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Brain insulin resistance triggers early onset Alzheimer disease in Down syndrome

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ABSTRACT

Dysregulation of insulin signaling pathway with reduced downstream neuronal survival and plasticity mechanisms is a fundamental abnormality observed in Alzheimer's disease (AD) brain. This phenomenon, known as brain insulin resistance, is associated with poor cognitive performance and is driven by the uncoupling of insulin receptor (IR) from its direct substrate (IRS1). Considering that Down syndrome (DS) and AD neuropathology share many common features, we investigated metabolic aspects of neurodegeneration, i.e., brain insulin resistance, in DS and whether it would contribute to early onset AD in DS population.

Changes of levels and activation of main brain proteins belonging to the insulin signaling pathway (i.e., IR, IRS1, PTEN, GSK3β, PKCζ, AS160, GLUT4) were evaluated. Furthermore, we analyzed whether changes of these proteins were associated with alterations of: (i) proteins regulating brain energy metabolism; (ii) APP cleavage; and (ii) regulation of synaptic plasticity mechanisms in post-mortem brain samples collected from people with DS before and after the development of AD pathology (DSAD) compared with their age-matched controls.

We found that DS cases were characterized by key markers of brain insulin resistance (reduced IR and increased IRS1 inhibition) early in life. Furthermore, downstream from IRS1, an overall uncoupling among the proteins of insulin signaling was observed. Dysregulated brain insulin signaling was associated with reduced hexokinase II (HKII) levels and proteins associated with mitochondrial complexes levels as well as with reduced levels of syntaxin in DS cases. Tellingly, these alterations precede the development of AD neuropathology and clinical presentations in DS.

We propose that markers of brain insulin resistance rise earlier with age in DS compared with the general population and may contribute to the cognitive impairment associated with the early development of AD in DS.

1. Background

Down syndrome (DS) is a genetic-related disorder - due to triplication of Chromosome 21 - characterized by intellectual disability and often between ages of 40–50 years by early onset Alzheimer-like dementia (AD). There are approximately 6 million people with DS worldwide, most of them without a completely independent life (Foundation, 2019). Moreover, improvements in care for children and adults with DS have led to significant extensions in life span and quality of life for people with (Lott and Head, 2019).

Abbreviations: Aβ, beta amyloid; AD, Alzheimer's disease; Akt, protein kinase B; AMPA, α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid; APP, amyloid precursor protein; AS160, Akt substrate 160; C83, APP C-terminal fragment 83; C99, APP C-terminal fragment 99; Ctr O, control old; Ctr Y, control young; DS, down syndrome; DSAD, down syndrome people with Alzheimer's disease pathology; DYRK1, dual-specificity tyrosine-phosphorylation regulated kinase 1A; ECL, Clarity enhanced chemiluminescence substrate; EDTA, ethylenediamine tetraacetic acid; GABA, gamma-aminobutyric acid; GLUT, glucose transporter; GSK3β, glycogen synthase-3 beta; HKII, hexokinase type II; IR, insulin receptor; IRS1, insulin receptor substrate 1; LTD, long-term depression; LTP, long-term potentiation; MCI, mild cognitive impairment; mTOR, mammalian target of rapamycin; NMDA, *N*-methyl-D-aspartate; NTFs, neurofibrillary tangles; PI3K, phosphatydilinositol-3 kinase; PIP3, phosphatidylinositol (3,4,5)-trisphosphate; PKCζ, atypical protein kinase C zeta; PMI, postmortem interval; PSD95, postsynaptic density protein 95; PTEN, phosphatase and tensin homolog; RIPA, Radioimmunoprecipitation assay buffer; SDS, sodium dodecyl sulfate; Ser, serine; TGS, Tris/Glycine/SDS; TNFα, tumor necrosis factor alpha; TTBS, Tween-20/Tris buffered saline; Tyr, tyrosine

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Intellectual disability in DS is due to alterations of brain development that can be traced back to foetal life stages. These alterations include widespread impairments in neurogenesis, excessive numbers of astrocytes, dendritic atrophy, and impaired connectivity (Bartesaghi et al., 2011). These deficits are distributed throughout the brain and, therefore, a constellation of brain functions is altered. A key challenge for adults with DS is an increased risk of developing clinical symptoms of AD as they age. Almost all adults with DS demonstrate AD-like neuropathology by age 40 years. In the affected DS elderly, extensive similarities with AD regarding clinical presentation, neuropathological findings and participation of genetic factors justifies the designation of AD in DS (Wiseman et al., 2015).

An increasing number of studies showed that molecules able to regulate glucose metabolism, such as insulin, insulin-like growth factor-1 and glucagon-like peptide-1, modulate different functions of the cortex and hippocampus – two key areas of the brain responsible of learning and memory – and are also involved in development and aging processes (Arnold et al., 2018; Biessels and Reagan, 2015). Detection of insulin in the brain reveals that the brain is a target organ for insulin. Indeed, insulin plays several functions in the central nervous system ranging from regulation of protein synthesis and cytoskeletal protein expression, to neurite outgrowth, migration, and differentiation, and synapse formation (Blazquez et al., 2014). All these functions participate in cognitive processes, including attention, executive functioning, learning, and memory (Blazquez et al., 2014). Insulin also has the ability to provide neuroprotection, acting mainly against apoptosis, amyloid-β (Aβ) toxicity, oxidative stress and ischemia (Ferreira et al., 2018).

It is now well-recognized that defects in insulin level/signaling may have a profound impact on the central nervous system function, and many authors have suggested that alterations of brain insulin signaling pathway and changes in glucose metabolism may increase the risk of developing dementia [reviewed in (Arnold et al., 2018)]. Examination of postmortem AD and amnestic mild cognitive impairment brains uncovered key signs of brain insulin resistance, i.e., reduced insulin receptor (IR) and increased serine phosphorylation (inhibitory) of insulin receptor substrate 1 (IRS1), particularly in the hippocampus, cortex and hypothalamus (Biessels and Reagan, 2015; De Felice, 2013; Tramutola et al., 2015). Higher levels of markers of insulin resistance are associated with poorer performance on cognitive tests of episodic and working memory, independent of the senile plaques (beta-amyloid – Aβ) and neurofibrillary tangles (Tau) load, thus suggesting a role for insulin signaling in neuronal functions (Biessels and Reagan, 2015). Moreover, it is important to emphasize that brain insulin resistance and impaired cerebral glucose metabolism are pathophysiological features that may even precede others neuropathological alterations by several decades (Chen and Zhong, 2013; Stanley et al., 2016). Understanding and sequencing the cascade of changes that eventually precedes Aβ and Tau aggregation is central to assess the “risk profile” and to identify novel therapeutic targets that can be used for preventative interventions.

No evidence is available on the expression and activation of the members of the insulin signaling machinery in the brains of people with DS. Given that the brains of individuals with DS share many common neuropathological features with AD in the general population, in the present study we hypothesized that brain insulin resistance develops in DS as a function of age and with the development of AD neuropathology and thus may contribute to the development of AD in DS.

2. Methods

2.1. Human subjects

Frontal cortex human brain samples were obtained from the University of California Alzheimer's Disease Research Center (UCI-ADRC) and the Institute for Memory Impairments and Neurological Disorders, the Eunice Kennedy Shriver NICHD Brain and Tissue Bank for Developmental Disorders, and the University of Kentucky Alzheimer's disease Center. Table 1 shows the characteristics and demographic data of the included cases in the study. DS cases were divided into two groups, with or without sufficient pathology for a neuropathological diagnosis of AD. The ages of DS cases without AD were under 40 years, while the cases with both DS and AD were over the age of 40 years. Likewise, controls were split into two groups: Controls Young (Ctr Y), to compare with DS group because they are ≤45 years; Controls Old (Ctr O), to compare with DSAD because they are older than 45 years at death. Although the range of the age within Ctr Y and DS subjects appears wide, this is quite similar between the two groups of subjects [Ctr Y: 1–44 y; DS: 9–39 y (Table 1)] thus limiting the impact of age when comparing DS vs Ctr Y. Moreover this in agreement with previous studies both from our groups and others (Cenini et al., 2012; Hamlett et al., 2017; Lott and Head, 2019; Perluigi et al., 2014; Tramutola et al., 2017; Wilcock et al., 2015).

Table 1
Subjects demographic data.

