Dysregulation of Insulin-Linked Metabolic Pathways in Alzheimer’s Disease: Co-Factor Role of Apolipoprotein E ε4

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Abstract.
Background: Brain insulin resistance and deficiency are well-recognized abnormalities in Alzheimer’s disease (AD) and likely mediators of impaired energy metabolism. Since apolipoprotein E (APOE) is a major risk factor for late-onset AD, it was of interest to examine its potential contribution to altered insulin-linked signaling networks in the brain.
Objective: The main goal was to evaluate the independent and interactive contributions of AD severity and APOE ε4 dose on brain expression of insulin-related polypeptides and inflammatory mediators of metabolic dysfunction.
Methods: Postmortem fresh frozen frontal lobe tissue from banked cases with known APOE genotypes and different AD Braak stages were used to measure insulin network polypeptide immunoreactivity with a commercial multiplex enzyme-linked immunosorbent assay (ELISA).
Results: Significant AD Braak stage and APOE genotype-related abnormalities in insulin, C-peptide, gastric inhibitory polypeptide (GIP), glucagon-like peptide-1 (GLP-1), leptin, ghrelin, glucagon, resistin, and plasminogen activator inhibitor-1 (PAI-1) were detected. The main factors inhibiting polypeptide expression and promoting neuro-inflammatory responses included AD Braak stage and APOE ε4/ε4 rather than ε3/ε4.
Conclusion: This study demonstrates an expanded role for impaired expression of insulin-related network polypeptides as well as neuroinflammatory mediators of brain insulin resistance in AD pathogenesis and progression. In addition, the findings show that APOE has independent and additive effects on these aberrations in brain polypeptide expression, but the impact is decidedly greater for APOE ε4/ε4 than ε3/ε4.

Keywords: Alzheimer’s disease, APOE, Braak stage, human brain, incretins, insulin resistance, leptin, multiplex ELISA, neurodegeneration, neuroinflammation

INTRODUCTION

Growing evidence supports the concept that Alzheimer’s disease (AD) is mechanistically linked to impairments in brain energy metabolism [1] marked...
by reduced glucose uptake and utilization from presymptomatic stages of disease [2]. Furthermore, in the prospective Baltimore Longitudinal Study, impairments in brain glucose uptake were correlated with reduced expression of the glucose transporter 3 (GLUT3) and subsequent development of AD [3]. Since glucose uptake and utilization in the brain and neuronal cells are stimulated by insulin [4–7], insulin deficiency or insulin resistance could dysregulate energy metabolism and contribute to the pathogenesis of AD [8–10]. Besides regulating energy metabolism, insulin stimulates working memory, cognition [11–14], and neuronal plasticity, and its receptors are abundantly expressed in brain regions that are most susceptible to AD-type neurodegeneration [15, 16]. Correspondingly, experimental inhibition of insulin-related signaling networks causes neurodegeneration with AD features [17–19]. Altogether, these findings point to dysregulation of insulin signaling networks as a fundamental mediator of neurodegeneration.

Human postmortem [9, 20] and clinical [10, 21, 22] studies have demonstrated brain insulin deficiency and insulin resistance in AD. Insulin deficiency is mainly manifested by reduced insulin levels in brain and cerebrospinal fluid (CSF), whereas insulin resistance is associated with reduced expression, tyrosine phosphorylation, and binding of the insulin receptor, activation of insulin receptor substrate downstream signaling through phosphoinositol-3-kinase (PI3K)-Akt pathways, and brain glucose levels [9, 16, 20, 23]. Disruption of these insulin-related networks adversely impacts neuronal survival, oligodendrocyte function, neuronal plasticity, and energy metabolism, and promotes neuro-inflammation, oxidative, nitrosative, and endoplasmic reticular stress, lipid peroxidation and cell death [8, 24–26]. In addition, impairments in brain insulin signaling have been linked to increased tau phosphorylation and amyloid-β 1–42 (Aβ1–42) accumulation/toxicity [20, 25, 27–29]. Therefore, apart from the specific AD-associated outcomes, the molecular, biochemical, and cytopathological consequences of insulin deficiency/resistance in the brain closely resemble those that occur in diabetes mellitus and other insulin resistance diseases [8, 30].

