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Impaired insulin signaling in an animal model of Niemann-Pick Type C disease

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ABSTRACT

Studies have shown similarities between the histopathological characteristics of NPC and Alzheimer's disease (AD) including amyloid and tau pathologies. While dysfunction in insulin signaling was widely detected in AD brain, the function of insulin signaling proteins has not been examined in NPC disease. In this study, we have examined the expression and phosphorylation of proteins linked to the insulin signaling pathway in the brain of 9 weeks old NPC^{nih} mice. Our results showed lower expression of insulin receptor substrate 2 (IRS2) in the NPC^{nih} mice, and insulin receptor substrate 1 (IRS1) expression was almost non-detectable in this NPC mouse model. This reduction was associated with the loss of expression for the regulatory p85 subunit of phosphatidylinositol 3-kinase (p85/PI3K). Interestingly, the impairment was observed to link to a greater reduction of Akt phosphorylation at residue T308 than S473. This aberrant Akt phosphorylation could be contributing to lower GSK3β phosphorylation detected in the NPC^{nih} mouse brain. To our knowledge, this is the first report documenting impaired insulin signaling in the brain of a NPC mouse model.

1. Introduction

Niemann-Pick Type C (NPC) disease is a fatal neurovisceral disorder characterized by progressive neurodegeneration [1]. This disease is autosomal recessive and is mostly caused by mutations in the *NPC1* gene [2]. NPC1 protein is an ubiquitously expressed transmembrane glycoprotein [3]. The presence of a sterol-sensing domain on the protein and the accumulation of cholesterol in NPC-1 deficient cells indicate a role for NPC1 in cholesterol homeostasis [4]. One of the most commonly used NPC animal model is the NPC^{nih} mice [5]. The spontaneous mutation was localized to mouse chromosome 18 in a region syntenic to the human NPC1 locus. This mouse model shares many of the clinical abnormalities observed in humans with NPC. They include increased levels of sphingomyelin and unesterified cholesterol in the liver.

Interestingly, increasingly numbers of studies have suggested similarities between the histopathological characteristics of NPC and Alzheimer's disease (AD) [6–9] including amyloid and tau pathologies [10,11]. We [12] and others [13–15] have observed these neuropathological features in the NPC^{nih} mice as well as other NPC mouse models.

Recent studies have shown a cross-talk between insulin signaling and cholesterol metabolism [16], and dysfunction in insulin signaling is widely detected in AD brain [17–19]. Although aberrant cholesterol metabolism is a major pathological event in NPC [20,21], the function of insulin signaling proteins has not been examined in NPC disease.

In this study, we have attempted to examine the expression and phosphorylation of proteins associated with the insulin signaling pathway in the brain of 9 weeks old NPC^{nih} mice. This age corresponds to after the onset of neuronal abnormalities in the NPC^{nih} mice [12]. Our results showed lower expression of insulin receptor substrate 2 (IRS2) in the NPC^{nih} mice. Insulin receptor substrate 1 (IRS1) expression was almost non-detectable in this NPC mouse model. This reduction was associated with the loss of expression for the regulatory p85 subunit of phosphatidylinositol 3-kinase (p85/PI3K) [22]. Interestingly, the impairment was observed to link to a greater reduction of Akt phosphorylation at residue T308 than S473. This aberrant Akt phosphorylation could be contributing to lower GSK3β phosphorylation detected in the NPC^{nih} mouse brain. To our knowledge, this is the first report documenting impaired insulin signaling in the brain of a NPC mouse model.

2. Materials and methods

2.1. Animals

The experimental protocol #010/06 involving the maintenance and euthanasia of laboratory mice was approved by the Institutional Animal Care and Use Committees (IACUC) at the National University of Singapore. Mice used in this study were homozygous mutant BALB/c NPC^{nih} mice [5] from Jackson Laboratories, and their wild-type littermates as controls. Homozygous NPC mice developed

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neurological abnormalities at 6–7 weeks of age and died within 10–12 weeks of age. Both homozygous NPC^{nih} and control mice were sacrificed and brains were collected at week 9, corresponding to *after* the onset of neuropathology. Three brains ($n = 3$) from each time point in each animal group were used in all analysis.

