8.63 and 22976.79 ± 1903.83 pg/mg protein (Mean ± SD), respectively (Figure 1A). The levels of Aβ42 in the ND and AD/CAA brain samples were 60.50 ± 1.87 and 210.79 ± 27.42 pg/mg protein, respectively (Figure 1B). Nuclear extracts were prepared from 6 ND and 6 AD/CAA brain samples, and the TranSignal Protein/DNA Arrays were used to detect the activities of insulin signaling-responsive transcription factors (TF) in the ND and AD/CAA brain tissues. Previous studies have demonstrated that insulin signaling can activate CREB (cAMP-responsive element binding protein) and NFκB (nuclear factor kappa B) pathways [35,36] and regulate insulin-responsive gene expression *via* SP1 (specificity protein 1) transcription factor [37,38]. In addition, nuclear receptor signaling, including thyroid hormone receptor (TR) and Peroxisome Proliferator-Activated Receptors (PPAR), can potentiate insulin signaling, crosstalk with insulin signaling or modulate insulin sensitivity [39-41]. Heat Shock Transcription Factor (HSF) that binds to the Heat Shock Sequence Elements (HSE) is also responsive to insulin/IGF-1-like signaling to regulate longevity and stress response [42]. These transcription factors were not previously investigated in human AD/CAA and ND brains although we reported JNK-AP1 signaling was activated but the levels of CREB and NFκB were lower in human AD brains as compared to ND brains [32]. The activities of the transcription factors, CREB, NFκB, SP1, TR, HSF, and PPAR in the ND and AD/CAA brain tissues were analyzed by TF arrays (Figures 1C and 1D). There are four dots for each of the transcription factors on the TF array blots (the bottom two dots contained 10 times less hybridization probe than the top two dots), and the four dots for each transcription factor were quantified for the activity of the transcription factor (Figures 1C and 1D). The activities of these 6 transcription factors were significantly decreased in AD/CAA brain samples as compared to those in ND brain tissues (Figure 1E; two-tailed student t-test, $^{**}p<0.01$; $^{****}p<0.0001$). This indicates that AD/CAA brains had low responsive transcription factors to insulin signaling as compared to ND brains. The presence of large amounts of Aβ40 and Aβ42 peptides in AD/CAA brain may interfere with insulin signaling and are responsible for reduced activities of these transcription factors that are involved or crosstalk with insulin signaling (Figure 1E).

N2a-AβPP cells produce beta-amyloid peptides and have basal dysregulation of insulin signalling proteins

Mouse neuroblastoma 2a (N2a) cells and the N2a cells stably transfected with a human gene encoding AβPP695 (N2a-AβPP) cells were then used to study Aβ-induced dysregulation of insulin signaling [31]. Immunocytochemical and phase contrast microscopy visualizations of the N2a and N2a-AβPP cells show that the general morphology of the two cell lines does not differ. Both cell lines were observed to have round morphology with short extending neurofilaments (Figure S2). The size of

both cell lines ranged from about 10 to 18 µm. More N2a-AβPP cells appear to have smaller diameter than parental N2a cells (Figure S2).

ELISA was used to determine the levels of Aβ1-40 and Aβ1-42 peptides secreted from the N2a-AβPP cells into supernatant culture medium at different time points. The secretion of Aβ1-40 peptides from the cells gradually increased from 1h and reached a peak at 6 h (1647.75 ± 33.51 pg/mL) and maintained at high levels at 12 h and 24 h (Figure 2A). The secretion of Aβ1-42 peptides from N2a-AβPP cells also reached a peak at 6h (2395.26 ± 401.77 pg/mL) and then decreased at 12 h (701.09 ± 90.63 pg/mL) (Figure 2B). It is known that Aβ1-42 forms oligomers. The ELISA kits used this study specifically detects either Aβ1-40 or Aβ1-42, but not Aβ oligomers. It is likely that most of Aβ1-42 in the supernatant medium may form Aβ oligomers at 12h. This may be the reason that the level of Aβ1-42 at 12 h was decreased as compared to that at 6 h in the cell medium. Further analysis by Western blotting using anti-Aβ (6E10) antibody detected Aβ monomers, oligomers and high molecular-weight aggregates in concentrated medium (data not shown). Secretion of Aβ1-40 and 1-42 peptides from parental N2a cells was not detected (data not shown).

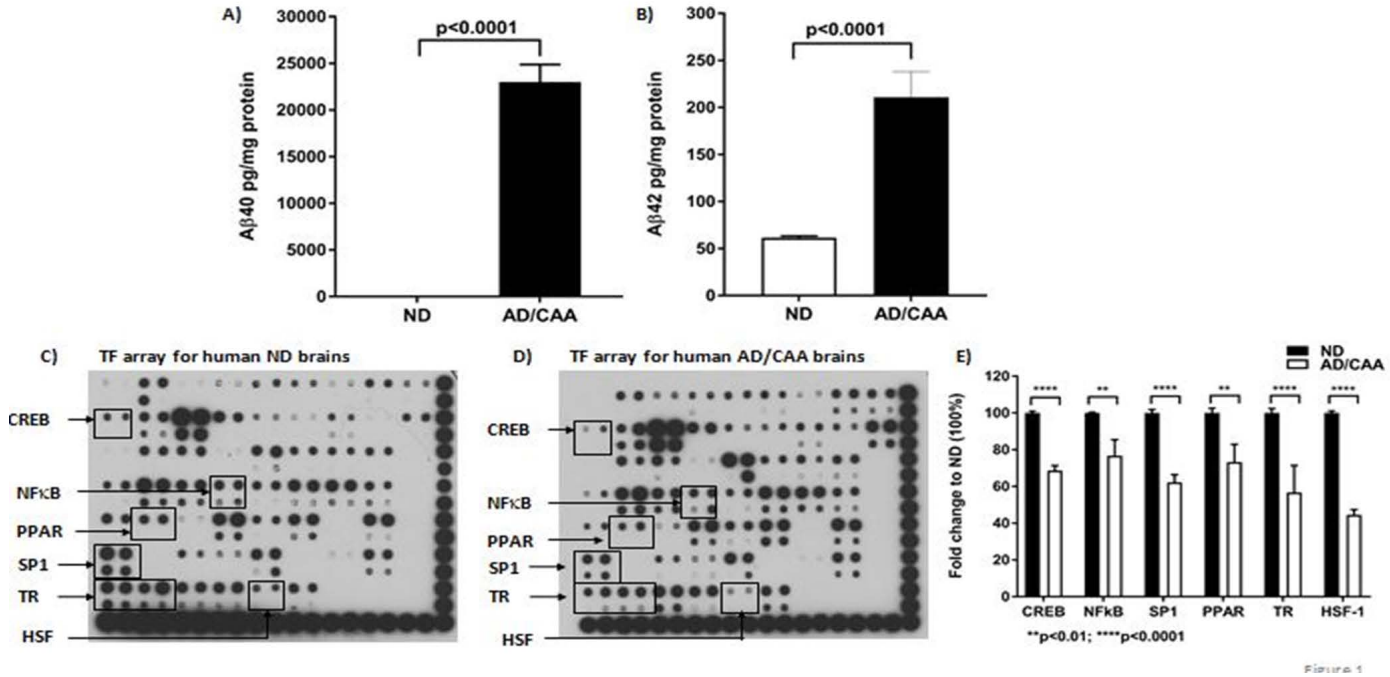

Figure 1. Aβ ELISA and transcription factor (TranSignal DNA/protein) array assays for human AD/CAA and ND brain tissues: A) ELISA to detect Aβ1-40 in ND and AD/CAA brain tissues. B) ELISA to detect Aβ1-42 in ND and AD/CAA brain tissues. C) and D) Transcription factor (TF) arrays for human ND and AD/CAA brain tissues, respectively. E) Densitometry analysis of TF array blots for AD/CAA brain tissues relative to ND brain tissues. Two-tailed student t-test was used to compare the data between ND and AD/CAA. (**p<0.01; ****p<0.0001).