The steadily increasing prevalence of AD over the past several decades and across all age groups [31] indicates that factors other than genetics can mediate AD neurodegeneration. Furthermore, the parallel increases in rates of diabetes mellitus and other insulin resistant states, the higher rates of cognitive impairment and AD in people with obesity or type 2 diabetes mellitus [31], and the increased risk of developing mild cognitive impairment (MCI) or AD in non-obese, non-diabetic people with elevated blood glucose [32, 33] suggest the drivers and mechanisms of peripheral insulin resistance and AD may be shared. Another way to consider the problem is that perhaps insulin resistant disease states are fundamentally related but differentially manifested due to variation in tissues, organs and systems targeted. For example, atherosclerosis is a single pathologic process that causes different diseases based on compromised flow through specific arteries. If AD is truly one of the progressive insulin resistance/insulin deficiency diseases in which the brain is selective or prominently involved, then therapeutic interventions developed for other related diseases may be extendable to AD. Already this concept has some validity since cognitive impairment in MCI and AD are positively responsive to intranasal insulin, insulin sensitizers, incretins, and lifestyle modifications that enhance insulin responsiveness [8, 11, 14, 34–38]. Furthermore, intranasal insulin has been shown in humans to increase brain energy, including levels of ATP and phosphocreatine using 31-P magnetic resonance spectroscopy to assess cerebral energy metabolism [39]. To delve deeper into the overarching question concerning insulin network dysfunction versus insulin resistance/deficiency as mediators of neurodegeneration, in this study we measured broad indices of insulin-regulated metabolic integrity in postmortem frontal cortex samples from controls and AD human subjects. The primary objective was to assess the presence and characteristics of central nervous system (CNS) dysregulated metabolic networks.

This study examined the contributions of both Braak stage histopathological grade of AD and apolipoprotein E (APOE) genotype on frontal cortex expression of insulin-related polypeptides in postmortem human brains using a commercial multiplex gut hormone panel. Previously, we used this approach to demonstrate insulin-related metabolic abnormalities in CSF and serum from patients with MCI or AD [40–43]. Furthermore, this study extends earlier work characterizing AD grade and APOE genotype (ε3/ε3, ε3/ε4, ε4/ε4) effects on brain expression of insulin degrading enzyme (IDE) and regulator of calcineurin 1 (RCAN1) [44]. That study demonstrated AD severity and APOE ε4 dose-dependent reductions in IDE and increases in RCAN1 expression. Those findings are relevant to the present study because IDE is a 110 kD thiol zinc metalloendopeptidase that
### Table 1

| Polypeptide                | Functions                                                                                                                                 |
|---------------------------|------------------------------------------------------------------------------------------------------------------------------------------|
| Insulin                   | Reduces blood glucose; regulates metabolism by increasing cell permeability to monosaccharides, amino acids and fatty acids; accelerates the pentose phosphate cycle and glycogen synthesis in the liver. |
| C-peptide: Connecting peptide | Stable by-product of pro-insulin cleavage to generate insulin; mediates efficient assembly, folding, and processing of insulin in the ER.          |
| GIP1: Gastric inhibitory polypeptide | Potent stimulator of insulin secretion; stimulates lipoprotein lipase; modulates fatty acid metabolism; poor inhibitor of gastric acid secretion |
| GLP-1: Glucagon-like peptide-1 | Potent stimulator of glucose-dependent insulin release; stimulates glucose disposal, independent insulin actions; suppresses plasma glucagon; modulates gastric motility; may suppress satiety; promotes growth of intestinal epithelium. neuroprotective. |
| Leptin                    | Critical regulator of energy balance by inhibiting food intake and promoting energy expenditure; helps regulate fat depots.                     |
| Ghrelin                   | Ligand for growth hormone secretagogue receptor type 1; induces growth hormone release from the pituitary-regulates growth; stimulates appetite; induces adiposity; stimulates gastric acid secretion. |
| Glucagon                  | Regulates glucose metabolism and homeostasis by increasing gluconeogenesis and decreasing glycolysis and counterregulatory to insulin; raises plasma glucose in response to insulin-induced hypoglycemia; initiates and maintains hyperglycemic conditions in diabetes mellitus. |
| Resistin                  | Promotes insulin resistance; suppresses insulin-stimulated glucose uptake in adipocytes; potentially links obesity to diabetes; increases hepatic production of LDL and degradation of LDL receptors, increasing risk of cardiovascular disease; promotes cytokine inflammatory responses and DNA transcription. |
| PAI-1: Plasminogen activator inhibitor-1 | Serine protease inhibitor that acts as a ‘bait’ for tissue plasminogen activator, urokinase, protein C and matriptase-3/TMPRSS7; regulates fibrinolysis. |
| Visfatin                  | Increases insulin sensitivity; promotes cytokine activation. However, more typically known to regulate circadian clock functions, promotes B-cell maturation, and inhibits neutrophil apoptosis. |

degradates insulin and other small polypeptides including atrial natriuretic peptide, transforming growth factor-alpha, amylin, bradykinin, kallidin and Aβ, and RCAN1 inhibits calcineurin causing increased glycogen synthase kinase 3β activation with attendant hyperphosphorylation of tau and neurofibrillary tangle formation. The present work broadens the analysis of dysregulated brain metabolic networks and the co-factor role of APOE4 dose as mediators of neurodegeneration in the pathogenesis of AD.