Subjects	PMI	Age	Sex	Race	Cause of death
Ctr Y 1	20	9	F	African American	Asthma
Ctr Y 2	28	13	M	African American	Accident, fire
Ctr Y 3	8	33	M	Caucasian	Cardiac arrythmia
Ctr Y 4	17	39	F	Caucasian	Car accident
Ctr Y 5	12	22	M	African American	Cardiac arrythmia
Ctr Y 6	10	11	F	Caucasian	Asthma
DS 1	12	2	M	Caucasian	Unknown
DS 2	15	23	M	African American	Pneumonia
DS 3	24	24	M	Caucasian	Cardiac arrythmia
DS 4	28	1	F	African American	Pneumonia
DS 5	13	44	F	Caucasian	Cardiac arrythmia
DS 6	12	39	F	Caucasian	Cancer
DS 7	10	40	M	African American	Hypertensive cardiovascular dis.
DS 8	14	20	M	Indian	Cardiopulmonary arrest
DSAD 1	10	66	M	Unknown	Cardiac arrest
DSAD 2	3.3	58	M	Unknown	Alzheimer disease
DSAD 3	11	61	M	Unknown	Unknown
DSAD 4	4.5	55	M	Unknown	Pneumonia
DSAD 5	3	63	F	Unknown	Respiratory problems
DSAD 6	6	63	F	Unknown	Unknown
DSAD 7	5.3	57	F	Unknown	Seizure disorder
DSAD 8	3	62	F	Unknown	Pneumonia
Ctr O 1	17	57	M	Caucasian	Hypertensive cardiovascular dis.
Ctr O 2	24	46	F	Caucasian	Bike acident
Ctr O 3	8	64	F	Unknown	Myocardial infarction
Ctr O 4	28	52	M	Caucasian	Ateriosclerotic Cardiovascular dis.
Ctr O 5	16	55	M	Caucasian	Ateriosclerotic Cardiovascular dis.
Ctr O 6	4.5	65	M	Unknown	Cardiac arrest
Ctr O 7	17	55	M	Caucasian	Car accident
Ctr O 8	2.7	67	M	Unknown	Cardiomyopathy

All the results obtained from human autopsy samples were analyzed by considering the difference in postmortem interval (PMI) among groups. Correlation analysis did not show any association between all the reported measures and PMI (Supplementary Table 1). A more limited sample was available for studies of syntaxin and PSD95 protein levels, as two samples in the DS group and 1 sample in DSAD group were no longer available, thus sample size for these analyses was smaller.

In some of the results showed throughout the paper there are samples that appears to be out of the mean range. In particular for Ctr Y and DS group, we acknowledge that these samples are neither the younger nor the oldest. All the samples were maintained in the analysis

because they are representative of the physiological variability characterizing human subjects. Indeed, the complexity of DS phenotypes is the result of significant individual variability, due to trisomy 21; thus, it is recommended to consider this aspect in sample collection and analysis (Lott and Head, 2019).

Finally, we acknowledge that the same autopsy cases were used in previous experiments measuring Aβ levels, p-Tau levels, total oxidation, and autophagy pathway as a function of age in DS (Cenini et al., 2012; Di Domenico et al., 2014; Perluigi et al., 2014).

2.2. Samples preparation

Total protein extracts from the frontal cortex were prepared in radioimmunoprecipitation assay (RIPA) buffer (pH 7.4) containing Tris-HCl (50 mM, pH 7.4), NaCl (150 mM), 1% NP-40, 0.25% sodium deoxycholate, ethylenediamine tetraacetic acid (EDTA) (1 mM), 0.1% sodium dodecyl sulfate (SDS), supplemented with protease inhibitors [phenylmethylsulfonyl fluoride (PMSF, 1 mM), sodium fluoride (NaF, 1 mM) and sodium orthovanadate (Na_3VO_4, 1 mM)]. Before clarification, brain tissues were homogenized by 20 passes with a Wheaton tissue homogenizer. Brain tissues homogenates were then centrifuged at 16,000 × *g* for 1 h at 4 °C. The supernatant was used to determine the total protein concentration by the Bradford assay (Pierce, Rockford, IL).

2.3. Western blot

For western blots, 20 μg of proteins were resolved on Criterion TGX Stain-Free 4–15% 18-well gel (Bio-Rad Laboratories, #5678084) in a Criterion large format electrophoresis cell (Bio-Rad Laboratories, #1656001) in Tris/Glycine/SDS (TGS) Running Buffer (Bio-Rad Laboratories, #1610772). Immediately after electrophoresis, the gel was then placed on a Chemi/UV/Stain-Free tray and then placed into a ChemiDoc MP imaging System (Bio-Rad Laboratories, #17001402) and UV-activated based on the appropriate settings with Image Lab Software (Bio-Rad Laboratories) to collect total protein load image.

Following electrophoresis and gel imaging, the proteins were transferred via the TransBlot Turbo semi-dry blotting apparatus (Bio-Rad Laboratories, #1704150) onto nitrocellulose membranes (Bio-Rad, Hercules, CA, USA, #162–0115) and membranes were blocked with 3% bovine serum albumin in 0.5% Tween-20/Tris-buffered saline (TTBS) and incubated overnight at 4 °C with the following antibodies: anti-IRβ (1:1000, Cell Signaling, Bioconcept, Allschwill, Switzerland, #3020), anti-phospho(Tyr1158/1162/1163)-IRβ (1:1000, Genetex, Irvine, CA, USA, #GTX25681), anti-IRS1 (1:1000, Cell Signaling, Bioconcept, Allschwill, Switzerland, #3407), anti-phospho(Ser636)-IRS1 (1:500, Santa Cruz, Santa Cruz, CA, USA, #sc-33,957), anti-phospho(Tyr632)-IRS1 (1:500, Santa Cruz, Santa Cruz, CA, USA, #sc-17,196), anti-PTEN (1:1000, Santa Cruz, Santa Cruz, CA, USA, #sc-7974), anti-phospho (Ser380/Thr382/383)-PTEN (1:500, Santa Cruz, Santa Cruz, CA, USA, #sc-101,789), anti-GSK3β (1:1000, Santa Cruz, Santa Cruz, CA, USA, #sc-377,213), anti-phospho(Ser9)-GSK3β (1:500, Santa Cruz, Santa Cruz, CA, USA, #sc-373,800), anti-phospho(Tyr216)-GSK3β (1:500, Santa Cruz, Santa Cruz, CA, USA, #sc-135,653), anti-GLUT4 (1:1000, Santa Cruz, Santa Cruz, CA, USA, #sc-53,566), anti-AS160 (1:1000, Thermo Fischer, Waltham, MA, USA), anti-phospho(Thr642)-AS160 (1:1000, GeneTex, Irvine, CA, USA), anti-PKCζ (1:1000, Santa Cruz, Santa Cruz, CA, USA, #sc-393,218), anti-hexokinase II (1:1000, Santa Cruz, Santa Cruz, CA, USA, #sc-130,358), anti-APP (1:10000, Sigma-Aldrich, St Louis, MO, USA, #A8717), anti-TNFα (1:1000, EMD Millipore, Billerica, MA, USA, #AB1837P), anti-syntaxin (1:1000, Genetex, Irvine, CA, USA, #GTX113559), anti-PSD95 (1:1000, abcam, Cambridge, United Kingdom, #ab18258). For analyses of mitochondrial complexes, a cocktail of anitbodies against each subunit was used (1:3000, abcam, Cambridge, United Kingdom, #ab110411). After 3 washes with Tween-20/Tris buffered saline (TTBS) buffer the membranes were incubated for 60 min at room temperature with anti-rabbit/mouse/goat IgG secondary antibody conjugated with horseradish peroxidase (1:5000; Sigma–Aldrich, St Louis, MO, USA). Membranes were developed with Clarity enhanced chemiluminescence (ECL) substrate (Bio-Rad Laboratories, #1705061) and then acquired with Chemi-Doc MP (Bio-Rad, Hercules, CA, USA) and analyzed using Image Lab software (Bio-Rad, Hercules, CA, USA) that permits the normalization of a specific protein signal with the β-actin signal in the same lane or total proteins load.

2.4. Statistical analyses

Data were calculated as a percentage of Ctr Y and are expressed as mean ± SEM. Data were first tested for equal variance and normality (Shapiro–Wilk test). Data were then analyzed by two-way analyses of variance (ANOVA) with diagnostic group (DS vs Ctr) and age (Old vs Young) as between-subject factors. Tukey's honestly significant difference (HSD) test was used for multiple *post-hoc* comparisons when required. All statistical tests were two-tailed and the level of significance was set at 0.05. Correlation analyses were calculated by Pearson's rank correlations All the analyses were performed using GraphPad Prism 7.0 software.

3. Results

3.1. Dysregulation of brain insulin signaling occurs early in DS

Changes of (i) levels and (ii) activation/inhibition state of proteins belonging to the insulin signaling pathway were evaluated in the frontal cortex of DS subjects before (DS, age < 40y) and after the development of AD pathology (DSAD, age > 40y) in comparison with age-matched controls (Ctr Y and Ctr O) (Table 1). No significant changes for IR protein levels and activation (pIR/IR) were observed among the 4 groups (Fig. 1 A-C). Notwithstanding the lack of statistical significance, IR protein levels showed a trend to be reduced in the younger DS without AD cases (−50% vs Ctr Y, Fig. 1B), as previously reported in AD (Barone et al., 2019; Batista et al., 2018; Bomfim et al., 2012; Frolich et al., 1998; Zhao et al., 2008). However, a significantly increased inhibition of IRS1 (evaluated as ratio between the two main inhibition/activation phosphorylation sites, Ser636/Tyr632) both in DS (+176%) and DSAD (+168%) subjects with respect to age-matched controls (Fig. 1A,D), was observed. Increased IRS1 inhibition in DS is mainly driven by increased Ser636 phosphorylation, since no significant changes were observed for total IRS1 protein levels or IRS1 activation (Tyr632 phosphorylation) (Supplementary Fig. 1A-C). To detect age and diagnostic group effects on changes in IR and IRS1 in the frontal cortex, a 2-way ANOVA analysis was used. IR protein levels showed a significant effect of age *x* diagnostic group interaction [$F(1,26) = 9.79$, $p = .004$, Table 2]. A linear regression analysis was used to evaluate age-associated changes in Ctr and DS populations and showed that IR protein levels were significantly decreased with age in Ctr subjects (Supplementary Fig. 1D), suggesting that observed losses of IR protein in DS (Fig. 1A,B) likely recapitulate age-associated defects. Furthermore, a deeper analysis performed by testing the association between IR levels and age in the 4 sub-groups of subjects (Ctr Y, DS, DSAD, Ctr O) showed that IR protein levels significantly decreased with age in younger DS (Fig. 1E). Similar analyses performed with regard to IR activation (pIR/IR vs age) showed no significant association (Supplementary Fig. 1E,F). With regard to IRS1 inhibition (Ser636/Tyr632), 2-way ANOVA analysis revealed a main effect of diagnostic group [$F(1,26) = 25.32$, $p < .0001$, Table 2]. A linear regression analysis showed no significant association between IRS1 inhibition and age either in Ctr and DS populations or in the 4 sub-groups of subjects (Supplementary Fig. 1G,H).