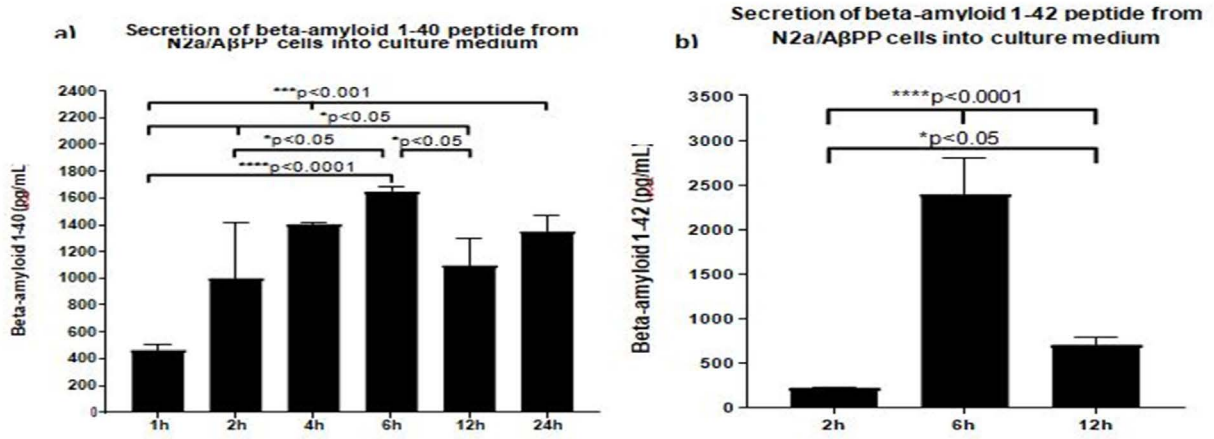

Figure 2. Secretion of Aβ1-40 and Aβ1-42 peptides from N2a-AβPP cells into culture medium: a) Secretion of Aβ1-40 peptides at 1 h, 2 h, 4 h, 6 h, 12 h and 24 h. The levels of Aβ1-40 secreted were significantly higher at 2 h and 12 h than 1 h (*p<0.05), at 4h and 24h than 1h (**p<0.001), and at 6 h than 1 h (****p<0.0001), 2 h and 12 h (*p<0.05). b) Secretion of Aβ1-42 peptides at 2 h, 6 h, and 12 h. The levels of Aβ1-42 were significantly higher at 6 h than 2 h and 12 h (****p<0.0001) and at 12 h than 2 h (*p<0.05) (One-way ANOVA). Parental N2a cells do not produce either Aβ1-40 or Aβ1-42 peptides.

To evaluate the effect of Aβ on insulin signaling pathway, the basal levels of insulin signaling proteins in N2a and N2a-AβPP cells were determined by Western blotting. The cells were plated and grown to ~ 90% confluence and then harvested as described above. This cell model is centered around the effect of Aβ on signal regulation; indeed, the only difference between the parental N2a cells and the transfected N2a-AβPP cells is the overexpression of AβPP at the membrane of the transfected N2a-AβPP cells; these transfected cells release Aβ extracellularly [31]. Western blotting was performed to detect total IRS1, p-IRS1 (ser 612), total Akt, p-Akt (ser 473), total GSK3β, p-GSK3β (ser 9), p-tau (ser 199/202), total mTOR and p-mTOR (ser 2448) which were then normalized to β-tubulin (Figure 3). The results show that there was a significant increase in the level of IRS1 in N2a-AβPP cells as compared to N2a cells at baseline but the level of p-IRS1 was significant lower in N2a-AβPP cells than that in N2a cells at baseline (Figure 3). At baseline, N2a-AβPP cells also significantly expressed higher levels of p-Akt and Akt as compared to those of N2a cells (Figure 3). These results suggest that some of the insulin signaling proteins were dysregulated in N2a-AβPP cells, in comparison with N2a cells, as a result of AβPP overexpression and the production of Aβ peptides.

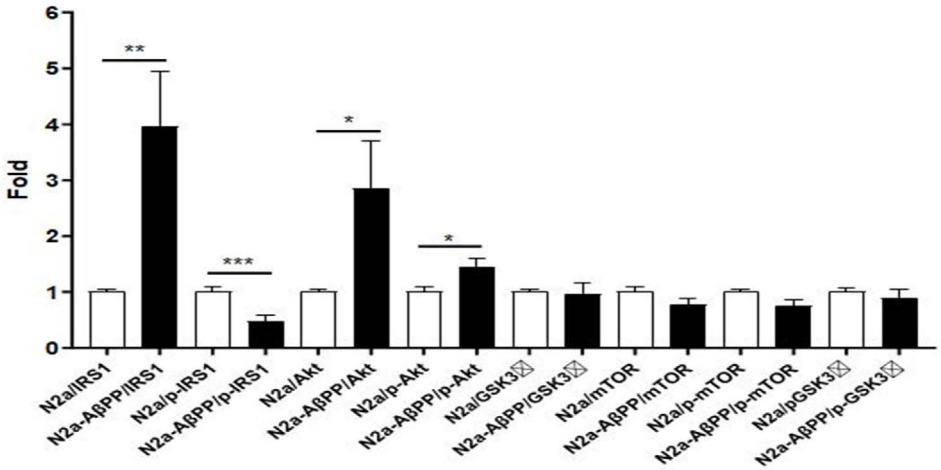

Figure 3. Basal levels of insulin signaling proteins in N2a and N2a-AβPP cells: Mouse neuroblastoma 2a (N2a) cells, and N2a-AβPP cells stably transfected with the gene encoding hAβPP695, were plated in serum-free medium for 24 hours, then collected without any applied treatment to determine the cell lines' basal protein levels. N2a-AβPP cells have significantly lower basal levels of p-IRS, while having significantly higher levels of total IRS1, total Akt, p-Akt, and p-tau as compared to those in parental N2a cells. The data presented were normalized against β-tubulin (One-way ANOVA, F=7.751, p<0.0001; two-tailed Student t-test, *p<0.05, **p<0.01, ***p<0.001; N ≥ 5).

N2a-AβPP cells show insulin resistance phenotype

To assess how the N2a-AβPP cells may respond differently from parental N2a cells to insulin, 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 (0.1 nM to 5 nM) and supra-physiological (100 nM) levels over a time course of 0, 15 min, 30 min, 1 h, 2 h, 4 h, and 8 h (Figure 4). The aim was to compare any fluctuations in the levels of signaling proteins on the insulin signaling pathway, and therefore any difference in insulin response between the two cell lines. Treatment of N2a cells with insulin significantly enhanced the phosphorylation of IRS1 at ser612 (Figures 4A and 4B). The total level of IRS1 was significantly increased in N2a-AβPP cells as compared to N2a cells (Figures 4A and 4C) at 30 min, 1 h and 2 h of 0.5 nM and 5 nM insulin treatments (Figures 4B and 4C). However, the IRS1 phosphorylation was almost not detected in N2a-AβPP cells as compared to N2a cells at 30 min, 1 h, and 2 h of 0.5 nM and 5 nM of insulin treatment (Figures 4A and 4C). Together, these results show that the effect of Aβ peptides on N2a cells is twofold: One, that Aβ induces an insulin resistant state and there was almost no IRS1 phosphorylation in N2a-APP cells when different concentrations of insulin were added, and two, that the Aβ-mediated induction of insulin resistance may also trigger the cell to overexpress IRS1 in an attempt to compensate for the lack of phosphorylation response. Since IRS1 is located at the upper stream of insulin signaling, dysregulation of IRS1 expression and phosphorylation disturbs the transmission of insulin signaling downstream (Figure 4). Our previous study observed that the level of IRS phosphorylation at ser612 was increased in N2a-AβPP cells at 24 h time point as compared to the observation from the current study (15 min to 8 h). This may result from different cell culture conditions and incubation time used in the previous study. It is known that IRS1 and IRS2 are regulated through a complex mechanism involving phosphorylation of over 50 serine/threonine sites in their tail regions [43,44]. The activation of insulin signaling at IRS1 may be a combinatory effect of multiple site phosphorylation on IRS1 rather than a single site phosphorylation.

Further down the insulin signaling pathway, mTOR (mammalian target of rapamycin) phosphorylation also displayed a degree of insulin resistance in N2a-AβPP cells, in that at the lower insulin treatment concentrations of 0.5 nM and 5 nM, the level of p-mTOR was significantly lower in N2a-AβPP cells than in N2a cells (Figures 5A-5C). However, unlike IRS1, the response was recovered with the application of higher 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 min to 8 h time course (Figures 5A and 5C) (One-way ANONA, *p<0.05).

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. p70S6K is a dual pathway kinase by signaling cell survival and growth through differential substrates, including mitochondrial BAD and the ribosomal subunit S6, respectively [45]. The phosphorylation of p70S6K at threonine 389 is a hallmark of activation by mTOR [46]. However, the phosphorylation of p70S6 kinase at threonine 389 in response to insulin was diminished in N2a-AβPP cells as compared to N2a cells treated with insulin, indicating decreased mTOR activity (Figure 5D) and insulin resistance in N2a-AβPP cells.