**METHODS**

**Human subjects**

Human postmortem fresh frozen frontal cortex samples from Brodmann Area 8/9 were provided by the Duke Kathleen Price Bryan Brain Bank and Biorepository (Durham, NC). The standardized brain banking protocol ensures storage of high-quality tissue for molecular and biochemical analyses and systematic review by neuropathologists to assign diagnoses and disease stage. In addition, all cases were APOE genotyped [e3/e3, e3/e4, e4/e4] (https://neurology.duke.edu/research/research-centers/joseph-and-kathleen-bryan-alzheimers-disease-research-center/brain-bank). However, apart from standard demographics, case de-identification precludes detailed correlative analysis of clinical data in relation to research findings. For this project, we obtained 72 fresh frozen brain samples from men and women who were grouped based on their Braak stage scores for AD severity (Braak 0–2; B0–2 = normal aging; B3–4 = moderate AD; B5–6 = severe or advanced AD) and APOE genotypes. The Lifespan Hospitals Institutional Review Board (IRB) approved the use of human postmortem de-identified brains for this research.

*Multiplex human gut hormone enzyme-linked immunosorbent assay (ELISA)*

For these studies, we used a Human Gut Hormone 10-Plex ™ Assay (Bio-Rad, Hercules, CA), which is based on a magnetic bead-based format for simultaneously measuring immunoreactivity to insulin, C-peptide, gastric inhibitory polypeptide (GIP), glucagon-like peptide-1 (GLP-1), leptin, ghrelin, glucagon, resistin, plasminogen activator inhibitor-1 (PAI-1), and visfatin in tissue homogenates. This assay was utilized to investigate a fuller spectrum of potential polypeptide abnormalities that could contribute to impairments in energy balance within the CNS (Table 1). The assays were performed in accordance with the manufacturer’s protocol. In brief, captured antigens were detected with biotinylated secondary antibodies followed by a streptavidin-phycocerythrin reporter conjugate.
Fluorescence intensity was measured in a MAGPIX (Bio-Rad, Hercules, CA) and hormone concentrations (pg/mL) were determined from standard curves using MAGPIX software.

Statistics

Results are graphed using scatter plots to depict median (horizontal bars) and within-group and between-group variability. Inter-group statistical comparisons were made by repeated measures two-way ANOVA tests with 1% false discovery corrections and post-hoc Tukey tests (GraphPad Prism 8 software, San Diego, CA). Statistical significance was defined as $p < 0.05$. Statistical trend was defined as $0.05 < p < 0.10$.

RESULTS

Study groups

Among the 72 cases evaluated, 38 were genotyped as APOE $\varepsilon3/e3$, 25 as APOE $\varepsilon3/e4$, and 9 as APOE $\varepsilon4/e4$ (Table 2). The gender distributions were balanced across APOE genotypes, except for APOE $\varepsilon3/e4$ which had 30% more males than females with B3–4 AD, and 2.5 times as many females as males with B5–6 AD. Mean ages ranged from 72 to 84 years within each subgroup. There were no significant differences in mean age among the sub-groups, except for APOE $\varepsilon3/e4$, B0–2 controls which were significantly younger than the corresponding AD groups ($p < 0.05$), but not APOE $\varepsilon3/e3$ controls. None of the B0–2 controls had an APOE $\varepsilon4/e4$ genotype. Post-hoc inter-group statistical comparisons made with respect to APOE $\varepsilon3/e3$ B0–2 controls are depicted in the figures, while all inter-group significant differences and trends are listed in Tables 3–11.

Insulin

Insulin promotes glucose uptake into cells, decreasing its concentrations in peripheral blood (Table 1). Two-way ANOVA revealed significant effects of Braak stage AD severity ($F = 8.78; p = 0.0007$), APOE genotype ($F = 7.27; p = 0.002$), and interactions between AD severity and APOE ($F = 2.83; p = 0.036$). Post hoc multiple comparison tests revealed significant reductions in frontal cortex insulin immunoreactivity in B5–6 APOE $\varepsilon3/e3$, and both B3–4 and B5–6 APOE $\varepsilon4/e4$ relative to B0–2 APOE $\varepsilon3/e3$ (Fig. 1A) and B0–2 APOE $\varepsilon3/e4$ controls (Table 3). In addition, post hoc testing revealed significant inhibitory effects of APOE $\varepsilon4$ dose and AD severity on brain insulin expression, and within B5–6, APOE dose-dependent inhibition of insulin expression (Table 3).

C-peptide

C-peptide is a component of pro-insulin that is co-generated with insulin upon cleavage of the precursor protein (Table 1). Due to its stability, C-peptide is often used to gauge insulin concentration and insulin resistance over time along with current glucose levels in peripheral blood. Two-way ANOVA demonstrated significant effects of APOE genotype ($F = 3.76; p = 0.03$) and a trend effect for AD severity ($F = 2.76; p = 0.07$) on C-peptide expression. Post hoc multiple comparisons testing demonstrated trend reductions in C-peptide in B5–6 APOE $\varepsilon3/e3$ relative to corresponding controls (Table 4), and significant reductions in C-peptide in both B3–4 and B5–6 APOE $\varepsilon4/e4$ relative to B0–2 APOE $\varepsilon3/e3$ (Fig. 1B), and B3–4 APOE $\varepsilon4/e4$ versus B3–4 APOE $\varepsilon3/e4$, i.e., an APOE dose effect (Fig. 1B and Table 4). These findings partially mimicked the insulin responses and demonstrated that APOE $\varepsilon4$ dose and Braak stage severity of AD had significant inhibitory effects on C-peptide expression in the brain.