Downstream from IRS1, we previously reported the hyperactivation of phosphatydilinositol-3 kinase/protein kinase B (PI3K/Akt) axis in the same autopsy cases used in the current study (Perluigi et al., 2014).

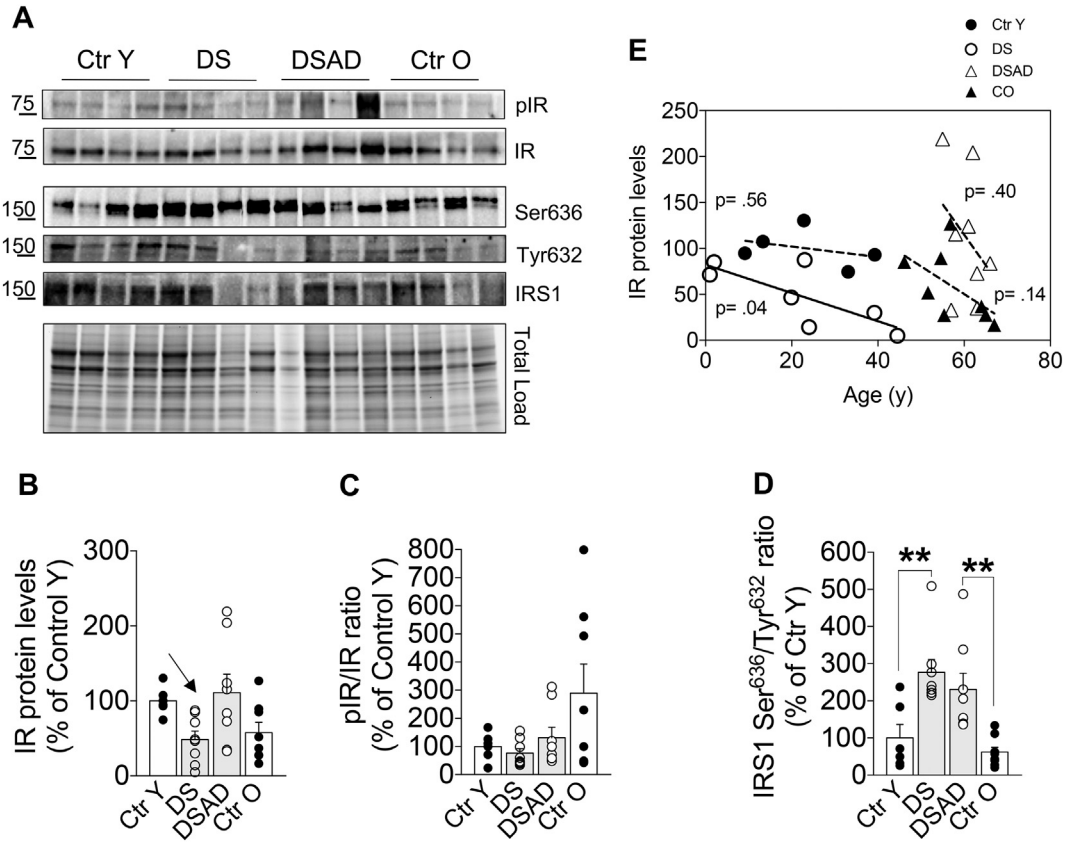

Fig. 1. Loss of IR along with increased IRS1 inhibition in DS brain. Changes of IR protein levels and activation as well as IRS1 inhibition were evaluated in the frontal cortex of DS individuals before (< 40 y, $n = 8$) and after the development of AD pathology (DSAD, > 40 y, n = 8) with respect to their age-matched controls [Ctr Y ($n = 6$) and Ctr O (n = 8)]. **(A)** Representative western blot images and densitometric evaluation of **(B)** IR protein levels, **(C)** IR activation (pIR/IR) and **(D)** IRS1 inhibition (IRS1 Ser636/Tyr632 ratio). Protein levels were normalized per total protein load. IR-, and IRS1-associated phosphorylations were normalized by taking into account the respective protein levels and are expressed as the ratio between the phosphorylated form and the total protein levels: Tyr1158/1162/1163 / IR and (Ser636/IRS1) / (Tyr632/IRS1). All densitometric values are given as percentage of Ctr Y set as 100%. Data are presented as means ± SEM, $^{**}p < .01$ (Two-way ANOVA with Bonferroni *post-hoc* test). **(E)** Linear regression analysis performed by evaluating changes of IR protein levels with respect to age in the 4 subgroups of subjects.

Here, we extended these findings regarding alterations of brain insulin signaling in DS population by evaluating changes of phosphatase and tensin homolog (PTEN) and glycogen synthase-3 beta (GSK3β) proteins. PTEN is a phosphatase, that regulates the activation of the insulin signaling pathway by reducing the PI3K-derived phosphatidylinositol (3,4,5)-trisphosphate (PIP_3), that,in turn, promotes Akt activation (Chen et al., 2016; Knafo and Esteban, 2017). GSK3β is one of the main targets of Akt in response to insulin (Hermida et al., 2017), known to be involved in neuro-protective and pro-survival mechanisms (Chong et al., 2005). PTEN protein levels are reduced in DS with respect to Ctr Y (−45%, Fig. 2A,B), while the evaluation of PTEN inhibitory phosphorylation sites reveal that PTEN is consistently activated in DS population after the development of AD pathology (pPTEN/PTEN, −90% in DSAD vs DS, Fig. 2A,C). With regard to GSK3β, we found a nearly significant reduced protein levels in DS (−50%, $p = .06$) relative to age-matched controls (Fig. 2A,D). GSK3β activation [evaluated as ratio between the inhibitory and activation phosphorylation sites (Ser9/Tyr416 ratio)] appears to be decreased following the development of AD pathology in DS. Indeed, GSK3β is significantly inhibited in the DSAD group with respect to both DS (+240%) and Ctr O (+362%) (Fig. 2A,E). This findings results from increased GSK3β inhibitory phosphorylation (Ser9) levels since no differences for the activation phosphorylation site (Tyr416) levels were observed (Supplementary Figs. 2A-C). A two-way ANOVA analysis reveals a significant effect of age *x* diagnostic group interaction with regard to changes of PTEN protein levels [$F(1,26) = 4.65$, $p = .04$, Table 2], while PTEN inhibition appears to be affected by both age [$F(1,26) = 14.21$, $p < .001$] and age *x* diagnostic group interaction [$F(1,26) = 7.13$, $p = .01$] (Table 2). Reduced PTEN levels in DS seem to reproduce in advance aging-associated alterations considering that a significant negative association between PTEN and age was found in Ctr subjects (Fig. 2F). No significant association between PTEN inhibition (pPTEN/PTEN) and age either in Ctr and DS populations or in the 4 sub-groups of subjects was found (Supplementary Fig. 2D,E).

In contrast, changes of GSK3β levels [$F(1,26) = 12.24$, $p = .001$] and activation [$F(1,26) = 10.2$, $p = .003$] are primarily associated with DS (Table 2).

Together, these data may suggest that alterations of brain insulin signaling occurs early in DS with respect to neurotypical controls and before the development of AD pathology.

3.2. Altered brain insulin signaling is associated with defects in glucose uptake in DS

To understand whether observed alterations of brain insulin signaling may affect glucose uptake, we evaluated changes of the insulin-dependent glucose transporter-4 (GLUT4) together with changes of Akt substrate 160 (AS160) and atypical protein kinase C zeta (PKCζ), which are responsible for translocation of GLUT4-containing vesicles to the plasma membrane (Bogan, 2012; Kim et al., 2003; Manna and Jain, 2013). No significant changes for GLUT4 protein levels were observed among the 4 groups (Fig. 3A,B). Levels (Supplementary Fig. 2E) and

Table 2
Two-way analyses of variance (ANOVA) with diagnostic group (Ctr vs DS) and age (young vs old) as between-subject factors (n = 6/8 per group).