2.2. Preparation of brain homogenates

The mouse brain tissues were snapped frozen in liquid nitrogen when harvested and the wet weight of the tissues (in mg) was determined using an electronic balance. Twenty percent (w/v) brain homogenates were prepared with 1× cell lysis buffer (Cell Signaling Technol., Danvers, USA) with protease inhibitors cocktail (Roche Diagnostic). The cell lysis buffer contains sodium orthovanadate, pyrophosphate and glycerophosphate, which can acts as phosphatase inhibitors. Lysates were then homogenized using a hand held motorized pestle (Sigma–Aldrich, St. Louis, USA) for 30 s on ice. Tissue lysates were subsequently centrifuged at 30,000*g* for 30 min under 4 °C. The soluble portion of the lysates was collected for analysis.

2.3. Protein quantification of lysates

Tissue lysates were quantified using the Pierce™ MicroBCA assay kit (ThermoFisher Scientific, Waltham, USA) in a 96-well microplate format. Lysates were diluted in PBS and the working reagent was prepared and added in accordance to the manufacturer's instructions. Samples were then incubated at 37 °C for 30 min before reading the absorbance values at 562 nm. Protein concentrations of samples were calculated based on a standard curve constructed from a range of BSA standards. The brain tissue lysates were aliquoted and stored at −80 °C.

2.4. Immunoblot analysis

Fifty micrograms (μg) of soluble brain proteins from lysate samples were heated at 95 °C for 5 min. Protein samples were then centrifuged at 14,000*g* for 2 min on a bench top centrifuge before they were loaded on a 7.5–10% Tris–glycine polyacrylamide gel. The Precision Plus protein™ standard (Bio-Rad Laboratories, Hercules, California, USA) was used as a molecular weight standard and run together with the samples on the same piece of gel.

The separated proteins were transferred onto a nitrocellulose membrane, probed with the respective antibodies and exposed to horseradish peroxidase (HRP)-conjugated secondary antibodies. The reactive protein bands were visualized by chemiluminescence on the Image Station 4000R (Carestream Health Inc.) using the SuperSignal® West Dura Substrate (Pierce) system.

Immunoblotting of β-actin using a rabbit polyclonal antibody that binds to the C-terminal of β-actin (Sigma) was included in