It appears that the total levels of Akt expression and phosphorylation in response to insulin treatment in N2a and N2a-AβPP seemed to be similar (Figure 6). However, distinct concentration and time-dependent patterns of sensitivity to insulin of both cell lines were noticed in the phosphorylation of Akt. With 5 nM and 100 nM of insulin treatment, the time-dependent phosphorylation of Akt was apparent as a response curve of fold change, in that both N2a and N2a-AβPP cells demonstrated significantly increased

Akt phosphorylation at 15 minutes of treatment as compared to the control, then subsequently higher fold increase of Akt phosphorylation at 30 minutes of treatment as compared to 15 minutes (Figure 6A). With 0.5 nM and 5 nM of insulin treatment, N2a cells' phosphorylation response to insulin peaked at 1 hour, at which p-Akt was significantly increased from the 30-minute treatment. With 0.5 nM and 5 nM of insulin, N2a-AβPP cells peaked at 2 hours of treatment (Figure 6B), which was delayed for 1 hour. However, with the highest insulin treatment concentration of 100 nM, both N2a and N2a-AβPP cells seemed to peak at 1 hour of treatment; in N2a-AβPP cells, p-Akt was significantly increased at 1 hour as compared to 30 minutes, but was significantly decreased at 2 hours as compared to the peak at 1 hour (Figure 6C). Weak response of Akt phosphorylation to insulin treatment (0.5 to 5 nM at 1 h treatment) in N2a-AβPP cells was also observed as compared to N2a cells in our previous study [43]. These results suggest that the response of Akt phosphorylation to insulin was delayed and the magnitude of the response was minimized in N2a-AβPP cells as compared to N2a cells. A previous study showed that serum-withdrawal resulted in Akt phosphorylation in N2a cells [47], but this was not observed in our study. The levels and phosphorylation of GSK3β in N2a-AβPP cells were not changed as compared to those of N2a cells (data not shown).

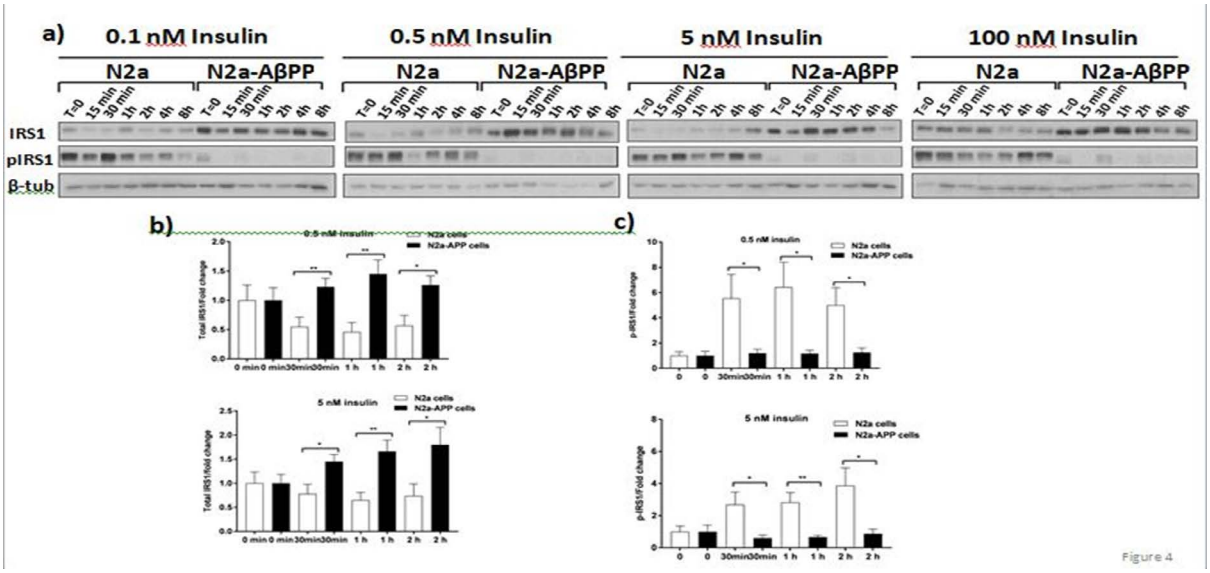

Figure 4. Effect of insulin on phosphorylation of IRS1 in N2a and N2a-AβPP cells: a) Phosphorylation of IRS1 in N2a and N2a-AβPP cells in response to insulin treatment at 0.1 nM, 0.5 nM, 5 nM, and 100 nM concentrations for 0, 15 min, 30 min, 1 h, 2 h, 4 h, and 8 hours. Quantification of total IRS1 (b) and p-IRS1 (c) in these treatments (One-way ANOVA: b for 0.5 nM insulin, F=3.725, **p=0.0027; b for 5 nM insulin, F=3.729, **p=0.0027; c for 0.5 nM insulin, F=4.618, ***p=0.0004; c for 5 nM insulin, F=4.694, ***p=0.0004; two-tailed t-test *p<0.05, **p<0.01; N ≥ 5).

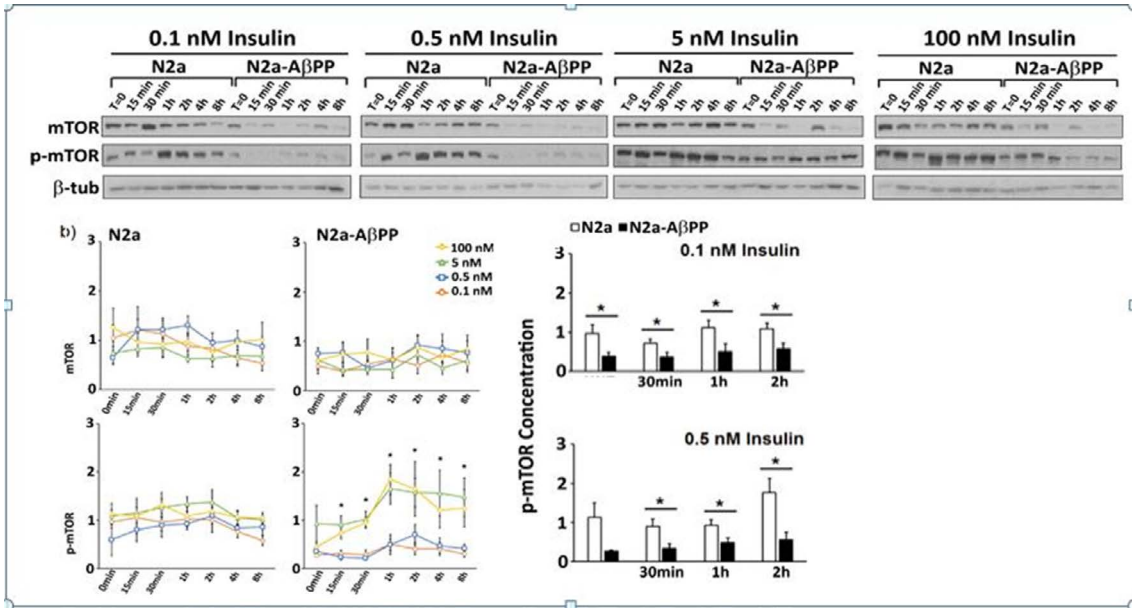

Figure 5. Effect of insulin on phosphorylation of mTOR in N2a and N2a-AβPP cells: a) Phosphorylation of p-mTOR in N2a and N2a-AβPP cells in response to insulin treatment at 0.1 nM, 0.5 nM, 5 nM, and 100 nM concentrations for 0, 15 min, 30 min, 1 h, 2 h, 4 h, and 8 hours. b) N2a-AβPP cells had significantly higher mTOR phosphorylation levels at 5 nM and 100 nM of insulin treatment as compared to 0.1 nM and 0.5 nM insulin treatments, across all time points, from 15 minutes to 8 hours (two-tailed t-test, *p<0.05). c) Quantification of p-mTOR in N2a and N2a-AβPP cells treated with 0.1 nM and 0.5 nM insulin for 30 min, 1 h and 2 h (two-tailed t-test, *p<0.05). N2a-AβPP cells had significantly less phosphorylation of mTOR as compared to N2a cells at 0.1 nM and 0.5 nM of insulin treatment, particularly at baseline, 30 min, 1 h, and 2 h of treatment. d) Phosphorylation of p70S6 kinase (p70S6K) in response to 0.5 nM and 5 nM insulin treatments for 30 min in N2a and N2a-AβPP cells. p70S6K was strongly phosphorylated in N2a cells treated with 0.5 nM and 5 nM insulin in comparison with untreated N2a cells (UNT). N2a-AβPP cells treated with 0.5 nM insulin had no phosphorylation of p70S6K as compared to untreated N2a-AβPP cells (UNT), but N2a-AβPP cells treated with 5 nM insulin had slightly increased phosphorylation of p70S6K as compared to UNT.