Parameter	Diagnostic group	Age	Interaction
IR	n.s.	n.s.	F(1,26) = 9.79, p = .004
pIR/IR	n.s.	n.s.	n.s.
IRS1	n.s.	n.s.	n.s.
Tyr632/IRS1	F(1,26) = 3.77, p = .06	n.s.	n.s.
Ser636/IRS1	F(1,25) = 26.8, p < .0001	F(1,25) = 4.25, p = .04	n.s.
Ser636/Tyr632	F(1,26) = 25.32, p < .0001	n.s.	n.s.
PTEN	n.s.	n.s.	F(1,26) = 4.65, p = .04
pPTEN/PTEN	n.s.	F(1,26) = 14.21, p < .001	F(1,26) = 7.13, p = .01
GSK3β	F(1,26) = 12.24, p = .001	n.s.	n.s.
Ser9/GSK3β	F(1,26) = 7.00, p = .01	n.s.	n.s.
Tyr416/GSK3β	n.s.	n.s.	n.s.
Ser9/Tyr416	F(1,26) = 10.2, p = .003	F(1,26) = 8.11, p = .008	n.s.
GLUT4	n.s.	n.s.	n.s.
AS160	n.s.	n.s.	n.s.
Thr642/AS160	n.s.	n.s.	n.s.
PKCζ	F(1,26) = 15.1, p = .0006	F(1,26) = 18.5, p = .0002	F(1,26) = 6.5, p = .01
HKII	F(1,25) = 5.51, p = .02	n.s.	n.s.
Complex II	F (1, 24) = 34.75, p < .0001	n.s.	F (1, 25) = 4.95, p = .03
Complex III	F (1, 25) = 18.83, p < .001	n.s.	F (1, 25) = 9.08, p < .01
Complex IV	F (1, 25) = 12.67, p < .01	n.s.	F (1, 25) = 10.8, p < .01
ATP synthase	F (1, 25) = 9.41, p < .01	n.s.	F (1, 25) = 8.15, p < .01
C83	n.s.	n.s.	F(1,25) = 5.72, p = .02
C99	n.s.	F(1,25) = 6.79, p = .01	n.s.
C99/C83	n.s.	n.s.	F(1,25) = 4.53, p = .04
TNFα	n.s.	F(1,26) = 8.48, p = .007	n.s.
Syntaxin	n.s.	F(1,23) = 11.44, p = .002	F(1,23) = 5.13, p = .03
PSD95	n.s.	F(1,23) = 5.63, p = .02	n.s.

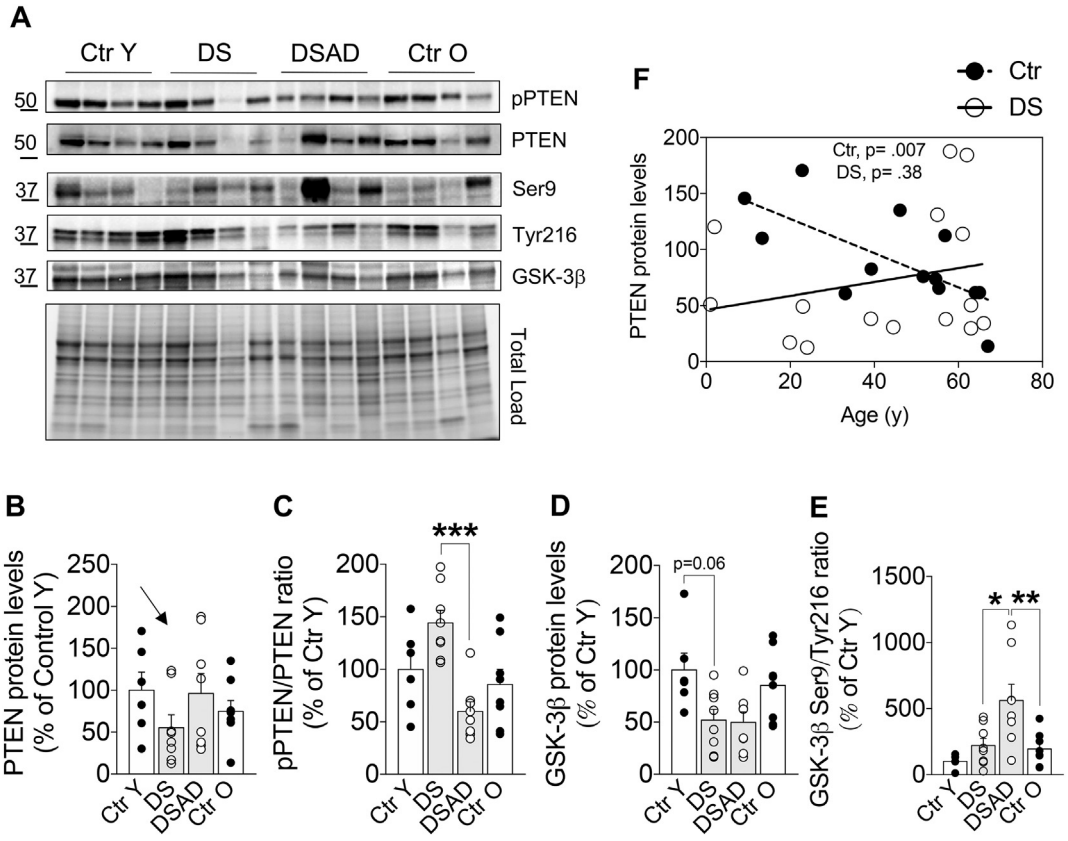

Fig. 2. Altered PTEN and GSK3β levels and activation in DS brain. Changes of PTEN and GSK3β protein levels and activation state were evaluated in the frontal cortex of DS individuals before (< 40 y, n = 8) and after the development of AD pathology (DSAD, > 40 y, n = 8) with respect to their age-matched controls [Ctr Y (n = 6) and Ctr O (n = 8)]. **(A)** Representative western blot images and densitometric evaluation of **(B)** PTEN protein levels, **(C)** PTEN inhibition (pPTEN/PTEN), **(D)** GSK3β protein levels and **(E)** GSK3β activation (GSK3β Ser9/Tyr216 ratio). Protein levels were normalized per total protein load. PTEN-, and GSK3β-associated phosphorylations were normalized by taking into account the respective protein levels and are expressed as the ratio between the phosphorylated form and the total protein levels: Ser380/Thr382/383 / PTEN and (Ser9/GSK3β) / (Tyr216/GSK3β). All densitometric values are given as percentage of Ctr Y set as 100%. Data are presented as means ± SEM, $*p < .05$, $**p < .01$ and $***p < .001$ (Two-way ANOVA with Bonferroni *post-hoc* test). **(F)** Linear regression analysis performed by evaluating changes of PTEN protein levels with respect to age in Control and DS populations.

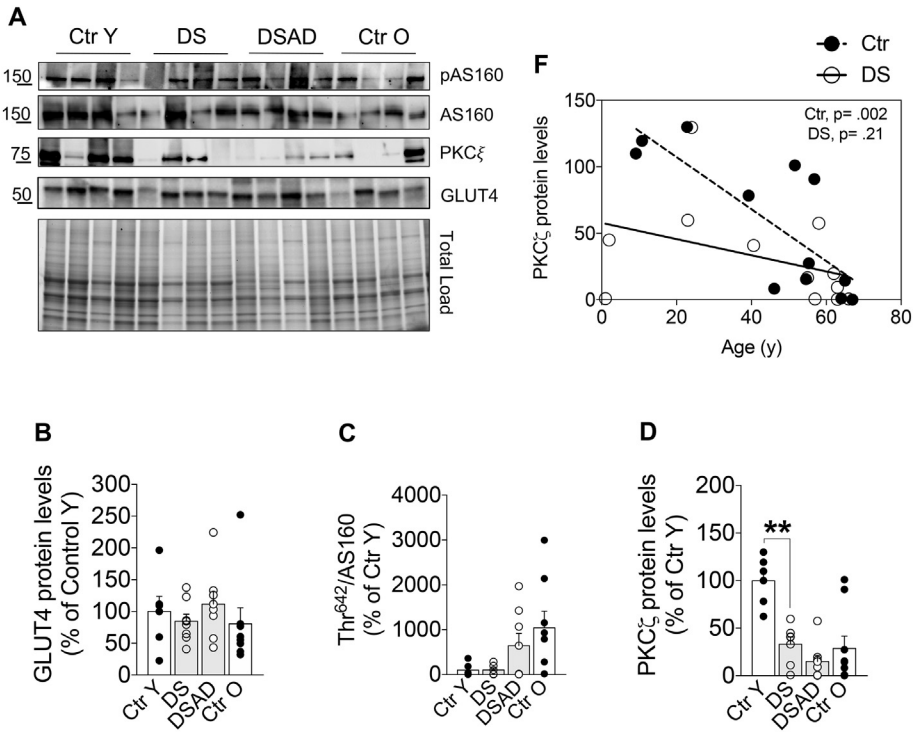

Fig. 3. DS brain shows alterations of proteins normally regulating glucose uptake and metabolism. Downstream from IR/IRS1 alterations of proteins normally favoring glucose uptake in response to insulin were evaluated in the frontal cortex of DS individuals before (< 40 y, n = 8) and after the development of AD pathology (DSAD, > 40 y, n = 8) with respect to their age-matched controls [Ctr Y (n = 6) and Ctr O (n = 8)]. **(A)** Representative western blot images and densitometric evaluation of **(B)** GLUT4 protein levels, **(C)** AS160 activation (Thr642/AS160) and **(D)** PKCζ protein levels. Protein levels were normalized per total protein load. AS160-associated phosphorylation was normalized by taking into account the respective protein levels and is expressed as the ratio between the phosphorylated form and the total protein levels: Thr642/AS160. All densitometric values are given as percentage of Ctr Y set as 100%. Data are presented as means ± SEM, *p < .05 and **p < .01 (Two-way ANOVA with Bonferroni *post-hoc* test). **(E)** Linear regression analysis performed by evaluating changes of PKCζ protein levels with respect to the age in Control and DS populations.

activation (Thr642/AS160, Fig. 3A,C) of AS160 were not different as well. In contrast, a significant reduction of PKCζ protein levels in DS (−70%) with respect to age-matched controls was observed (Fig. 3A,D). A linear regression analysis revealed a negative association between PKCζ and age in Ctr subjects (Fig. 3F), highlighting loss of PKCζ in DS as an early event in the aging process. When the same analysis was performed by considering the 4 sub-groups separately, we did not observe significant associations (Supplementary Fig. 2F).