Gastric inhibitory peptide 1 (GIP-1)

GIP-1 is an incretin with potent stimulatory effects on insulin secretion and a modulator of fatty acid metabolism (Table 1). Two-way ANOVA revealed significant effects of AD Braak stage ($F = 8.0$;
Table 3
INSULIN: Two-way ANOVA Results

| Control | APOE | Braak Stage | APOE AD | p     |
|---------|------|-------------|---------|-------|
| B0–2    | e3/e3| B5–6        | e3/e3   | 0.01  |
| B0–2    | e3/e4| B3–4        | e4/e4   | 0.004 |
| B0–2    | e3/e4| B5–6        | e3/e3   | 0.01  |
| B0–2    | e3/e4| B5–6        | e3/e4   | 0.001 |
| B0–2    | e3/e4| B3–4        | e4/e4   | 0.0005|
| B0–2    | e3/e4| B5–6        | e4/e4   | 0.001 |

Table 4
C-PEPTIDE: Two-way ANOVA Results

| Control | APOE | Braak Stage | APOE AD | p     |
|---------|------|-------------|---------|-------|
| B0–2    | e3/e3| B5–6        | e3/e3   | 0.06  |
| B0–2    | e3/e4| B3–4        | e4/e4   | 0.001 |
| B0–2    | e3/e4| B5–6        | e4/e4   | 0.006 |
| B0–2    | e3/e4| B3–4        | e4/e4   | 0.03  |
| Alzheimer | APOE | Braak Stage | APOE AD | p     |
| B3–4    | e3/e3| B3–4        | e4/e4   | 0.004 |
| B3–4    | e3/e4| B3–4        | e4/e4   | 0.01  |
| B3–4    | e3/e4| B5–6        | e3/e3   | 0.0006|
| B3–4    | e3/e4| B5–6        | e4/e4   | 0.006 |
| B3–4    | e3/e4| B5–6        | e3/e4   | 0.03  |
| B3–4    | e3/e4| B5–6        | e4/e4   | 0.002 |
| B5–6    | e3/e4| B5–6        | e4/e4   | 0.01  |

Glucagon-like peptide 1 (GLP-1)

The GLP-1 incretin has potent stimulatory effects on glucose-dependent insulin release leading to increased glucose disposal and suppression of plasma glucagon (Table 1). Two-way ANOVA demonstrated significant effects of APOE genotype (F = 6.34; p = 0.004) but not AD severity or AD x APOE interactions. Figure 2B clearly depicts how GLP-1 was modulated with APOE dose in AD. The main effects were that GLP-1 expression was significantly reduced in B3–4 and B5–6 APOE e4/e4 relative to B0–2 and B3–4 APOE e3/e3 and APOE e3/e4. In addition, GLP-1 expression was significantly lower in B5–6 APOE e4/e4 than in B5–6 APOE e3/e4, reflecting an inhibitory impact of APOE4 dose (Table 6).

Leptin

Leptin inhibits food intake and promotes energy expenditure (Table 1). Two-way ANOVA revealed
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Fig. 2. Incretins (GIP-1 and GLP-1): A multiplex ELISA measured A) GIP and B) GLP-1 immunoreactivity in 72 postmortem human frontal cortex samples from patients with established APOE genotypes (APOE ε3/ε3; APOE ε3/ε4; APOE ε4/ε4). Routine histological studies were used to grade Braak stage (B) severities of AD: B0–2 corresponds to normal aging; B3–4 represents moderate AD; B5–6 is severe AD. Graphs depict the mean ± S.E.M. levels (pg/mL) of immunoreactivity. Two-way ANOVA with post-hoc Tukey multiple comparison tests were used for intergroup comparisons. ** p < 0.01; *** p < 0.005; **** p < 0.0001 relative to B0–2 APOE ε3/ε3 controls. Other significant inter-group differences are provided in Tables 5 and 6.