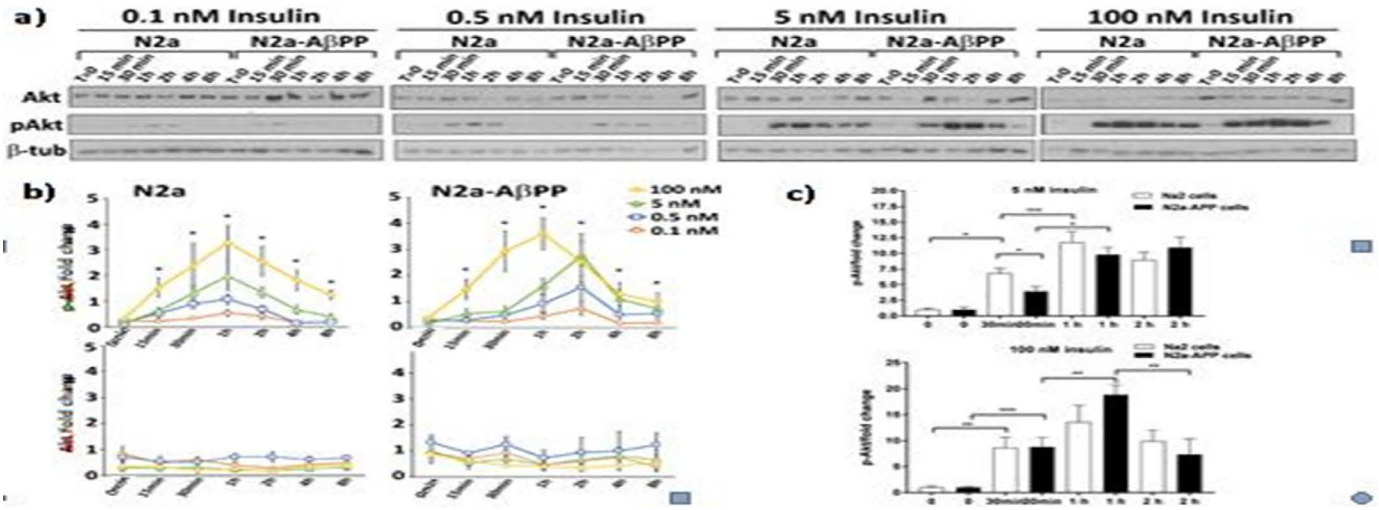

Figure 6. Effect of insulin on phosphorylation of Akt in N2a and N2a-AβPP cells. a) Phosphorylation of Akt in N2a and N2a-AβPP cells in response to insulin treatment at 0.1 nM, 0.5 nM, 5 nM, and 100 nM concentrations for 0, 15min, 30 min, 1 h, 2 h, 4 h and 8 h. b) Akt phosphorylation in N2a cells was peaked in response to all concentrations of insulin at 1 h treatment, while Akt phosphorylation in N2a-AβPP cells was delayed and peaked at 2 hours of treatment with 0.1 nM, 0.5 nM, and 5 nM of insulin treatments. At the highest treatment concentration of 100 nM, however, the peak of Akt phosphorylation remained at 1 hour in N2a-AβPP cells (two-tailed t-test, *p<0.05). c) Quantification of Akt phosphorylation in N2a and N2a-AβPP cells in response to 5 nM and 100 nM insulin treatments for 0, 30 min, 1 h and 2 h (One-way ANOVA analysis: c for 5 nM insulin, F=14.63, ****p<0.0001; c for 100nM insulin, F=8.096, ****p<0.0001; *p < 0.05, **p<0.01, ***p<0.001).

In summary, the altered levels of IRS and p-IRS, Akt and p-Akt, mTOR and p-mTOR, and p70S6K observed in N2a-AβPP cells in response to insulin treatment as compared to parental N2a cells indicate the presence of altered insulin signaling or insulin resistance in N2a-AβPP cells.

Amyloid-beta binding peptide (ABP) pre-treatment of N2a-AβPP cells enhanced insulin signalling response

Now that the effect of Aβ on insulin signaling dysregulation has been established in N2a-AβPP cells, it is important also to determine whether clearing Aβ in the extracellular environment of N2a-AβPP cells can relieve insulin resistance or improve insulin signaling. To this end, N2a and N2a-AβPP cells were treated with 0.5 nM and 5 nM insulin for 30 minutes, 1 hour, and 2 hours. Before the insulin treatment, N2a-AβPP cells were pre-treated with 0.44 μM Aβ-binding peptide (ABP) [27,28] at a concentration of ~ 500 times of extracellular Aβ. ABP is a 5 kDa synthetic peptide consisting of a region of the 228 kDa human pericentrioloar material-1 (PCM-1) protein [28]. No toxicity of ABP at 0.44 μM concentration was observed in the cells [27,28]. ABP has been previously shown to selectively bind Aß1-42 oligomers and reduce Aß1-42-induced cytotoxicity in human neuroblastoma cells [28]. Furthermore, ABP binds Aß deposits in AD transgenic mouse brain when directly injected into live animal AD models as well as in the human AD brains *in vitro* [27-29]. ABP fusion protein with a BBB carrier FC5 (KG207-M) can substantially reduce the brain Aβ levels and increase the Aβ42/40 ratio in the CSF in a rat Alzheimer's model [29]. N2a and N2a-AβPP cell protein was extracted and probed *via* Western blot for p-IRS1 (ser 612), total IRS1, p-Akt (ser 473), total Akt, p-GSK3β (ser 9), total GSK3β, p-tau (ser 199/202), and p-mTOR (ser 2448), total mTOR, p70S6 kinase, 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. Treatment of N2a cells with ABP was not toxic to the cells and did not affect insulin signaling (data not shown).

At the beginning of the insulin signaling pathway, there was an immediate significant increase of p-IRS1 in N2a-AβPP cells treated with ABP followed by 0.5 nM insulin for 30 minutes as compared to N2a-AβPP cells treated only with insulin (Figure 7A). In N2a-AβPP cells, p-IRS1 was 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-AβPP cells treated only with insulin (Figures 7A and 7C). With regards to total IRS1 levels, an increase in N2a-AβPP cells was also observed at 30 minutes of 0.5 nM insulin after 24 hours of ABP pre-treatment as compared to insulin treatment only; the increase in IRS1 seemed 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 5 nM treatment (Figures 7A and 7B). 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 found that the upregulation of IRS1 in N2a-AβPP cells may occur in compensation of the decreased phosphorylation of IRS1, yet if some phosphorylation was being recovered with ABP treatment, it would also be expected that IRS1 expression would decrease since the compensation was not needed. However, ABP pre-treatment seemed to additionally increase IRS1 response. The reason for this may be that the cells were 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 p-IRS, pre-treatment of N2a-AβPP cells with ABP for 24 hours did significantly trigger stronger increased levels of p-Akt in response to insulin than the cells treated only with 5 nM insulin for 30 minutes (Figures 7D and 7E). Similarly, with 48 h ABP pre-treatment, the same significant increased at 30 minutes of 5nM insulin was seen. ABP might be enhancing insulin signaling for the phosphorylation of Akt in these conditions.

Further downstream in the insulin signaling pathway, phosphorylation of mTOR at ser 2448 was also increased in trend with 24 hours of ABP pre-treatment followed by 5 nM insulin as compared to N2a-AβPP cells only treated with 5 nM insulin (Figures 8A and 8B). The phosphorylation of p70S6 kinase is a direct indicator of the level of mTORC1 activity. Pre-treatment with ABP significantly increased the phosphorylation of p70S6 kinase in N2a-AβPP cells in response to insulin as compared to non-ABP-treated N2a-AβPP cells, specifically with 24 hours of ABP pre-treatment followed by 0.5 nM or 5 nM insulin (Figures 8C and 8D). This is consistent with the aforementioned trend of increase in p-mTOR and mTOR.

Analysis of the phosphorylation of GSK3β (at ser 9) and tau did not show

any significant trends in changing response to insulin in the cells. It is known that there are about 79 phosphorylation sites of serine and threonine on tau protein, therefore the phosphorylation sites of serine 199/202 targeted in this experiment may not be the one affected in these treatments.