We used a 2-way ANOVA analysis to determine whether age and/or diagnostic group affects PKCζ. We found that changes of PKCζ protein levels are dependent upon age [$F(1,26) = 18.5$, $p = .0002$], DS status [$F(1,26) = 15.1$, $p = .0006$] and their interaction [$F(1,26) = 6.5$, $p = .01$] (Table 2).

Overall, these data suggest that the DS brain is characterized by an impairment of insulin-mediated glucose uptake.

3.3. Altered brain insulin signaling is associated with defects in brain energy metabolism in DS

We hypothesized that defects of the insulin signaling pathway and glucose uptake were associated with an impaired brain energy metabolism. Thus, we evaluated changes of hexokinase-II (HKII) protein levels along with changes of mitochondrial complexes and ATP synthase levels. HKII is a rate-limiting enzyme in glucose metabolism, whose expression normally increases in response to insulin via Akt (Burcelin et al., 1993; Chehtane and Khaled, 2010; Culbert and Tavare, 2002; Duarte et al., 2008; Katzen, 1966; Katzen et al., 1970; Osawa et al., 1996; Printz et al., 1993). Our results show a significant reduction of HKII protein levels in DS with respect to Ctr Y (−53%, Fig. 3E). A two-way ANOVA analysis highlights that observed differences are mainly due to an effect of diagnostic group [$F(1,25) = 5.51$, $p = .02$, Table 2] with DS showing lower levels of HKII. With regard to mitochondria, we were able to detect measurable protein levels of all the complexes (II, III and IV) except complex I. We found reduced complex II levels in DS with respect to Ctr Y that were close to significance (−40%, $p = .07$, Fig. 4A,C). All the mitochondrial complexes were significantly reduced in the DSAD group relative to Ctr O (Complex II: −80%; Complex III: −130%; Complex IV: −260%) (Fig. 4A-E). Also, ATP synthase levels were significantly reduced in DSAD group with repsect to age-matched controls (−120%, Fig. 4A,F). A two-way ANOVA analysis highlights that differences primarily driven by lower levels being observed in DS as well as an interaction with age (Table 2).

Together, these observations highlight that the DS brain is characterized by an impairment of proteins involved in brain energy metabolism, which becomes consistent with the development of AD pathology.

3.4. Altered brain insulin signaling is associated with an increased amyloidogenic cleavage of APP and a rise of TNFa levels in DS

DS is characterized by the triplication of the *APP* gene that favors the amyloidogenic pathway in the brain (Lott and Head, 2019). In addition, an increase in amyloidogenic APP processing was associated with brain insulin resistance in AD (de la Monte, 2012; Rivera et al., 2005; Steen et al., 2005; Talbot et al., 2012; Triani et al., 2018). Therefore, we sought to evaluate whether a defective APP cleavage also characterizes our cohort of subjects. We evaluated APP C83 and C99 C-terminal fragments, which result from the cleavage of APP by alpha-secretase (non-amyloidogenic) and beta-secretase (amyloidogenic), respectively (Chen and Mobley, 2019).

Our results show no significant changes in the levels of C83 or C99 levels across the 4 groups of subjects (Fig. 5A-C). Examination of the percentage of C99 to C83 ratio, showed higher C99/C83 ratios in DS vs Ctr Y groups (+75%, $p = .08$, Fig. 5D) and in Ctr O vs Ctr Y groups (+120%, $p = .07$, Fig. 5D). A two-way ANOVA analysis revealed a main effect of age *x* diagnostic group interaction with regards to changes in the C99/C83 ratio [$F(1,25) = 4.53$, $p = .04$] (Table 2). A linear regression analysis shows that the percentage of the C99/C83 ratio increases with age in Control subjects ($p = .008$, Supplementary Fig. 3), suggesting again that the observed trend in DS could reflect defects associated with an aging process. Furthermore, when we looked at the 4 groups separately, the contribution of age appears to be primarily within the DS group in cases below the age of 40 years (Fig. 5E). When the C99/C83 ratio and age are analyzed in this subgroup of subjects there is a significant increase in the C99/C83 ratio with age ($p = .03$).

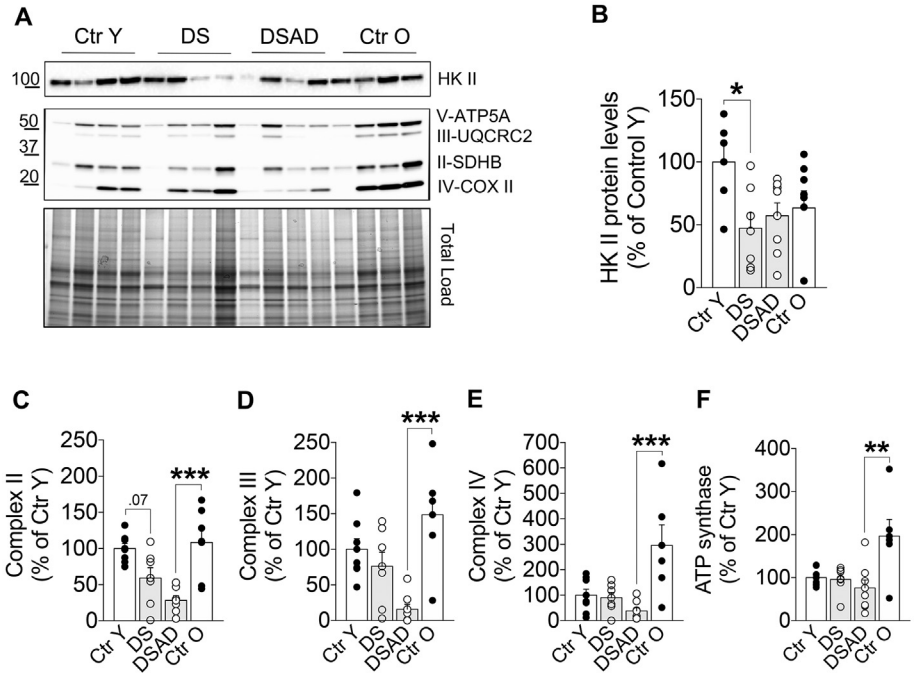

Fig. 4. DS brain shows early alterations of proteins regulating energy metabolism. Changes in levels of HKII and mitochondrial complexes were evaluated in the frontal cortex of DS individuals before (< 40 y, n = 8) and after the development of AD pathology (DSAD, > 40 y, n = 8) with respect to their age-matched controls [Ctr Y (n = 6) and Ctr O (n = 8)]. **(A)** Representative western blot images and densitometric evaluation of **(B)** HK II, **(C-E)** mitochondrial complexes and **(E)** ATP synthase protein levels. Protein levels were normalized per total protein load. All densitometric values are given as percentage of Ctr Y set as 100%. Data are presented as means ± SEM, *p < .05 and **p < .01 (Two-way ANOVA with Bonferroni *post-hoc* test).

Furthermore, levels of TNFα, a pro-inflammatory cytokine known to favor IRS1 phosphorylation on Ser636 (and thus inhibition) in AD (Bomfim et al., 2012; Lourenco et al., 2013) were evaluated. TNFα levels were significantly elevated in DS with respect to Ctr Y (+168%, Fig. 5F,G). A consistent elevation of TNFα also occurs in Ctr O vs Ctr Y (+680%, Fig. 5F,G). A two-way ANOVA showed a main effect of age [$F(1,26) = 8.48$, $p = .007$]. A linear regression analysis further highlights a significant association for increased TNFα levels and age in Ctr subjects (Fig. 5H).

3.5. Altered brain insulin signaling is associated with loss of synaptic proteins early in DS

Brain insulin resistance was reported to impair synaptic plasticity and thus favoring learning and memory impairment in AD (Arnold et al., 2018). To check whether alterations of brain insulin signaling also were associated with synaptic defects in DS, changes of two main synaptic proteins, i.e., syntaxin (pre-synaptic) and PSD95 (post-synaptic) were evaluated.

Our results show a significant reduction for syntaxin protein levels in Ctr O vs Ctr Y (Fig. 6A,B). DS cases show an average reduction of about 60% with respect to Ctr Y, which was nearly to significance ($p = .06$, Fig. 6A,B). No significant changes for PSD95 were observed across all groups (Fig. 6A,C). A two-way ANOVA analysis shows that changes observed for syntaxin were affected both by age [$F(1,23) = 11.44$, $p = .002$] and age *x* diagnostic group interaction [$F(1,23) = 5.13$, $p = .03$], while the overall differences observed for PSD95 are mainly due to an effect of age [$F(1,23) = 5.63$, $p = .02$] (Table 2). Syntaxin levels are reduced with age in Ctr subjects (Fig. 6D), further suggesting that lower syntaxin protein levels observed in DS represent an early defect.

Because most of the observed changes occur early in DS even before the development of AD pathology, we looked for any possible association between alterations of brain insulin signaling proteins and changes of syntaxin or PSD95 between Ctr Y and DS. As shown in Table 3, both syntaxin and PSD95 positively correlate with IR, IRS1, GSK3β, AS160 as well as proteins involved in brain energy metabolism (HKII, Complexes II-IV). In contrast, there were no significant correlations found with TNFα or APP C-terminal fragments (Table 3).