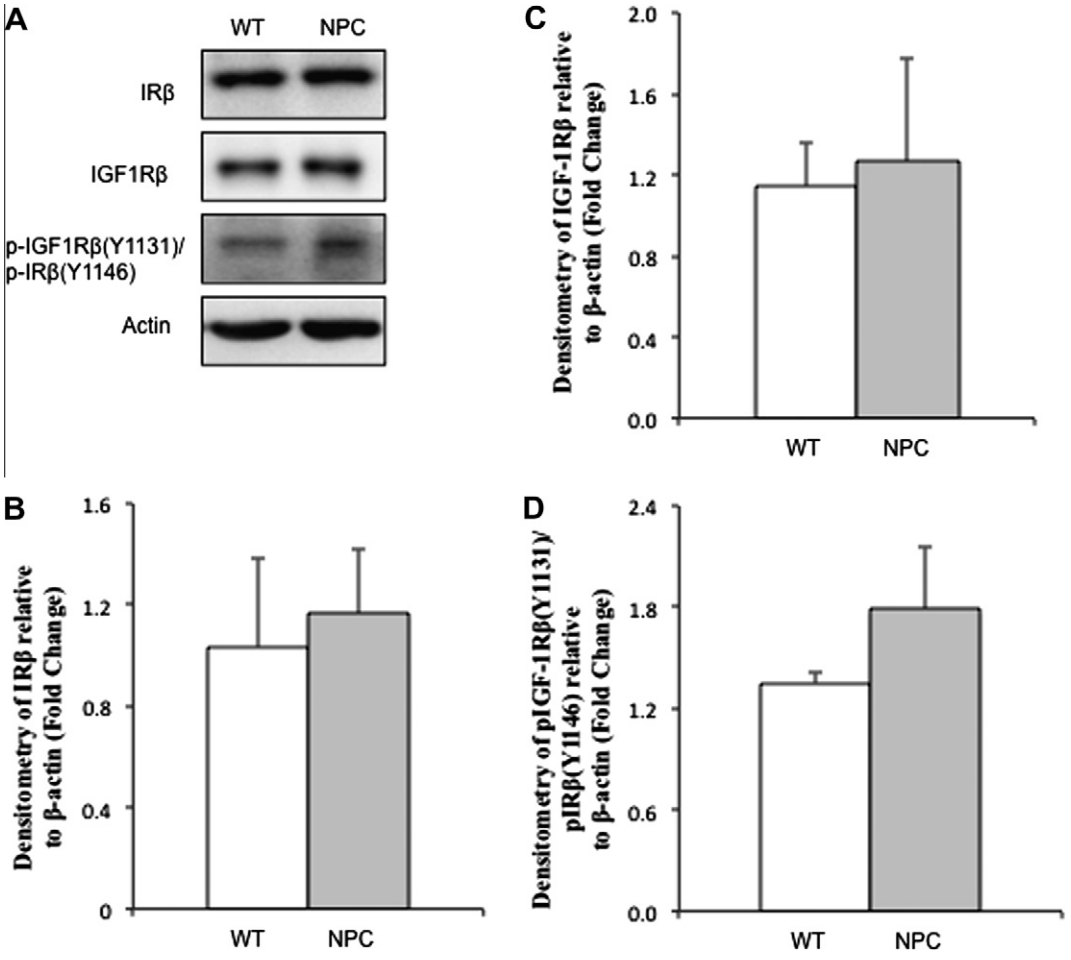

Fig. 1. Analysis of insulin receptor and insulin growth factor 1 receptor expression and phosphorylation in wild-type and NPC^{nih} mice. (A) Western Blot analysis of insulin receptor β-subunit (IRβ), insulin growth factor 1 receptor β-subunit (IGF1Rβ), phosphorylated IGF1Rβ (Y1131) and phosphorylated IRβ (Y1146) levels in wild-type (WT) and NPC^{nih} mice at 9 weeks of age. β-actin was immunoblotted to ensure similar gel loading of the starting material in each sample. Densitometry analysis of (B) IRβ, (C) IGF1Rβ and (D) phosphorylated IGF1Rβ (Y1131) and phosphorylated IRβ (Y1146) level relative to β-actin level in 9 weeks old WT (white bar) and NPC^{nih} (grey bar) mice was performed using the NIH ImageJ software. Each value represents the mean ± SEM for individual mouse brain sample ($n \leqslant 3$ at each time point for each mouse line).

all Western Blot analysis to ensure comparable protein loading. The primary antibodies used include anti-IRβ (Cell Signaling Technol., Cat# 3020), anti-IGF1Rβ (Cell Signaling Technol., Cat# 3018), anti-pIGF1Rβ (Y1131)/pIRβ (Y1146) (Cell Signaling Technol., Cat# 3021), anti-IRS1 (Cell Signaling Technol., Cat# 2382), anti-IRS2 (Cell Signaling Technol., Cat# 4502), anti-PI3K (p85 subunit) (Cell Signaling Technol., Cat# 4292), anti-Akt (Cell Signaling Technol., Cat# 4691), anti-pAkt(S473) (Cell Signaling Technol., Cat# 4060), anti-pAkt(T308) (Cell Signaling Technol., Cat# 2965), anti-GSK3β (Cell Signaling Technol., Cat# 9315), and anti-GSK3β(S9) (Cell Signaling Technol.), Cat# 9323.

Densitometry analysis was performed [23] by measuring the optical densities of the targeted protein bands relative to the endogenous β-actin level from the same brain sample. For protein phosphorylation, the optical densities of the phosphorylated protein bands were measured relative to the targeted total protein level from the same brain sample. The analysis was performed using the NIH ImageJ software.

2.5. Statistical analysis

Significant differences were analyzed using two-tailed Student's *t*-test. A p value of <0.05 is considered significant.

3. Results

3.1. Altered phosphorylation of insulin receptor and insulin growth factor 1 receptor in NPC^{nih} mice

Studies have suggested similarities between the histopathological characteristics of NPC disease and Alzheimer's disease (AD) [6–9]. Since impaired insulin signaling is well documented in AD [24,25], we therefore decided to examine if the expression and phosphorylation of two insulin-binding transmembrane receptors [26] are affected in the NPC^{nih} mice.

As shown in Fig. 1A, we did not detect any apparent change in insulin receptor β-subunit (IRβ) and insulin growth factor-1 β-subunit (IGF1Rβ) levels in wild-type (WT) and the NPC mouse lines using immunoblotting. Using an antibody recognizing both phosphorylated IRβ (Y1131) and IGF-1Rβ (Y1146), a ~30% increased reactivity was detected in the NPC^{nih} mice as compared to WT mice (Fig. 1D).