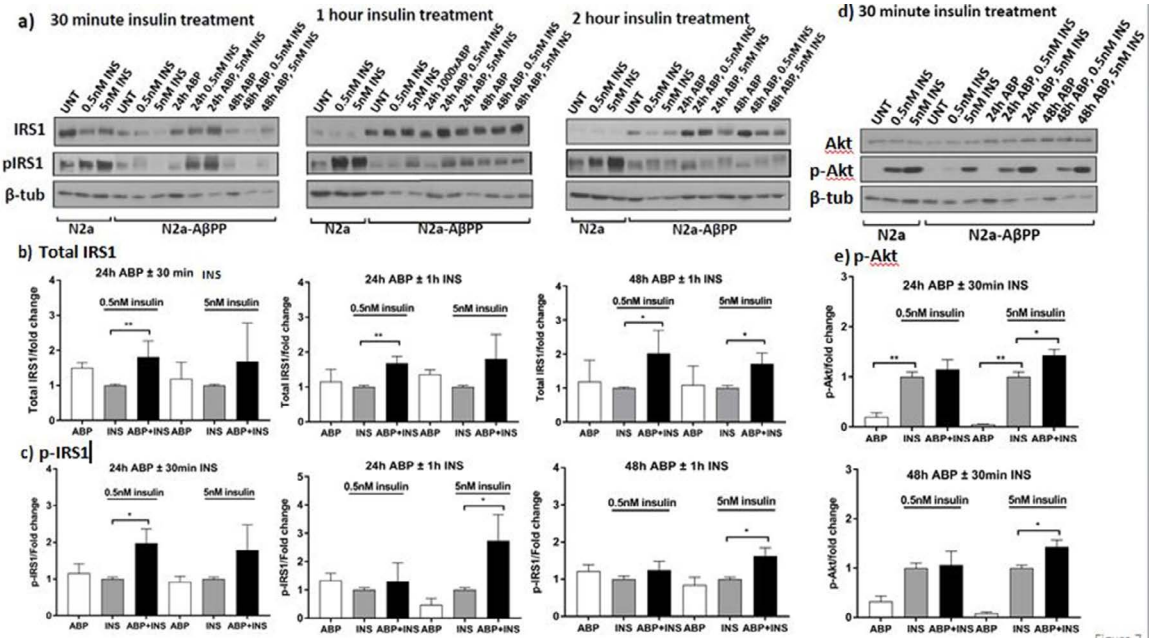

Figure 7. Effects of amyloid-β binding peptide (ABP) on the phosphorylation and expression of IRS1 and Akt in response to insulin. (a) N2a and N2a-AβPP cells were treated with 0.5 nM and 5 nM of insulin for 30 minutes, 1 h and 2 hours. N2a-AβPP cells were also pre-treated with ABP for 24 or 48 hours. The medium was 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), and Akt, and then normalized with β-tubulin. The ABP+insulin conditions were presented relative to the insulin treatment only conditions. Quantification of total IRS1 (b) and p-IRS1 (c) (One-way ANOVA: b for 24h ABP+30 min insulin, F=3.255, p=0.0238; b for 24h ABP+1 h insulin, F=3.368, p=0.0394; b for 48h ABP+1 h insulin, F=3.707, p=0.0264; c for 24 h ABP+30 min insulin, F=2.839, p=0.0413; c for 24 h ABP+1 h insulin, F=5.969, p=0.0082; c for 48 h ABP+1 h insulin, F=3.044, p=0.0458; *p < 0.05; **p < 0.01). d) Effect of ABP on the phosphorylation of Akt in response to insulin. e) Quantification of p-Akt (One-way ANOVA: e for 24 h ABP+30 min insulin, F=33.24, p<0.0001; e for 48 h ABP+30 min insulin, F=16.94, p<0.0001; *p < 0.05, ** p< 0.01).

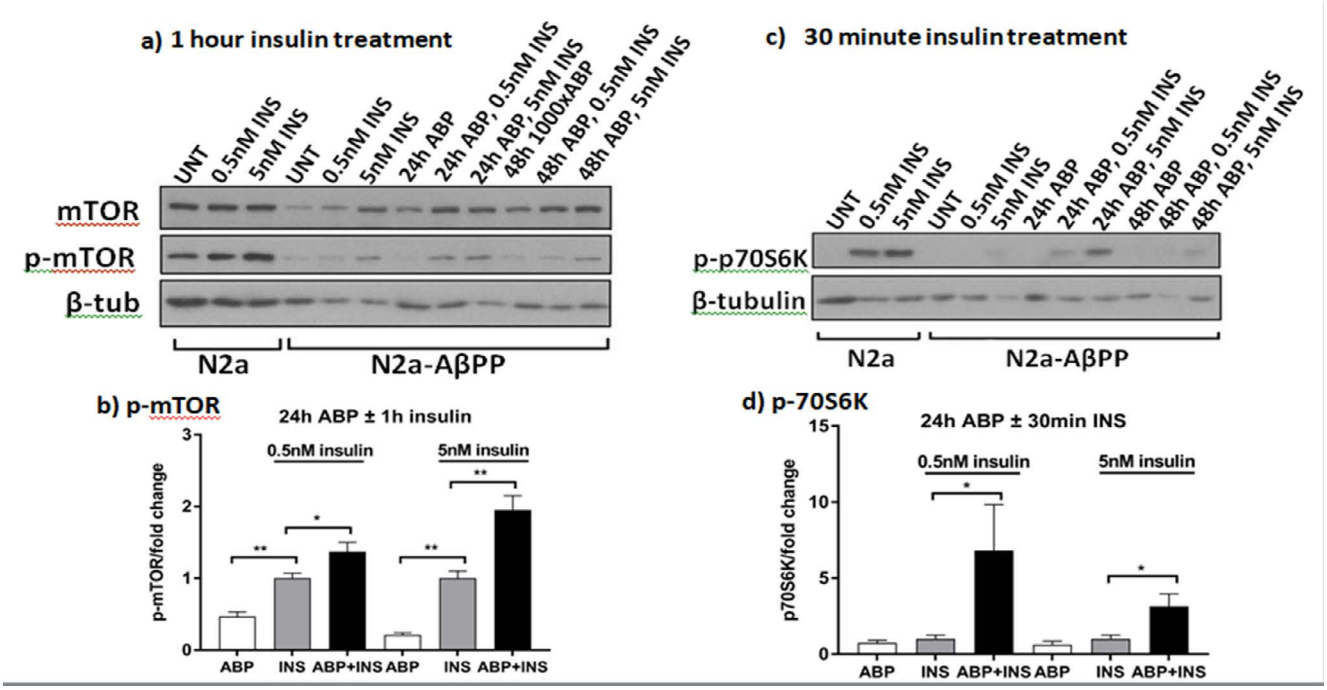

Figure 8. Effects of ABP on the phosphorylation of mTOR and p70S6K in response to insulin.

a) The phosphorylation of mTOR in N2a and N2a-AβPP cells and in N2a-AβPP cells pre-treated with ABP in response to 0.5nM and 5nM insulin treatment for 1 h. b) Quantification of p-mTOR in these cells of above treatments (One-way ANOVA, F=29.85, p<0.0001; *p < 0.05, **p < 0.01). c) The phosphorylation of p70S6K in N2a and N2a-AβPP cells and in N2a-AβPP cells pre-treated with ABP in response to 0.5 or 5nM insulin treatment for 30 min, and d) quantification of phosphorylated p70S6K in these cells (One-way ANOVA, F=5.66, p=0.0023; *p<0.05).

Discussion

Our analysis confirmed that the human AD/CAA brain tissues accumulated large amounts of Aβ40 and Aβ42 peptides and revealed significantly decreased activities of the transcription factors responsive to insulin signaling as compared to age-matched non-demented control (ND) brain tissues. Our observations of dysregulated insulin signaling are consistent with what is reported in the literature [8-10,17]. It is known that the transcription factors CREB, NFκB, PPAR, SP1, HSF and TR are responsive or crosstalk to insulin signaling. This is the first report that the activities of these transcription factors were significantly decreased or inhibited in human AD/CAA brains. Our findings provide further evidence that insulin signaling is dysregulated or inhibited in human AD brain possibly as a result of accumulation of large amounts of Aβ peptides or oligomers. Studies demonstrated that insulin signaling is important to brain function [48] and that intranasal insulin administration improved cognitive performance in early-stage AD patients [34,49-51], indicating that enhancing insulin signaling may improve brain function in AD patients. It is thus necessary to investigate the dysregulated insulin signaling and develop therapeutic strategies to relieve insulin resistance and improve insulin signaling in AD.