Table 5

|                | Control 1 | Control 2 | APOE 1 | APOE 2 | Braak Stage | APOE AD | p     |
|----------------|-----------|-----------|--------|--------|-------------|---------|-------|
| B0–2           | ε3/ε3     | ε3/ε3     | ε3/ε3  | ε3/ε3  | B5–6        | ε3/ε3   | 0.006 |
| B0–2           | ε3/ε3     | ε3/ε3     | ε4/ε4  | ε4/ε4  | B3–4        | ε4/ε4   | 0.01  |
| B0–2           | ε3/ε4     | ε3/ε4     | ε4/ε4  | ε4/ε4  | B3–4        | ε4/ε4   | 0.01  |
| B0–2           | ε3/ε4     | ε3/ε4     | ε4/ε4  | ε4/ε4  | B5–6        | ε4/ε4   | 0.008 |
| B0–2           | ε3/ε4     | ε3/ε4     | ε3/ε3  | ε3/ε3  | B5–6        | ε3/ε3   | 0.005 |
| B0–2           | ε3/ε4     | ε3/ε4     | ε3/ε4  | ε3/ε4  | B5–6        | ε3/ε4   | 0.009 |
| Alzheimer      | APOE 1    | APOE 2    | APOE 3 | APOE 4 | Braak Stage | APOE AD | p     |
| B3–4           | ε3/ε4     | ε3/ε4     | ε4/ε4  | ε4/ε4  | B3–4        | ε4/ε4   | 0.004 |
| B3–4           | ε3/ε4     | ε3/ε4     | ε3/ε3  | ε3/ε3  | B5–6        | ε3/ε3   | 0.0009|
| B3–4           | ε3/ε4     | ε3/ε4     | ε4/ε4  | ε4/ε4  | B5–6        | ε4/ε4   | 0.003 |
| B3–4           | ε4/ε4     | ε4/ε4     | ε3/ε3  | ε3/ε3  | B5–6        | ε3/ε3   | 0.08  |
| B5–6           | ε3/ε3     | ε3/ε3     | ε4/ε4  | ε4/ε4  | B5–6        | ε4/ε4   | 0.07  |

Table 6

|                | APOE 1 | APOE 2 | Braak Stage | APOE AD | p     |
|----------------|--------|--------|-------------|---------|-------|
| Control        | APOE 1 | APOE 2 | Braak Stage | APOE AD | p     |
| B0–2           | ε3/ε3  | ε3/ε3  | B5–6        | ε3/ε3   | 0.006 |
| B0–2           | ε3/ε3  | ε3/ε3  | B3–4        | ε4/ε4   | 0.01  |
| B0–2           | ε3/ε4  | ε3/ε4  | B3–4        | ε4/ε4   | 0.01  |
| B0–2           | ε3/ε4  | ε3/ε4  | B5–6        | ε4/ε4   | 0.008 |
| B0–2           | ε3/ε4  | ε3/ε4  | B3–4        | ε3/ε3   | 0.005 |
| B0–2           | ε3/ε4  | ε3/ε4  | B5–6        | ε3/ε3   | 0.009 |
| Alzheimer      | APOE 1 | APOE 2 | Braak Stage | APOE AD | p     |
| B3–4           | ε3/ε4  | ε3/ε4  | B3–4        | ε4/ε4   | 0.004 |
| B3–4           | ε3/ε4  | ε3/ε4  | ε3/ε3       | ε3/ε3   | 0.0009|
| B3–4           | ε3/ε4  | ε3/ε4  | ε4/ε4       | ε4/ε4   | 0.003 |
| B3–4           | ε4/ε4  | ε4/ε4  | ε3/ε3       | ε3/ε3   | 0.08  |
| B5–6           | ε3/ε3  | ε3/ε3  | ε4/ε4       | ε4/ε4   | 0.07  |

significant effects of AD Braak stage (F = 19.84; p < 0.0001), APOE (F = 7.19; p = 0.002), and Braak stage × APOE interactions (F = 9.0; p < 0.0001). As shown in Fig. 3A, the mean frontal lobe levels of leptin were significantly reduced in all groups relative to B0–2 APOE ε3/ε3 (p < 0.0001) (also see Table 7). In addition, within the APOE ε3/ε3, Leptin expression was lower in B5–6 than B3–4 (p = 0.02) reflecting an AD Braak stage severity effect.

Ghrelin

Ghrelin increases food intake and reduces energy expenditure, opposing the actions of leptin (Table 1). Two-way ANOVA demonstrated significant effects of Braak stage (F = 3.9; p = 0.02) and APOE (F = 5.74; p = 0.006), and trend effects of Braak stage × APOE (F = 2.25; p = 0.08). Figure 3B and Table 8 show the main effects of AD severity and APOE4 dose, namely that ghrelin expression was significantly reduced in B3–4 and B5–6 APOE ε4/ε4 relative to B0–2 and B3–4 APOE ε3/ε3 and APOE ε3/ε4. Furthermore, the negative impact of APOE4 dose is shown by the lower levels of ghrelin in B3–4 and B5–6 APOE ε4/ε4 relative to B3–4 and B5–6 APOE ε3/ε3 or APOE ε3/ε4 (Table 8).