Hence, these results are suggestive of the hypothesis that alterations of brain insulin signaling are associated with loss of synaptic proteins early in DS.

4. Discussion

Brain insulin resistance may precede the appearance of AD neuropathology, thus contributing to the long preclinical period during which often only subtle clinical symptoms are evident (Arnold et al., 2018). In particular, insulin normally mediates fundamental actions in the brain ranging from the regulation of cellular metabolism to the promotion of synaptic plasticity involved in learning and memory functions (Arnold et al., 2018).

Our results highlight for the first time that markers of brain insulin resistance, i.e., reduced IR and increased IRS1 inhibition, are evident in DS brain even before the development of AD pathology. These data are particularly helpful to better understand the molecular mechanisms associated with intellectual disability, as well as the early onset of AD in people with DS (Lott and Head, 2019). Remarkably, similar to previous observations in AD (Talbot et al., 2012; Tramutola et al., 2015), DS brain shows at a young age an overall uncoupling of the members of insulin signaling, as indicated by the inhibition of IRS1 but increased activation of the PI3K/Akt axis (Perluigi et al., 2014) along with loss of Akt-mediated regulation of: (i) GSK3β inhibition (no changes); (ii) AS160 activation (no changes); (iii) PKCζ levels (reduced); and (iv) HKII (reduced) levels (Fig. 7).

Upstream in the pathway, reduction of IR levels in young DS was observed, that is further exacerbated as function of age. This event appears to be a feature of DS brain and might reflect ongoing neuropathological changes normally associated with aging and/or neurodegeneration. Indeed, similar changes were observed in Ctr subjects with age. Furthermore, reduced IR levels were previously reported to be associated with the development of AD neuropathology (Barone et al., 2019; Batista et al., 2018; Bomfim et al., 2012; Frolich et al., 1998; Zhao et al., 2008). Nevertheless, loss of IR is not evident in the brain of DS subjects who developed AD (DSAD group). Rather, we observed increased IRS1 inhibition both in DS and DSAD suggesting that brain insulin resistance develops in young DS and persists with the development of AD pathology. Intriguingly, in this study we strengthened the

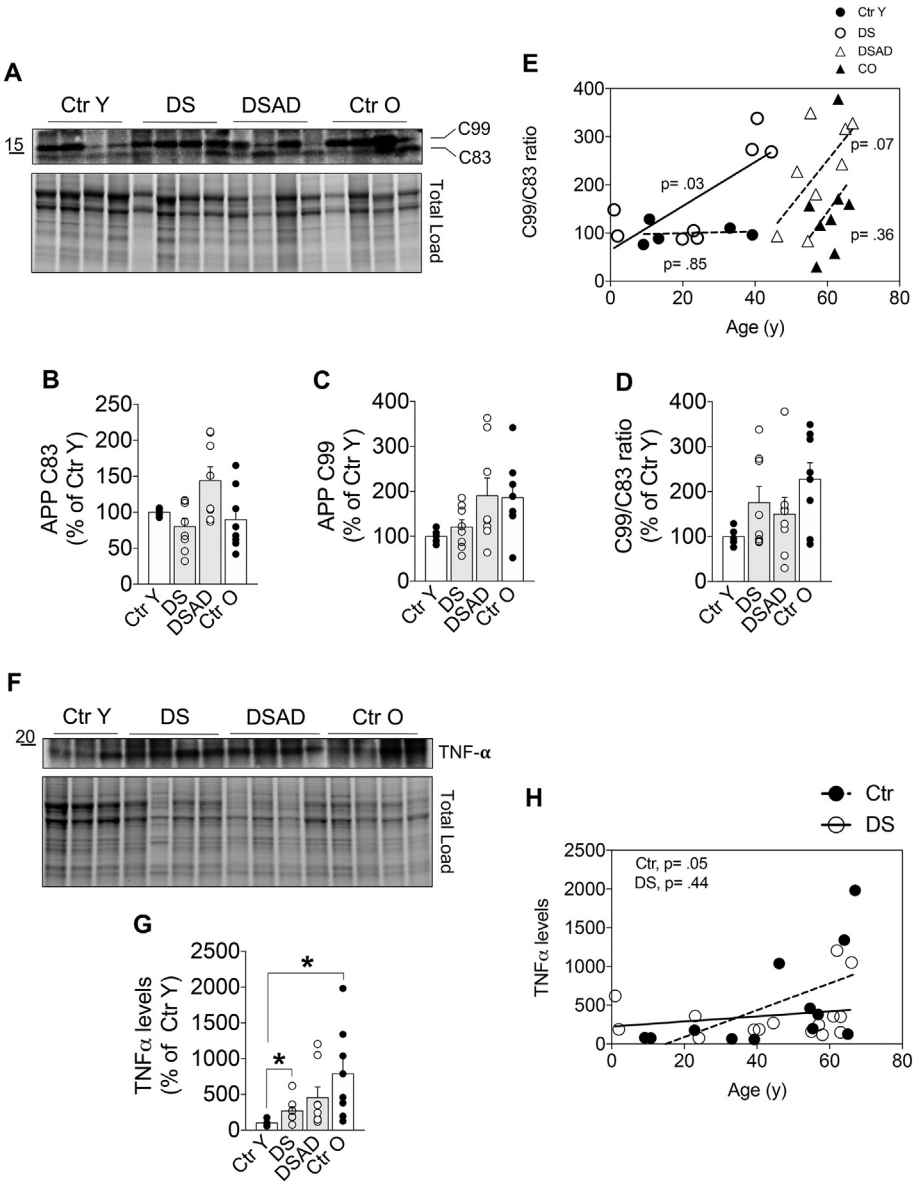

Fig. 5. Increased levels of APP cleavage products and TNFα in DS brain. APP C-terminal fragments (C83 and C99) and TNFα levels were evaluated in the frontal cortex of DS individuals before (< 40 y, n = 8) and after the development of AD pathology (DSAD, > 40 y, n = 8) with respect to their age-matched controls [Ctr Y (n = 6) and Ctr O (n = 8)]. **(A)** Representative western blot images and densitometric evaluation of **(B)** C83 (product from α-secretase-mediated cleavage, non-amyloidogenic), **(C)** C99 (product from β-secretase-mediated cleavage, amyloidogenic) and **(D)** C99/C83 ratio. **(E)** Linear regression analysis performed by evaluating changes of C99/C83 ratio with respect to the age in the 4 subgroups of subjects. **(F)** Representative western blot images and densitometric evaluation of **(G)** TNFα levels. **(H)** Linear regression analysis performed by evaluating changes of TNFα protein levels with respect to age in Control and DS populations. Protein levels were normalized per total protein load. All densitometric values are given as percentage of Ctr Y set as 100%. Data are presented as means ± SEM, *p < .05 (Two-way ANOVA with Bonferroni *post-hoc* test).

hypothesis that DS brain early shows signs of brain insulin resistance. Indeed, in a previous study from our group performed on the same cohort of subjects we observed increased IRS1 Ser307 phosphorylation both in DS and DSAD (Perluigi et al., 2014). Here, we looked at other two sites, specifically Tyr632 and Ser636, which, other than Ser307,are the most studied residues implicated in the activation and inhibition of IRS1, respectively (Copps and White, 2012). The reason is that IRS1 possesses multiple phosphorylation sites normally regulating its activation (in response to IR) or its inhibition (Copps and White, 2012) and therefore increasing the experimental observations about the phosphorylation state of these amino acidic residues could provide a better image of the activation or inhibition of IRS1. Our results clearly show that Ser636 phosphorylation significantly increases in DS and DSAD without consistent changes of Tyr632, thus confirming that IRS1 is inhibited in DS brain before and after the development of AD pathology.

Taking in mind that inhibition of IRS1 is responsible for the uncoupling between IR and IRS1, it is conceivable that brain cells in DS are unresponsive to the effects on insulin. Under physiological conditions insulin enhances neurite outgrowth, modulates catecholamine release and uptake, regulates trafficking of ligand-gated ion channels, regulates expression and localization of GABA, *N*-methyl-D-aspartate (NMDA) and α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA) receptors and modulates activity-dependent synaptic plasticity [that is, long-term potentiation (LTP) and long-term depression (LTD)] (Arnold et al., 2018; Spinelli et al., 2019). In addition, insulin has a crucial role in the development and maintenance of excitatory synapses and has been shown to promote dendritic spine formation and excitatory synapse development (Arnold et al., 2018; Spinelli et al., 2019). Finally, by inhibiting apoptosis insulin promotes neuronal survival (Arnold et al., 2018; Spinelli et al., 2019). Hence, we hypothesize that development of brain insulin resistance in DS impairs all these functions and contributes to the development of AD pathology.