Aiming to further characterize Aβ-mediated dysregulation of insulin signaling, a neural cell model was used in which the levels of insulin signaling proteins and signaling response were compared between the parental N2a and the N2a-AβPP lines. No previous study employed such a neural cell model that produces Aβ peptides to study dysregulated insulin signaling in AD. After analyses, we found that N2a-AβPP cells displayed altered basal levels of insulin signaling proteins and also had dysfunctional insulin responses at several points in the insulin signaling pathway when compared to the parental N2a cells. The basal levels of IRS and pIRS in the absence of insulin stimulation were significantly increased and decreased, respectively, in N2a-AβPP cells while the basal levels of Akt and pAkt were significantly increased in N2a-AβPP cells as compared to N2a cells. All of these suggest that the presence of AβOs or Aβ itself affected the basal insulin signaling in the cells. There was significantly reduced p-IRS1 signaling but increased IRS1 expression in the absence of insulin or at all insulin treatment concentrations in N2a-AβPP cells as compared to parental N2a cells. Interestingly, we observed previously that acute treatment of SH-SY5Y cells with exogenous Aβ42 peptide (5 μmol/L) enhanced the phosphorylation of IRS as compared to SH-SY5Y cells treated with scrambled Aβ42 peptide at the same concentration. We also observed that different culture conditions may affect the phosphorylation of IRS in N2a-AβPP cells [43]. The above observations imply that the persistent presence of Aβ peptides/oligomers is responsible for dysregulation of insulin signaling in N2a-AβPP cells.

In addition to decreased phosphorylation of IRS, the peak level of Akt phosphorylation in N2a-AβPP cells after treatment with a physiological concentration of insulin (5 nM) was delayed for one hour as compared to that of N2a cells treated with the same concentration of insulin. In addition, the phosphorylation of mTOR in N2a-AβPP cells exhibited a significant lack of response to insulin at lower concentrations of insulin treatment. This would support the hypothesis that the interaction between Akt and the mTOR pathways is not a straightforward progression of response and effect. If the Akt phosphorylation response to insulin was preserved in N2a-AβPP 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 was not consistent with the conserved Akt phosphorylation levels. The conclusion then follows that Akt-mediated mTOR phosphorylation was subject to AβO-mediated insulin resistance that required higher concentrations of insulin treatment to overcome. This further demonstrates that the signaling response to insulin treatment in N2a-AβPP cells was dysregulated as compared to N2a cells. This also establishes the link of Aβ-mediated dysregulation of insulin signaling.

Several clinical studies have shown that intranasal administration of insulin improved cognitive performance of AD patients. Alternatively, removal of Aβ peptides or oligomers may also improve insulin signaling. Investigation was then conducted to test the strategy by clearing the extracellular AβOs or/and Aβ peptides by pre-treating the N2a-AβPP cells with amyloid-beta binding peptide (ABP) [27-29]. In binding extracellular Aβ with ABP, enhancement of insulin response was observed along the signaling 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 as compared to the N2a-AβPP cells without ABP pre-treatment. Given that the ABP preferentially binds the oligomeric forms of Aβ42, the effects of the ABP pre-treatment were likely a result of lessening the load of AβOs that would otherwise interact with the insulin receptors [27-29]. Removal of AβOs indeed promoted the recovery of p-IRS1 phosphorylation. However, ABP pre-treatment seemed to further increase IRS1 expression in response to insulin in N2a-AβPP cells, which already expressed significantly more IRS in response to insulin than N2a cells. It is possible that IRS1 expression was being further upregulated in immediate response to the clearance of AβOs from the culture medium in an attempt to adapt to the increased availability of insulin. Recent studies have shown that ABP fused with a Blood-Brain Barrier (BBB) carrier FC5 (known as KG207) was capable of reducing brain Aβ levels in a rat AD model post intravenous injection and increased the cerebrospinal fluid (CSF) Aβ42/40 ratio [29]. Five-week treatment with KG207 rectified the CSF neurofilament light chain concentrations assayed, resting-state functional connectivity and hippocampal atrophy measured by magnetic resonance imaging [29]. The studies have further demonstrated that ABP is capable of binding and removing Aβ or/and Aβ oligomers in animal studies.

A number of studies have shown that the regulation of insulin signaling and IRS1 phosphorylation is very complex, particularly in Alzheimer's insulin resistance [9,52-58]. Some studies suggest that the phosphorylation at some serine sites on IRS1 (such as mouse ser612 and human ser616) has an inhibitory effect on insulin signaling [9,55]. In our current study, we have observed that treatment of N2a cells with insulin significantly enhanced the phosphorylation of IRS1 at ser612 and there was no phosphorylation of IRS1 at ser612 in N2a-AβPP cells post insulin treatment. This is an objective observation, indicating that insulin can enhance the phosphorylation of IRS1 at ser612 in N2a cells. There are multiple phosphorylation sites (serine/threonine) on IRS1 which can be phosphorylated following binding of insulin to INSR or by other kinases (such as JNK, ERK). The activation of insulin signaling at IRS1 may be a combinatory effect of multiple site phosphorylation rather than a single site phosphorylation, possibly including some unknown sites [59].

Downstream of IRS1, the observed increase of p-Akt, p-mTOR, and p-p70S6K is consistent with the expected effect of increased phosphorylation of IRS1, which is an indication of recovered insulin response in N2a-AβPP cells by ABP treatment. These results are promising considerations for targeting AβOs to relieve insulin resistance, since the results suggest that signaling may be recovered throughout the insulin signaling pathway by reducing the extracellular AβOs that would competitively bind to insulin receptors. Of particular interest was the ABP-mediated stimulation of Akt and mTOR phosphorylation, which were downregulated in the AD state. The observation that GSK3β and p-GSK3β remains unchanged in N2a-AβPP cells suggests that some protective mechanism maintained normal active levels of GSK3β in the face of AβO pathology and treatments in this model. The ABP pre-treatment with subsequent 0.5 or 5 nM insulin treatment was able to recover mTOR signaling at certain degree. The timing of this response recovery is important to consider in how mTOR signaling functions in this context. Significant recovery of mTOR phosphorylation came 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. This implies that Akt is located upstream of the mTOR pathway, and that most likely the effect of the ABP pre-treatment was first exerted upon the mTORC1 complex, which is downstream of Akt. The increase in p70S6K phosphorylation was also an indication of increased mTORC1 activity. Whether the mTORC2 complex also further phosphorylated additional Akt is unclear.

These findings establish that, in N2a-AβPP cells, AβOs or Aβ peptides disturbed insulin signaling and induced insulin resistance by interacting with the insulin receptor. Furthermore, clearing AβOs or Aβ peptides relieved insulin resistance and improved insulin signaling in the N2a-AβPP cells. The presence of excess Aβ in the N2a-AβPP culture medium, by binding to the insulin receptor and subsequently reducing IRS1 phosphorylation, affected 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 then reduced the interactions of Aβ or AβO with insulin receptors, relieved insulin resistance and improved insulin signaling. 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 or Aβ peptides extracellularly in the brain may reduce insulin resistance and brain malfunction if at early stage; Akt phosphorylation can be enhanced by agents such as ABPs. Whether treatments like ABP can be developed as preventative measures remains to be further investigated.

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. Excess soluble AβO induced a state of insulin resistance observed as a pronounced decrease in serine phosphorylation of IRS1 in the N2a-AβPP cells at basal levels as well as a reduced response to insulin, and also as dysregulation of basal levels and phosphorylation of Akt and mTOR. Furthermore, the binding of AβO from the culture medium with ABP, which prevented competitive binding of AβO or Aβ peptides on insulin receptors, enhanced PI3K/Akt/mTOR signaling upon insulin treatment. The effects of AβO or Aβ peptides may directly affect insulin signaling or may bypass the upstream insulin signaling pathways to affect downstream signaling, which is entirely plausible since Akt and mTOR are effectors involved in other receptor-mediated pathways. The findings from the cell model provide the supporting evidence that the presence of Aβ peptides or Aβ oligomers are responsible for the dysregulation of insulin signaling or low responsiveness of brain tissues/neural cells to insulin signaling in human AD brain. The fact that ABP relieved insulin resistance and improved insulin signaling in the N2a-AβPP cell model further validates the findings that AβO and/or Aβ are responsible for insulin resistance and the dysregulation of insulin signaling. Our study also suggests that clearance of AβO and/or Aβ by APB may be further investigated as potential therapeutics to relieve insulin resistance and improve insulin signaling in Alzheimer's disease.