3.2. Brain insulin signaling proteins expression

The increased phosphorylation of the insulin-binding receptors in the NPC^{nih} mouse brain was associated with reduced expression of both insulin receptor substrate 1 and 2 (IRS-1 and IRS-2)

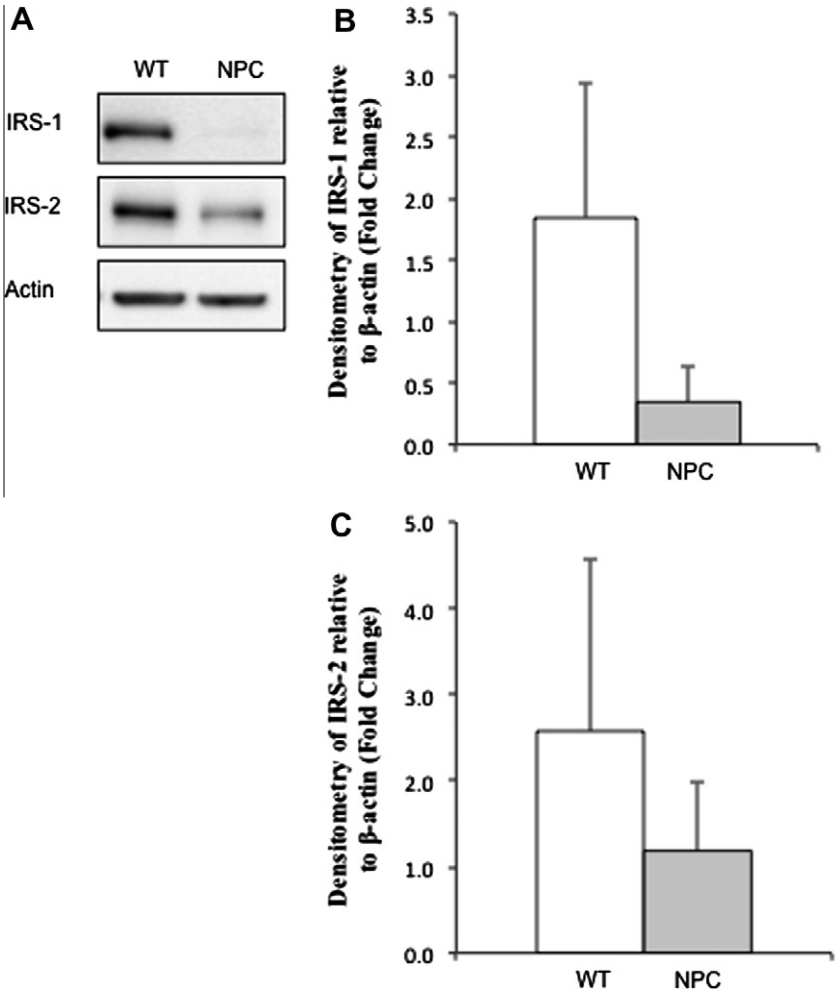

Fig. 2. Insulin receptor substrate proteins expression in wild-type and NPC^{nih} mice. (A) Western Blot analysis of insulin receptor substrate-1 and -2 (IRS-1 and IRS-2) levels in wild-type (WT) and NPC^{nih} mice at 9 weeks of age. β-actin was immunoblotted to ensure similar gel loading of the starting material in each sample. Densitometry analysis of (B) IRS-1 and (C) IRS-2 level relative to β-actin level in 9 weeks old WT (white bar) and NPC^{nih} (grey bar) mice was performed using the NIH ImageJ software. Each value represents the mean ± SEM for individual mouse brain sample ($n \leqslant 3$ at each time point for each mouse line).

(Fig. 2A). IRS1 was apparently not detectable by immunoblotting in the brain of the 9 weeks old NPC^{nih} mice. But for IRS2 expression, densitometric analysis indicates a ~50% reduction in NPC^{nih} mice as compared to WT mice (Fig. 2C).

3.3. Reduced Akt phosphorylation in the brain of NPC^{NIH} mice

We next decide to determine if the aberrant IRS expression will affect the downstream signaling partners. In Fig. 3, we were able to detect the presence of the p85 subunit of PI3K (p85/PI3K) in WT mice but not in the NPC^{nih} mice. In contrast, we did not observe any change in Akt expression between WT and NPC^{nih} mice. However, when we examined Akt phosphorylation, we observed an apparent greater reduction (~38%) in phosphorylation at Thr-308 than phosphorylation at Ser-473 (~17% reduction) in NPC^{nih} mice as compared to WT mice (Fig. 3).