From a molecular point of view, these processes are mediated by the PI3K/Akt axis downstream from IRS1 (Arnold et al., 2018; Butterfield et al., 2014a; Haeusler et al., 2018). An intriguing aspect is that despite increased IRS1 inhibition, a hyperactivation of the PI3K/Akt axis was observed in the same DS autopsy cases (Perluigi et al., 2014), further supporting the hypothesis of an uncoupling among the proteins of the insulin signaling. Results from the current study might provide novel insights to explain this phenomenon. Specifically, we found reduced activation of PTEN in young DS, as a result of both reduced protein

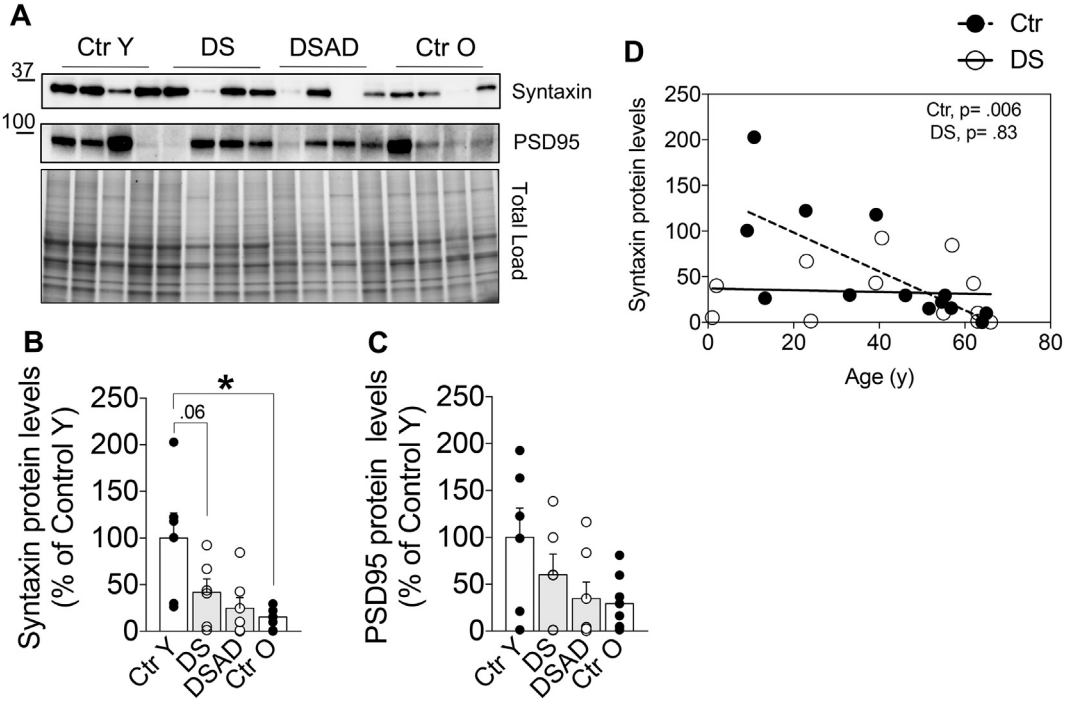

Fig. 6. Loss of synaptic proteins DS brain. Changes of syntaxin (pre-synaptic) and PSD95 (post-synaptic) protein levels were evaluated in the frontal cortex of DS individuals before (< 40 y, n = 6) and after the development of AD pathology (DSAD, > 40 y, *n* = 7) with respect to their age-matched controls [Ctr Y (n = 6) and Ctr O (n = 8)]. **(A)** Representative western blot images and densitometric evaluation of **(B)** syntaxin and **(C)** PSD95. **(D)** Linear regression analysis performed by evaluating changes of syntaxin protein levels with respect to age in Control and DS populations. Protein levels were normalized per total protein load. All densitometric values are given as percentage of Ctr Y set as 100%. Data are presented as means ± SEM, *p < .05 (Two-way ANOVA with Bonferroni *post-hoc* test).

Table 3
Significant Pearson correlations between syntaxin or PSD95 protein levels and proteins of the insulin signaling pathway found in young subjects (Ctr Y and DS).

Protein	Syntaxin		PSD95	
	r =	p =	r =	p =
IR	0.66	0.03	0.61	0.05
IRS1	0.58	0.06	0.61	0.04
GSK3β	0.67	0.02	0.68	0.02
AS160	0.80	0.003	0.81	0.002
HKII	0.66	0.03	0.60	0.06
Complex II	0.83	0.003	0.74	0.01
Complex III	0.70	0.02	0.72	0.01
Complex IV	0.72	0.01	0.78	0.007
TNFα	0.33	0.41	0.12	0.77
C83	0.06	0.84	0.05	0.88
C99	0.09	0.78	0.13	0.70
C99/C83	0.09	0.79	0.13	0.70

levels and increased inhibition, that agree with the observed hyper-activation of Akt, likely due to an accumulation of PIP3. Conversely, in DSAD subjects we observed increased PTEN activation along with sustained Akt activation, which may be the result of chronically impaired pathways in DSAD (Lott and Head, 2019). Observed alterations for PTEN agree with its role in synaptic plasticity; indeed, phosphorylation of a cluster of Ser/Thr residues (amino acids 380–385) on the C-terminal tail serves to alter the conformational state of PTEN from an open active state to a closed inhibited state. In addition to inhibiting PTEN phosphatase activity, phosphorylation also precludes the exposure of the PDZ domain (localized in the C-terminal fragment), through which PTEN interacts with a number of proteins involved in synaptic plasticity mechanisms (Chen et al., 2016; Knafo and Esteban, 2017; Vazquez et al., 2001). In particular, it was reported that PTEN interacts with and regulates the NMDA receptor during LTD (Jurado et al., 2010; Knafo et al., 2017). Paradoxically, Aβ triggers a PDZ-dependent recruitment of PTEN (meaning reduced PTEN phosphorylation) into the postsynaptic compartment along with a sustained LTD, ultimately resulting in impaired synaptic plasticity processes in AD (Jurado et al., 2010; Knafo et al., 2017). In light of these considerations and noting the imbalance between LTP and LTD previously observed in animal models of DS (Roncace et al., 2017; Schulz et al., 2019; Tovar et al., 2018), we hypothesize that loss of PTEN in young DS and hyperactivation of PTEN (less phosphorylated) in DSAD, both contribute to synaptic defects observed in DS.

The hypothesized uncoupling is further evident when we examined GSK3β. Hyper-active Akt is not associated with a significant inhibition of GSK3β in young DS, while the opposite is found in DSAD. These observations are in line with our previous data collected in post-mortem brain from mild cognitive impairment (MCI) and AD subjects, in which we identified an early phase characterized by the increased activation of GSK3β independently from Akt (in MCI), followed by a late phase when the inhibition of GSK3β was prominent and was associated with increased Akt activation (in AD) (Sharma et al., 2019; Tramutola et al., 2015). In both cases, the impairment of GSK3β parallels increased Tau phosphorylation (Griffin et al., 2005; Medina et al., 2011; Nicolia et al., 2017; Pei et al., 1999; Pei et al., 2003; Sharma et al., 2019; Tramutola et al., 2015; Yarchoan et al., 2014). Furthermore, defects in GSK3β activation were reported to alter neuronal survival and synaptic plasticity mechanisms, thus contributing to the development of AD neuropathology (Aceto et al., 2019; Anderton et al., 2001; Arnold et al., 2018; Mandelkow et al., 1992; Pei et al., 1999; van der Heide et al., 2005). Interestingly, early Tau pathological changes were observed in DS (Hamlett et al., 2017; Head et al., 2003), with subsequent findings of aggregated Tau into neurofibrillary tangles (NTFs) (Hof et al., 1995; Hyman et al., 1995; Mann and Esiri, 1989; Mann et al., 1990), which show a higher density with respect to AD (Hof et al., 1995; Lott and Head, 2019). A pivotal role for dual-specificity tyrosine-phosphorylation regulated kinase 1A (DYRK1A) and RCAN proteins in favoring Tau