Limitations

It is important to note that we did not analyze the levels of IRS, pIRS, AKT, pAKT, mTOR, pmTOR, p70S6K, and phosphorylated p70S6K in human AD/CAA and ND brain samples due to limited amounts of human brain tissue samples and also due to the fact that several published studies demonstrated that there were defective insulin signalling and decreased responsiveness to insulin signaling in human AD brain. N2a cell is a mouse neuroblastoma line, and the N2a-AβPP cell is stably transfected with the human gene encoding the Amyloid Precursor Protein (APP). There is no relevant human neuronal cell line expressing APP and producing Aβ peptides for our study. Thus, the interpretation and translation of the study results into human should be cautious. In addition, due to limited scope of our study, the animal models of Alzheimer's disease were not used in this investigation.

Conclusion

Our study shows that human AD/CAA brains had higher levels of Aβ40 and Aβ42 peptides and showed dysregulation of insulin signaling with low activities of insulin signaling-responsive transcription factors as compared to age-matched non-demented control brains. N2a-AβPP cells secrete excessive Aβ40 and Aβ42 peptides into culture medium and show insulin resistance in response to insulin stimulation as compared to parental N2a cells. ABP can bind to Aβ oligomers in the *in vitro* studies and bind to Aβ deposits in human and mouse AD brains as described in previous studies. Treatment of N2a-AβPP cells with ABP significantly improved insulin signaling and relieved insulin resistant phenotype. Our study suggests that Aβ oligomers/peptides may be responsible for inducing the insulin-resistant phenotype in N2a-AβPP cells and that ABP may be further investigated for its therapeutic potential to relieve Aβ-induced insulin resistance and improve insulin signaling.

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Conflict of Interest

The authors declare that Dr. Balu Chakravarty is the inventor and patent holder of the amyloid-beta binding peptide (ABP), and that the rest of the authors have no competing interest.

Contributions

YS designed and conducted the experiments, analyzed the data and wrote/revised the manuscript. BC provided the ABP for the experiments and revised the manuscript. DC conducted Aβ ELISA and TF array for human AD/CAA and ND brain tissues. QL and WZ conceived the idea, designed the framework of the experiments, examined the data and revised the manuscript.

Data Availability

The data presented are availability upon request.

References

1. Selkoe, DJ and Hardy J. "The Amyloid Hypothesis of Alzheimer's Disease at 25 Years." *EMBO Mol Med* 8(2016): 595–608.
2. Cline, EN, Bicca MA, Viola KL and Klein WL. "The Amyloid-B Oligomer Hypothesis: The Beginning of the Third Decade." *J Alzheimers Dis* 64(2008): S567–S610.
3. Sakono, M and Zako T. "Amyloid Oligomers: Formation and Toxicity of Abeta Oligomers." *FEBS J* 277(2010): 1348-1358.
4. Ledo, JH, Azevedo EP, Clarke JR and Ribeiro FC, et al. "Amyloid-β Oligomers Link Depressive-Like Behavior and Cognitive Deficits in Mice." *Mol Psychiatry* 18(2003): 1053-1054.
5. Walsh, DM, Klyubin I, Fadeeva JV and Cullen WK, et al. "Naturally Secreted Oligomers of Amyloid β Protein Potently Inhibit Hippocampal Long-Term Potentiation *In vivo*." *Nature* 416(2002): 535–539.
6. Li, S, Hong S, Shepardson NE and Walsh DM, et al. "Soluble Oligomers of Amyloid B Protein Facilitate Hippocampal Long-Term Depression by Disrupting Neuronal Glutamate Uptake." *Neuron* 62(2009): 788–801.
7. Heras-Sandoval, D, Ferrera P and Arias C. "Amyloid-β Protein

Modulates Insulin Signaling Inpresynaptic Terminals." *Neurochem Res* 37(2012): 1879-1885.

8. Steen, E, Terry BM, Rivera EJ and Cannon JL, et al. "Impaired Insulin and Insulin-Like Growth Factor Expression and Signaling Mechanisms in Alzheimer's Disease: Is this Type 3 Diabetes?" *J Alzheimers Dis* 7(2005): 63-80.

9. Talbot, K, Wang H, Kazi H and Han L, et al. "Demonstrated Brain Insulin Resistance in Alzheimer's Disease patients is Associated with IGF-1 Resistance, IRS1 Dysregulation and Cognitive Decline." *J Clin Invest* 122(2012): 1316-1338.

10. Najem, D, Bamji-Mirza M, Chang N and Liu QY, et al. "Insulin Resistance, Neuroinflammation and Alzheimer's Disease." *Rev Neurosci* 25(2014): 509-525.

11. Readhead, B, Haure-Mirande JV, Funk CC and Richards MA, et al. "Multiscale Analysis of Independent Alzheimer's Cohorts Finds Disruption of Molecular, Genetic and Clinical Networks by Human Herpesvirus." *Neuron* 99(2018): 64-82.

12. Chen, VC, Wu SI, Huang KY and Yang YH, Kuo et al. "Herpes Zoster and Dementia: A Nationwide Population-Based Cohort Study." *J Clin Psychiatry* 79(2018): 16m11312.

13. Harris, SA and Harris EA. "Herpes Simplex Virus Type 1 and other Pathogens are Key Causative Factors in Sporadic Alzheimer's Disease." *J Alzheimers Dis* 48(2015): 319-353.

14. Eimer, WA, Kumar DKV, Shanmugam NKN and Rodriguez AS, et al. "Alzheimer's Disease Associated β-amyloid is Rapidly Seeded by Herpesviridae to Protect against Brain Infection." *Neuron* 99(2018): 56-63.

15. Mullins, RJ, Diehl TC, Chia CW and Kapogiannis D. "Insulin Resistance as a Link between Amyloid-Beta and Tau Pathologies in Alzheimer's Disease." *Front Aging Neurosci* 9(2017): 118.

16. Alves, SS, Silva-Junior RMPD, Servilha-Menezes G and Homolak J, et al. "Insulin Resistance as a Common Link between Current Alzheimer's Disease Hypotheses." *J Alzheimers Dis* 82(2021): 71-105.

17. Griffin, RJ, Moloney A, Kelliher M and Johnston, et al. "Activation of Akt/PKB, Increased Phosphorylation of Akt Substrates and Loss and Altered Distribution of Akt and PTEN are Features of Alzheimer's Disease Pathology." *J Neurochem* 93(2005): 105–117.

18. Chatterjee, S and Mudher A. "Alzheimer's Disease and Type 2 Diabetes: A Critical Assessment of the Shared Pathological Traits." *Front Neurosci* 12(2018): 383.

19. Sun, XJ, Rothenberg PL, Kahn CR and Backer JM, et al. "The Structure of the Insulin Receptor Substrate IRS-1 Defines a Unique Signal Transduction Protein." *Nature* 352(1991): 73-77.

20. Dudek, H, Datta SR, Franke TF and Birnbaum MJ, et al. "Regulation of Neuronal Survival by the Serine-Threonine Protein Kinase Akt." *Science* 275(1997): 661-665.

21. Song, G, Ouyang G and Bao S. "The Activation of Akt/PKB Signaling Pathway and Cell Survival." *J Cell Mol Med* 9(2005): 59–71.

22. Faissner, A, Heck N, Dobbertin A and Garwood J. "DSD-1-Proteoglycan/Phosphacan and Receptor Protein Tyrosine Phosphatase-Beta Isoforms during Development and Regeneration of Neural Tissues." *Adv Exp Med Biol* 557(2006): 25–53.

23. Grundke-Iqbal, I, Iqbal K, Quinlan M and Tung YC, et al. "Microtubule-Associated Protein Tau: A Component of Alzheimer Paired Helical Filaments." *J Biol Chem* 261(1986): 6084-6089.

24. Eldar-Finkelman, H. "Glycogen Synthase Kinase 3: An Emerging Therapeutic Target." *Trends Mol Med* 8(2002): 126-132.

25. Inoki, K, Li Y, Xu T and Guan KL. "Rheb GTPase is a Direct Target of TSC2 GAP Activity and Regulates mTOR Signaling." *Genes Dev* 17(2003): 1829–1834.

26. Tee, AR, Manning BD, Roux PP and Cantley LC, et al. "Tuberous Sclerosis Complex Gene Products, Tuberin and Hamartin, Control mTOR Signaling by Acting as a GTPase-activating Protein Complex Toward Rheb." *Curr Biol* 13(2003): 1259–1268.