3.4. Lower GSK3β phosphorylation in NPC^{nih} mouse brain

Impaired PI3K/Akt signaling is known to affect GSK3β phosphorylation [27], but it is unclear if this is also apparent in the NPC^{nih} mice.

Using immunoblotting and densitometric analysis, we observed an apparent reduction of phosphorylated Ser9 on GSK3β in the NPC^{nih} mice (Fig. 4).

4. Discussion

Increasingly number of studies have observed striking parallels between NPC disease and AD [6–9] including amyloid and tau pathologies [10–12]. These neuropathological features are also observed in several NPC animal models [13–15] including the NPC^{nih} mice used in our current study. However, the factor(s) contributing to the development of these pathological hallmarks is unknown.

Although impaired insulin signaling is well documented in AD [24,25], this signaling cascade has not been examined in NPC disease. In this study, we observed lower GSK3β phosphorylation in the NPC^{nih} mice. This reduction is associated with impairment in insulin signaling, including lower Akt phosphorylation and decreased expression of p85/PI3K, IRS1 and IRS2.

It is also noteworthy that a previous study has reported conflicting observation in PI3K/Akt signaling using similar NPC mouse model [28]. However, their observation of higher PI3K, Akt and GSK3β phosphorylation were made using 2 and 4 weeks old NPC^{nih} mice. These time points corresponding to period before the onset of neurodegeneration and they were earlier than the one we are using our current study. Furthermore, PI3K is only one of several proteins that are able to regulate Akt phosphorylation [22]. A recent study has shown that Akt can also be activated using cAMP analog via protein kinase A (PKA) [29].