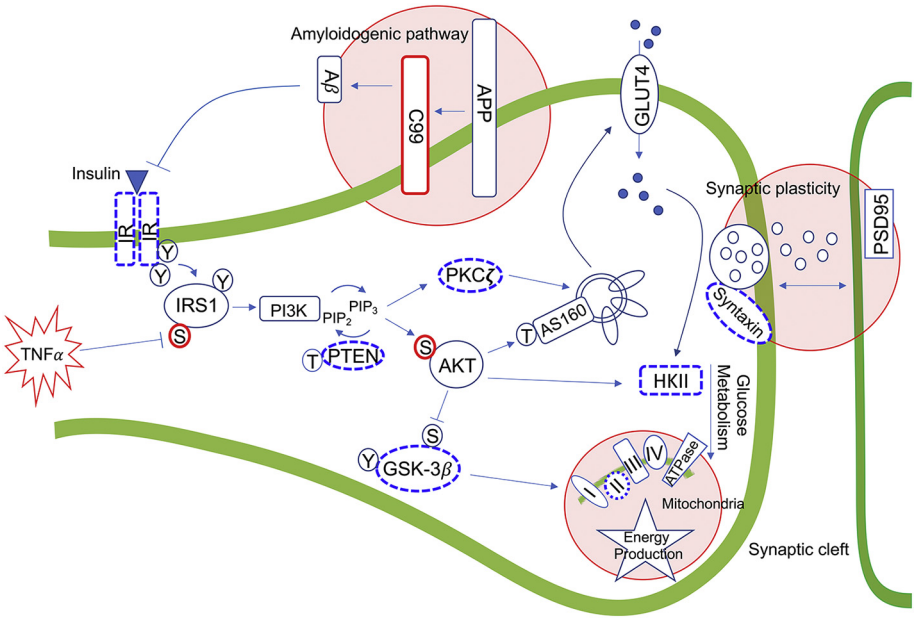

Fig. 7. Schematic representation of insulin signaling pathway with highlighted proteins found to be early altered in DS. Under physiological conditions, the activation of insulin signaling requires the binding of insulin to the insulin receptor (IR), which autophosphorylates on Tyr residues (e.g., Tyr1158/1162/1163) and promotes the receptor tyrosine kinase-mediated phosphorylation of its substrate (IRS1) on specific Tyr residues (e.g. 632). Once activated, IRS1 works as a scaffold protein, driving the activation of the PI3K/Akt axis, that is critical for linking upstream effectors (IR and IRS1) with downstream proteins mediating insulin neurotrophic outcomes. Activation of the PI3K/Akt axis is regulated by the phosphatase PTEN, which reduces PIP3 levels required for Akt activation as well as for increasing the expression of PKCζ. Akt promotes the phosphorylation of several targets among which are: (1) GSK3β (on Ser9, inhibitory site) and (2) AS160 (on Thr642, activating site). This latter, together with PKCζ, are responsible for the translocation of GLUT4-containing vesicles to the plasma membrane to mediate glucose uptake. Furthermore, Akt stimulates the upregulation of HKII, which is a pivotal enzyme involved in glucose metabolism and thus energy production. During the development of brain insulin resistance, a dysregulation of a number of these proteins was observed. In particular, the brain insulin resistance phenomenon shows key markers such as reduced IR protein levels and/or increased IRS1 inhibitory phosphorylation levels (e.g., Ser636), that are responsible for the uncoupling between IR and IRS1. As a result, despite that insulin binds to IR, reduced activation of its downstream effectors was found. TNFα was reported to favors IRS1 inhibition. Moreover, brain insulin resistance was associated with an increased amyloidogenic pathway, with alterations of proteins associated with synaptic plasticity and with reduced energy metabolism, that taken together contribute to the development of AD pathology (highlighted in red circles). For proteins analyzed in the current and previous study (Perluigi et al., 2014): blue dotted lines (reduced levels); red lines (increased levels). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

phosphorylation in DS was reported (Lott and Head, 2019). Our findings further support a role for impaired brain insulin signaling, which could favor increased Tau phosphorylation through GSK3β early in DS brain. As we previously reported in the same autopsy cases used in the current work (Perluigi et al., 2014), increased Tau Ser404 phosphorylation [specific target for GSK3β activity (Hanger and Noble, 2011; Leroy et al., 2010)] in young DS and DSAD subjects was evident.

Altered brain insulin signaling also impairs glucose uptake in DS. In particular, our data suggest the existence of defects in the mechanisms responsible for GLUT4 translocation to the plasma membrane. Although most glucose uptake in neurons occurs via GLUT3 (Duelli and Kuschinsky, 2001), insulin-regulated GLUT4 is also co-expressed with GLUT3 in brain regions related to cognitive behavior (Apelt et al., 1999). Activation by insulin induces GLUT4 translocation to the neuronal cell membrane via an Akt-dependent mechanism (Grillo et al., 2009; McEwen and Reagan, 2004) and is thought to improve glucose flux into neurons during periods of high metabolic demand, such as learning (Pearson-Leary and McNay, 2016). We found that both AS160 and PKCζ - that normally regulate translocation of GLUT4-containing vesicles to the plasma membrane (Bogan, 2012; Kim et al., 2003; Manna and Jain, 2013) – do not correlate with increased Akt activation in DS. We found reduced PKCζ protein levels early in DS. This is an intriguing result because in addition to mediating GLUT4 translocation in response to insulin, PKCζ also contributes to the maintenance of LTP in neurons thus maintaining long-term memory (Sacktor and Hell, 2017). This occurs mainly through a PKCζ cleavage products known as PKMζ, which is autonomously active because it lacks the autoinhibitory pseudosubstrate in the ζ regulatory domain (Hernandez et al., 2003; Sacktor and Hell, 2017; Sacktor et al., 1993). Therefore, significantly reduced PKCζ protein levels early in DS could impair neuronal functions through both defects in glucose uptake as well as directly at the levels of the synaptic cleft.

Reduced glucose utilization mainly results from defects of the glycolytic pathway and mitochondrial respiration. Our results show reduced HKII protein levels in young DS, that coupled with impaired GLUT4 translocation, may strengthen the role for metabolic defects in the development of AD-like neurodegeneration in DS. HKII is a constitutively active kinase, which possesses pro-survival effects and whose expression undergoes dynamic changes in various diseases (Roberts and Miyamoto, 2015). Activation of the Akt/mTOR axis in response to insulin was demonstrated to increase HKII protein levels (Burcelin et al., 1993; Chehtane and Khaled, 2010; Culbert and Tavare, 2002; Duarte et al., 2008; Katzen, 1966; Katzen et al., 1970; Osawa et al., 1996; Printz et al., 1993). Hence, our current and previous results show reduced HKII protein levels in spite of the hyper-activation of the Akt/mTOR axis early in DS, further supporting the idea of a general uncoupling between the insulin signaling pathway and energy metabolism in DS. The reduced glycolytic flux is also coupled with reduction of protein levels of respiratory chain complexes, which suggest reduced oxidative metabolism in the mitochondria in DSAD. These results are in agreement with our previous studies showing by redox proteomics the irreversible oxidative modification of enzymes involved in energy metabolism (i.e. glyceraldehyde 3-phosphate dehydrogenase (GAPDH), enolase, malate dehydrogenase, creatine kinase, ATP synthase), that likely impairs their activity, both in DS and AD post mortem brain (Barone et al., 2017; Butterfield et al., 2014b).

Moreover, alterations of brain insulin signaling in DS parallel increased APP cleavage and TNFα levels, found to favor the development of brain insulin resistance in AD (Bomfim et al., 2012; De Felice et al., 2009; de la Monte, 2012; Lourenco et al., 2013; Rivera et al., 2005; Steen et al., 2005; Talbot et al., 2012). With regard to the accumulation of AD neuropathological hallmarks in DS, defects of autophagy process were reported to have a role (Ahmed et al., 2013; Bordi et al., 2019; Colacurcio et al., 2018; Perluigi et al., 2014). Our group showed the hyper-activation of the PI3K/Akt axis along with increased mTOR activation and reduced LC3 II/I levels in the frontal cortex of both DS and DSAD subjects (Perluigi et al., 2014). In addition, we found that soluble and insoluble Aβ peptide and oligomers increase as a function of age in the brain of DS individuals (Cenini et al., 2012). Similar observations were obtained for p-Tau (Perluigi et al., 2014). All the above-mentioned

analyses were performed in the same autopsy cases used in the current study, thus highlighting that brain insulin resistance is associated with defects of autophagic flux that finally result in the accumulation of AD neuropathological hallmarks early in DS. In support of this hypothesis, we found that intranasal administration of rapamycin (a well-known inhibitor of mTOR) in a mouse model of DS rescue mTOR hyper-activation and reduces APP cleavage products in the brain (Di Domenico et al., 2019; Tramutola et al., 2018).

As reported in AD, increased Aβ levels and defects of brain insulin signaling show a mutual interaction. In particular, it was proposed that, as in a vicious cycle, brain insulin resistance favors the production and accumulation of Aβ (de la Monte, 2012; Rivera et al., 2005; Steen et al., 2005; Talbot et al., 2012; Triani et al., 2018), whereby increased Aβ generation further exacerbates brain insulin resistance through different mechanisms including either reduced IR levels or increased TNFα-mediated inhibition of IRS1 (Bomfim et al., 2012; De Felice et al., 2009; Lourenco et al., 2013). Our findings agree with this paradigm since DS brain is characterized by a significant cleavage of APP and by a pro-inflammatory state (Lott and Head, 2019; Nistor et al., 2007; Wilcock et al., 2015), which could contribute to the observed reduced IR levels and increased IRS1 inhibition, respectively. However, we cannot precisely determine "*who does what*". Notwithstanding this concern, the present collected data provide for the first time evidence that alterations of brain insulin signaling parallel the accumulation of AD neuropathological hallmarks in DS and that this is already evident in young DS subjects.

Finally, we acknowledge that all the above reported alterations are associated with loss of synaptic proteins, mainly syntaxin, which normally regulates neurotransmitter-containing vesicles exocytosis at the pre-synaptic level (Vardar et al., 2016). Loss of syntaxin severely compromised neuronal viability in vivo and in vitro, indicating an obligatory role of syntaxin for maintenance of developing and mature neurons (Vardar et al., 2016). This aspect appears of interest due to the observation that DS brain morphology continue to diverge compared with typically developing individuals (Lott, 2012). In particular, cortical layer thickness, dendritic branching, synapse formation, brain size and overall brain weight are all reduced (Aziz et al., 2018). Further, as explained above insulin signaling contributes to the maintenance of synapses (Arnold et al., 2018), thereby supporting the notion that defects of brain insulin signaling likely contribute to synaptic defects observed in DS subjects.

In conclusion, data reported in the current paper highlight that DS subjects show signs of brain insulin resistance early in life. These alterations resemble those previously described for subjects developing AD supporting a role for alterations of brain insulin signaling pathway in the development of AD in DS (Fig. 7).

Author contributions

EB and MP designed the study. EH provided post-mortem human brain samples. AT and CL performed the experiments. EB and FDD performed the statistical analyses. EB and FDD analyzed the data. EB, MP, EH and DAB interpreted the data. EB and MP drafted the manuscript. All authors revised the manuscript critically for important intellectual content and approved the final version.

Declaration of Competing Interest

The authors declare no conflicts of interest exist.

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Appendix A. Supplementary data

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