Glucagon

Glucagon mediates gluconeogenesis, raising blood glucose levels and opposing the actions of insulin. Two-way ANOVA showed that the main significant effect was APOE genotype (F = 6.3; p = 0.004). Post hoc Tukey tests demonstrated a trend reduction in brain glucagon levels in B5–6 APOE ε3/ε3 relative to B0–2 APOE ε3/ε3, and significant reductions in B3–4 and B5–6 APOE ε4/ε4 relative to B0–2...
Fig. 3. Leptin and Ghrelin: A multiplex ELISA was used to measure A) leptin and B) ghrelin immunoreactivity in 72 postmortem human frontal cortex samples from patients with known APOE genotypes (APOE ε3/ε3; APOE ε3/ε4; APOE ε4/ε4). Formalin fixed paraffin-embedded histological sections were used to assign Braak stage (B) severities of AD: B0–2 corresponds to normal aging; B3–4 represents moderate AD; B5–6 is severe AD. The Braak stage (B) severities of AD: B0–2 corresponds to normal aging; B3–4 represents moderate AD; B5–6 is severe AD. The graphs depict the mean ± S.E.M. levels (pg/mL) of immunoreactivity. Two-way ANOVA with post-hoc Tukey multiple comparison tests were used for intergroup comparisons. "**"p < 0.005; "****"p < 0.0001 relative to B0–2 APOE ε3/ε3 controls. Other significant inter-group differences are provided in Tables 7 and 8.

Table 7

| Control | APOE | Braak Stage | APOE AD | p   |
|---------|------|-------------|---------|-----|
| B0–2    | ε3/ε3| B0–2        | ε3/ε4   | p < 0.0001 |
| B0–2    | ε3/ε3| B0–2        | ε4/ε4   | p < 0.0001 |
| B0–2    | ε3/ε3| B3–4        | ε3/ε3   | p < 0.0001 |
| B0–2    | ε3/ε3| B3–4        | ε3/ε4   | p < 0.0001 |
| B0–2    | ε3/ε3| B3–4        | ε3/ε4   | p < 0.0001 |
| B0–2    | ε3/ε3| B3–4        | ε4/ε4   | p < 0.0001 |
| B0–2    | ε3/ε3| B5–6        | ε3/ε3   | p < 0.0001 |
| B0–2    | ε3/ε3| B5–6        | ε3/ε4   | p < 0.0001 |
| B0–2    | ε3/ε3| B5–6        | ε4/ε4   | p < 0.0001 |
| B0–2    | ε3/ε3| B3–4        | ε3/ε4   | p = 0.047   |
| B0–2    | ε3/ε3| B5–6        | ε3/ε3   | p = 0.02    |

Alzheimer APOE | Braak Stage | APOE AD | p   |
|---------------|-------------|---------|-----|
| B3–4          | ε3/ε3       | B5–6    | ε3/ε3 | p = 0.02    |
| B3–4          | ε3/ε3       | B5–6    | ε3/ε4 | p = 0.02    |
| B3–4          | ε3/ε3       | B5–6    | ε3/ε4 | p = 0.07    |

Resistin

Resistin promotes insulin resistance by suppressing insulin-stimulated glucose uptake into adipocytes (Table 1). Two-way ANOVA demonstrated significant AD Braak stage × APOE interactive effects (F = 3.83; p < 0.01) and a trend effect for APOE (F = 2.74; p = 0.08). For APOE ε3/ε3, resistin expression declined with increasing AD Braak stage resulting in significantly reduced levels in B5–6 relative to B0–2 (p = 0.003) (Fig. 4B, Table 10). In addition, resistin expression was significantly reduced in B3–4 APOE ε4/ε4 relative to B0–2 APOE ε3/ε3 (p = 0.01) (Fig. 4B, Table 10). B3–4 APOE ε3/ε4 had sharply elevated levels of resistin compared with all other groups (Fig. 4B), accounting for many of the significant differences detected (Table 10).

Plasminogen activator inhibitor-1 (PAI-1)

PAI-1 is a multi-functional serine protease inhibitor that regulates fibrinolysis, and therefore, may be critical for preventing vascular occlusions and ischemic injury (Table 1). Two-way ANOVA demonstrated significant AD Braak stage × APOE effects (F = 4.28; p = 0.005). In contrast to polypeptides that modulate insulin or insulin-related actions, PAI-1 expression in cases with an APOE ε3/ε3 genotype...
was elevated in B3–4 (p = 0.01) and B5–6; p = 0.008) relative to B0–2. In addition, the levels in B0–2 and B3–4 with an APOE e3/e4 genotype, and B5–6 with an APOE e4/e4 genotype were elevated compared with B0–2 APOE e3/e3 (Fig. 5A, Table 11), reflecting AD stage and APOE genotype effects. For APOE e3/e3 and APOE e3/e4, PAI-1 expression was similarly elevated by B3–4 and B5–6, whereas for APOE e4/e4, PAI-1 was significantly higher in B5–6 than B3–4, reflecting AD \times APOE4 dose interactive effects on PAI-1 expression.