27. Chakravarthy, B, Menard M, Brown L and Hewitt M, et al. "A Synthetic Peptide Corresponding to a Region of the Human Periocentriolar Material 1 (PCM-1) Protein Binds B-Amyloid." *J Neurochem* 126(2013): 415-424.

28. Chakravarthy, B, Ito S, Atkinson T and Gaudet C, et al. "Evidence that a Synthetic Amyloid-ß Oligomer-Binding Peptide (ABP) Targets Amyloid-ß Deposits in Transgenic Mouse Brain and Human Alzheimer's Disease Brain." *Biochem Biophys Res Commun* 445(2014): 656-660.

29. Kang, MS, Shin M, Ottoy J and Aliaga AA, et al. "Preclinical *In vivo* Longitudinal Assessment of KG207-M as a Disease-Modifying Alzheimer's Disease Therapeutic." *J Cereb Blood Flow Metab* 42(2022): 788-801.

30. Olichney, JM, Hansen LA, Galasko D and Saitoh T, et al. "The Apolipoprotein E Epsilon 4 Allele is Associated with Increased Neuritic Plaques and Cerebral Amyloid Angiopathy in Alzheimer's Disease and Lewy Body Variant." *Neurology* 47(1996): 190-196.

31. Xiong, H, Callaghan D, Jones A and Walker D, et al. "Cholesterol Retention in Alzheimer's Brain is Responsible for High β-and γ-Secretase Activities and Aβ Production." *Neurobiol Dis* 29(2008): 422-437.

32. Vukic V, Callaghan D, Walker D and Lue LF, et al. "Expression of Inflammatory Genes Induced by Beta-Amyloid Peptides in Human Brain Endothelial Cells and in Alzheimer's Brain is Mediated by the JNK-AP1 Signaling Pathway." *Neurobiol Dis* 34(2009): 95-106.

33. Dorey, E, Bamji-Mirza M, Najem D and Li Y, et al. "Apolipoprotein E Isoforms Differentially Regulate Alzheimer's and Aβ-Induced Inflammatory Response *In vivo* and *In vitro*." *J Alzheimers Disease* 57(2017): 1265-1279.

34. Craft, S, Baker LD, Montine TJ and Minoshima S, et al. "Intranasal insulin Therapy for Alzheimer Disease and Amnestic Mild Cognitive Impairment: A Pilot Clinical Trial." *Arch Neurol* 69(2012): 29-38.

35. Bertrand, F, Philippe C, Antoine PJ and Baud L, et al. "Insulin Activates Nuclear Factor Kappa B in Mammalian Cells through a Raf-1-Mediated Pathway." *J Biol Chem* 270(1995): 24435-24441.

36. Klemm, DJ, Roesler WJ, Boras T and Colton LA, et al. "Insulin Stimulates CAMP-Response Element Binding Protein Activity in HepG2 and 3T3-L1 Cell Lines." *J Biol Chem* 273(1998): 917-923.

37. Mounier, C and Posner BI. "Transcriptional Regulation by Insulin: From the Receptor to the Gene." *Can J Physiol Pharmacol* 84(2006): 713–724.

38. Solomon, SS, Majumdar G, Martinez-Hernandez A and Raghow R. "A Critical Role of Sp1 Transcription Factor in Regulating Gene Expression in Response to Insulin and Other Hormones." *Life Sci* 83(2008): 305-312.

39. Lin, Y and Sun Z. "Thyroid Hormone Potentiates Insulin Signaling and Attenuates Hyperglycemia and Insulin Resistance in a Mouse Model of Type 2 Diabetes." *Br J Pharmacol*162 (2011): 597-610.

40. Leonardini, A, Laviola L, Perrini S and Natalicchio A, et al. "Cross-Talk between PPARgamma and Insulin Signaling and Modulation of Insulin Sensitivity." *PPAR Res* 2009 (2009): 818945.

41. Cho, YM, Kim DH, Lee KH and Jeong SW, et al. "The IRE1α-XBP1s Pathway Promotes Insulin-Stimulated Glucose Uptake in Adipocytes by Increasing PPARγ Activity." *Exp Mol Med* 50(2018): 102.

42. Chiang, WC, Ching TT, Lee HC and Mousigian C, et al. "HSF-1 Regulators DDL-1/2 Link Insulin-Like Signaling to Heat-Shock Responses and Modulation of Longevity." *Cell* 148(2012): 322-334.

43. Najem, D, Bamji-Mirza M, Yang, Z, Zhang, W. "Characterization of Aβ-Induced Insulin Resistance and the Effects of Insulin on Cholesterol Synthesis Pathway and Aβ Secretion in Neural Cells." *Neurosci Bulletin* 32(2016): 227-238.

44. Copps, KD and White MF. "Regulation of Insulin Sensitivity by Serine/Threonine Phosphorylation of Insulin Receptor Substrate Proteins IRS1 and IRS2." *Diabetologia* 55(2012): 2565-2582.

45. Harada, H, Andersen JS, Mann M and Terada N, et al. "P70S6 Kinase Signals Cell Survival as well as Growth, Inactivating the Pro-Apoptotic Molecule BAD." *Proc Natl Acad Sci USA* 98(2001): 9666-9670.

46. Rosner, M and Hengstschlager M. "Nucleocytoplasmic Localization of p70 S6K1, but not of its Isoforms p85 and p31, is Regulated by TSC2/mTOR." *Oncogene* 30 (2011): 4509–4522.

47. Evangelopoulos, ME, Weis J and Krüttgen A. "Signalling Pathways Leading to Neuroblastoma Differentiation after Serum Withdrawal: HDL Blocks Neuroblastoma Differentiation by Inhibition of EGFR." *Oncogene* 24(2005): 3309-3318.

48. Soto, M, Cai W, Konishi M and Kahn CR. "Insulin Signaling in the Hippocampus and Amygdala Regulates Metabolism and Neurobehavior." *Proc Natl Acad Sci USA* 116(2019): 6379-6384.

49. Reger, MA, Watson GS, Green PS and Wilkinson CW, et al. "Intranasal Insulin Improves Cognition and Modulates β-Amyloid in Early AD." *Neurology* 70(2008): 440–448.

50. Craft, S, Baker LD, Montine TJ and Minoshima S, et al. "Intranasal insulin Therapy for Alzheimer Disease and Amnestic Mild Cognitive Impairment: A Pilot Clinical Trial." *Arch Neurol* 69(2012): 29-38.

51. Morris, JK and Burns JM. "Insulin: An Emerging Treatment for Alzheimer's Disease Dementia?" *Curr Neurol Neurosci Rep* 12(2012): 520–527.

52. Zhao, WQ, De Felice FG, Fernandez S and Chen H, et al. "Amyloid Beta Oligomers Induce Impairment of Neuronal Insulin Receptors." *FASEB J* 22(2008): 246-260.

53. Boura-Halfon, S and Zick Y. "Serine Kinases of Insulin Receptor Substrate Proteins." *Vitam Horm* 80(2009): 313-349.

54. Boura-Halfon, S and Zick Y. "Phosphorylation of IRS Proteins, Insulin Action and Insulin Resistance." *Am J Physiol Endocrinol Metab* 296(2009): E581-E591.

55. Bomfim, TR, Forny-Germano L, Sathler LB and Brito-Moreira J, et al. "An Anti-Diabetes Agent Protects the Mouse Brain from Defective Insulin Signaling caused by Alzheimer's Disease-Associated Abeta Oligomers." *J Clin Invest* 122(2012): 1339–1353.

56. Yarchoan, M and Arnold SE. "Repurposing Diabetes Drugs for Brain Insulin Resistance in Alzheimer Disease." *Diabetes* 63(2014): 2253-2261.

57. Ferreira, LSS, Fernandes CS, Vieira MNN and De Felice FG. "Insulin Resistance in Alzheimer's Disease". *Front Neurosci* 12(2018): 830.

58. Barone, E, Di Domenico F, Perluigi M and Butterfield DA. "The Interplay among Oxidative Stress, Brain Insulin Resistance and AMPK Dysfunction Contribute to Neurodegeneration in Type 2 Diabetes and Alzheimer Disease." *Free Radic Biol Med* 176(2021): 16-33.

59. Gual, P, Marchand-Brustel LY and Tanti JF. "Positive and Negative Regulation of Insulin Signaling through IRS-1 Phosphorylation." *Biochimie* 87(2005): 99-109.

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