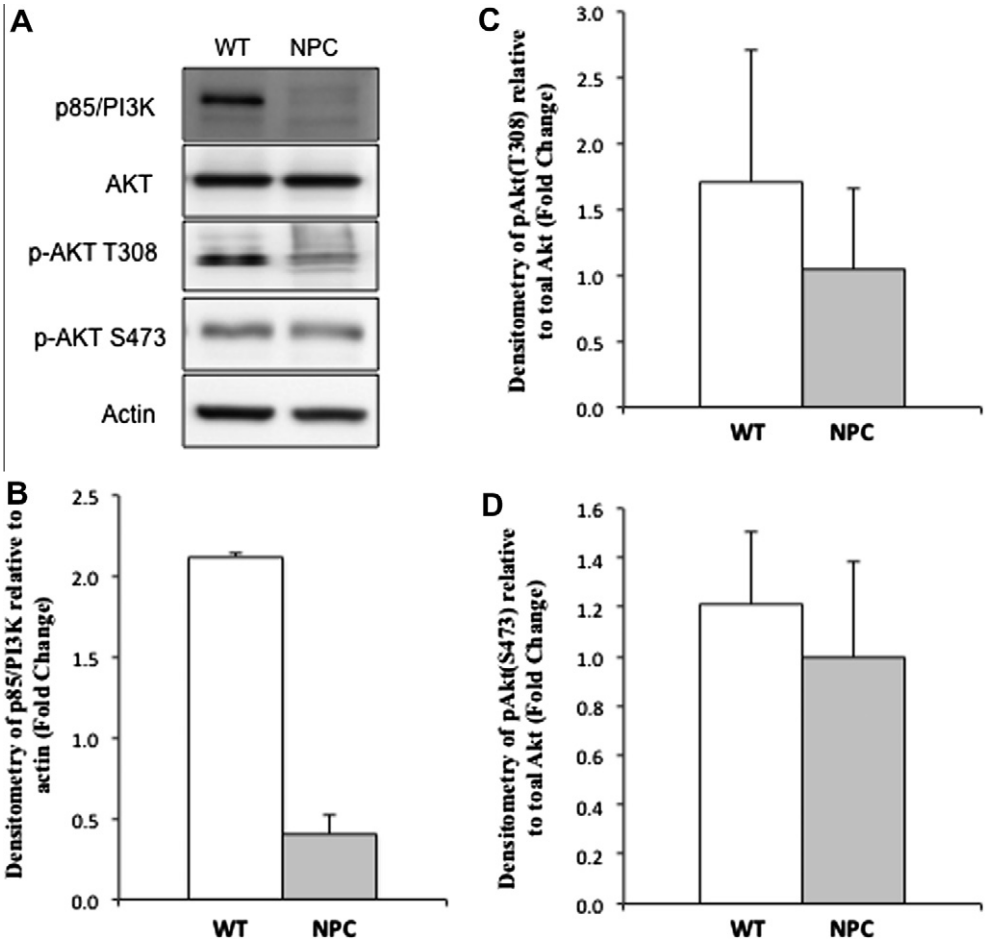

Fig. 3. The expression level of insulin signaling proteins in NPC^{nih} mice. (A) Immunoblotting of p85/PI3K, total Akt, phosphorylated Akt (T308) and phosphorylated Akt (S473) in wild-type (WT) and NPC^{nih} mice at 9 weeks of age. β-actin was immunoblotted to ensure similar gel loading of the starting material in each sample. Densitometry analysis of (B) p85/PI3K level relative to β-actin level, (C) phosphorylated Akt(T308) and (D) phosphorylated Akt(S473) level relative to total Akt level, in 9 weeks old WT (white bar) and NPC^{nih} (grey bar) mice was performed using the NIH ImageJ software. Each value represents the mean ± SEM for individual mouse brain sample ($n \leqslant 3$ at each time point for each mouse line).

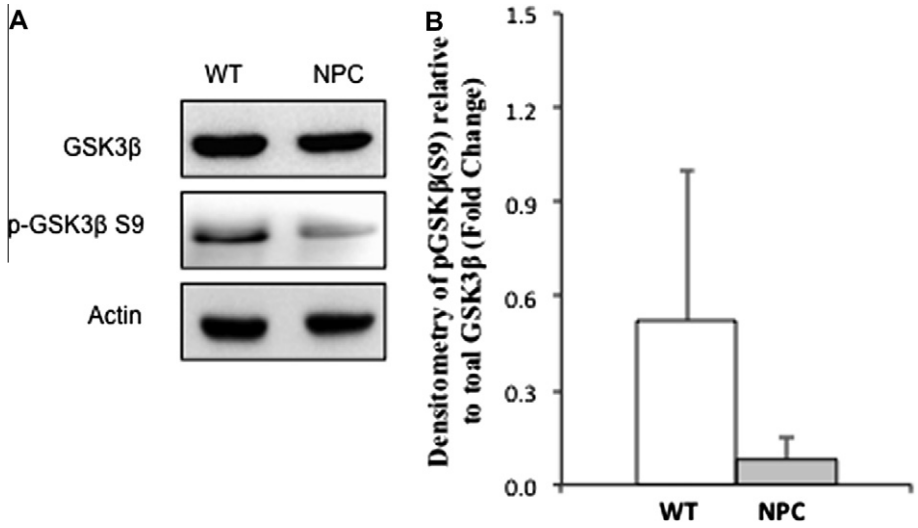

Fig. 4. GSK3 expression in NPC^{nih} mice. (A) Immunoblotting of GSK3 and phosphorylated GSK3 (S9) level in wild-type (WT) and NPC^{nih} mice at 9 weeks of age. β-actin was immunoblotted to ensure similar gel loading of the starting material in each sample. (B) Densitometry analysis of phosphorylated GSK3 (S9) level relative to total GSK3 level in 9 weeks old WT (white bar) and NPC^{nih} (grey bar) mice was performed using the NIH ImageJ software. Each value represents the mean ± SEM for individual mouse brain sample ($n \leqslant 3$ at each time point for each mouse line).

Here in the NPC^{nih} mice, we found lower IRS1 and IRS2 expression. IRS1 and IRS2 are known to signal through the Akt pathway [30] and it is possible that the reduction in PI3K expression and Akt phosphorylation could due to lower IRS1 and IRS2 levels. The reduced Akt phosphorylation in the NPC^{nih} mice also lead to increased GSK3β activity (via lower GSK3β phosphorylation). This increased GSK3β activation could be contributing to increased tau phosphorylation and subsequent development of tau pathology reported in several NPC mouse models [10,11,13–15].

In summary, our study shows for the first time that insulin signaling is impaired in the NPC^{nih} mice. This observation has also demonstrated another parallel between NPC disease and AD. However, the exact mechanism of action caused by impaired insulin signaling to the development of NPC neuropathology requires further investigation.

Author Contributions

Q.R.O., M.L.L. and C.C.C. performed the experiments. Q.R.O. and B.S.W. analyzed the data. Q.R.O. and B.S.W. conceived and designed the experiments. Q.R.O., S.N.C. and B.S.W. wrote the paper.

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