Visfatin

Visfatin is a peripheral blood adipokine that like PAI-1, impacts vascular function and ultimately tissue perfusion. Visfatin promotes vascular smooth muscle cell maturation and has insulin-mimetic effects that improve insulin sensitivity (Table 1). Two-way ANOVA demonstrated no statistically significant effects of APOE, AD Braak stage severity, or APOE x AD interactions. Correspondingly, post hoc Tukey tests were negative (Fig. 5B).

DISCUSSION

This study utilized human postmortem brains to examine the independent and interactive relationships between AD severity and APOE4 on frontal lobe expression of insulin-related polypeptides and inflammatory mediators that impact insulin responsiveness. The premise was that, although

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**Table 9**

| Control | APOE | Braak Stage | APOE AD | p       |
|---------|------|-------------|---------|---------|
| B0–2    | e3/e3| B5–6        | e3/e3   | p = 0.09|
| B0–2    | e3/e3| B3–4        | e3/e4   | p = 0.0007|
| B0–2    | e3/e4| B5–6        | e4/e4   | p = 0.009|
| B0–2    | e3/e4| B3–4        | e4/e4   | p = 0.009|
| B0–2    | e3/e4| B5–6        | e4/e4   | p = 0.08|
| Alzheimer APOE | Braak Stage | APOE AD | p       |
| B3–4    | e3/e3| B3–4        | e4/e4   | p = 0.002|
| B3–4    | e3/e4| B5–6        | e3/e3   | p = 0.02|
| B3–4    | e3/e4| B5–6        | e3/e3   | p = 0.06|
| B3–4    | e3/e4| B3–4        | e4/e4   | p = 0.0004|
| B3–4    | e3/e4| B5–6        | e4/e4   | p = 0.005|
| B3–4    | e3/e4| B5–6        | e3/e3   | p = 0.03|
| B3–4    | e4/e4| B5–6        | e3/e3   | p = 0.0007|
| B5–6    | e3/e4| B3–4        | e3/e4   | p = 0.09|
| B5–6    | e3/e4| B5–6        | e3/e4   | p = 0.009|

**Table 10**

| Control | APOE | Braak Stage | APOE AD | p       |
|---------|------|-------------|---------|---------|
| B0–2    | e3/e3| B3–4        | e4/e4   | p = 0.01|
| B0–2    | e3/e4| B5–6        | e3/e3   | p = 0.003|
| B0–2    | e3/e4| B3–4        | e3/e4   | p = 0.029|
| Alzheimer APOE | Braak Stage | APOE AD | p       |
| B3–4    | e3/e3| B3–4        | e4/e4   | p = 0.006|
| B3–4    | e3/e4| B3–4        | e4/e4   | p = 0.0004|
| B3–4    | e3/e4| B5–6        | e3/e3   | p < 0.0001|
| B3–4    | e4/e4| B5–6        | e3/e4   | p = 0.009|
| B3–4    | e4/e4| B3–4        | e4/e4   | p = 0.006|
| B5–6    | e3/e3| B5–6        | e3/e4   | p = 0.09|

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Fig. 4. Glucagon and Resistin: A commercial multiplex ELISA was used to measure A) glucagon and B) resistin immunoreactivity in 72 human postmortem frontal cortex tissue samples from patients with known APOE genotypes (APOE e3/e3; APOE e3/e4; APOE e4/e4). Standardized formalin fixed paraffin-embedded histological sections were used to assign Braak stage (B) severities of AD: B0–2 represents normal aging; B3–4 represents moderate AD; B5–6 is severe AD. The graphs depict the mean ± S.E.M. levels (pg/mL) of immunoreactivity. Two-way ANOVA with post-hoc Tukey multiple comparison tests were used for intergroup comparisons. *p < 0.05; **p < 0.01; ***p < 0.005; ****p < 0.0001 relative to B0–2 APOE e3/e3 controls. Other significant inter-group differences are provided in Tables 9 and 10.
imperfections in insulin expression and signaling have been solidly linked to many aspects of AD pathogenesis and progression, growing evidence suggests that the entire network mediating insulin-related metabolic functions is impaired. The significance of this concept is that more than one target should be considered for effective disease remediation. This work builds on our prior investigations that demonstrated significant and early AD-associated abnormalities in CSF and serum levels of insulin-related polypeptides [40–43], and interactive effects of APOE and AD severity on IDE and RCAN1 expression in the brain [44].

ApoE, a 34 kDa protein, has three major isoforms (e2, e3, e4) that differ by single amino acid substitutions at residues 112 and 158 [45]. The inclusion of APOE genotype as a variable in these analyses was prompted by the facts that: 1) the e4 allele is the strongest genetic risk factor for late onset sporadic AD [46]; 2) e4 carriers account for over 50% of AD cases [47]; 3) AD risk is increased by 3- or 4-fold among e4 carriers and 15-fold in homozygous individuals (e4/e4); and 4) besides its role in Aβ accumulation, APOE4 is associated with reduced brain glucose metabolism in the pre-clinical stages of disease [48], ultimately impairing signal transduction through the insulin receptor with attendant reduction of Aβ clearance and increased Aβ aggregation [49]. In addition, APOE e4’s contribution to AD pathogenesis is likely linked to the associated impairments in synaptic plasticity mediated by alterations in endocytic recycling [50]. The mechanism may involve the inhibition of Reelin signaling through APOE and attendant regulation of N-methyl-D-aspartate (NMDA) receptor activity, stabilization of microtubules, and prevention of neurofibrillary tangle-associated tau hyperphosphorylation [51].

Although it is not known how APOE4 impairs insulin signaling, the mechanism may involve alterations in cholesterol homeostasis since APOE is an important regulator of cholesterol synthesis and receptor-mediated uptake [52], and previous studies showed that alterations in neuronal membrane cholesterol content lead to impaired insulin signaling and glucose uptake [53]. Correspondingly, experimental over-loading of neuronal membranes with cholesterol increases amyloid-β protein precursor (AβPP) internalization and Aβ generation [54]. In addition to its roles in lipoprotein metabolism,
expression, suggesting that a contribution to AD neuromodulation with earlier Braak stage reductions in brain insulin expression. APOE and B5–6 were comparable to that measured in B5–6 insulin expression. In contrast, homozygous APOE out knowledge of corresponding with previous observations made within the absence of an APOE e3/e3, e3/e4, or e4/e4 genotype. There were no normal aged brains from APOE e4/e4 cases, perhaps due to the strong propensity for such individuals to have developed AD by the time they reach 70–90 years of age. It is also noteworthy that a skewed percentage of the APOE e4/e4 cases had B5–6 AD whereas those with an APOE e3/e3 or e3/e4 genotype predominantly (68% or 70%) had B3–4 stage AD. One limitation of this study was the lack of APOE e4/e4 controls for comparison with AD cases of the same genotype. Nonetheless, using two-way ANOVA tests with post hoc comparisons it was possible to ascertain APOE e4 dose effect in relation to AD severity.

Insulin decreases blood glucose, increases cell permeability to monosaccharides, and accelerates glycolysis, the pentose phosphate cycle, and glycan synthesis. The decline in brain insulin with increasing severity of AD in the APOE e3/e3 group corresponds with previous observations made without knowledge of APOE status [16, 20, 41]. The absence of an APOE e3/e4 effect suggests that APOE4 carrier status does not adversely impact brain insulin expression. In contrast, homozygous APOE e4/e4 was associated with significantly reduced brain insulin expression such that the mean levels in B3–4 and B5–6 were comparable to that measured in B5–6 APOE e3/e3. Therefore, APOE e4/e4 was associated with earlier Braak stage reductions in brain insulin expression, suggesting that a contribution to AD neuropathology is exacerbation of brain metabolic dysfunction via insulin deficiency.

Although it has been suggested that AD-associated reductions in brain insulin could be attributed to increased expression of IDE [59], a previous analysis of these cases revealed primarily reduced expression of IDE mRNA and protein [44]. Alternatively, impairments in local synthesis of insulin [16, 20] or its uptake from the peripheral circulation [60] could account for the reduced CNS levels of insulin with AD progression. One argument in favor of AD and APOE e4-mediated impairments in local insulin synthesis was the finding of reduced C-peptide expression in patterns that mimicked changes in insulin. C-peptide is a cleavage product of pro-insulin and generated with insulin. Its longer half-life provides a more reliable in vivo index of insulin production.

Incretins, including GIP-1 and GLP-1, are potent stimulators of glucose-dependent insulin secretion and modulators of fatty acid metabolism [61]. GLP-1 suppresses plasma glucagon, stimulates glucose disposal, and has neuroprotective actions that may benefit individuals with AD [62, 63]. In the APOE e3/e3 cases, GIP expression was reduced with AD severity whereas GLP-1 was not, but with an APOE e4/e4, frontal lobe GIP and GLP-1 were similarly reduced in B3–4 and B5–6 relative to B0–2 controls. Again, the presence of a single e4 allele was not associated with significantly altered incretin expression in either control or AD cases, supporting the concept that APOE e4/e4 is the principal driver of brain metabolic dysfunction, and that its adverse effects include impairments in brain incretin expression and function.

Leptin and ghrelin have opposing effects in that leptin regulates fat depots by inhibiting food intake and regulating energy expenditure while ghrelin stimulates appetite and induces adiposity in addition to inducing growth hormone release from the pituitary gland [64]. This study demonstrated that frontal lobe leptin was strikingly reduced in moderate and severe AD regardless of APOE genotype, as well as in APOE e3/e4 controls. Downregulation of leptin may reflect an adaptive response to insulin resistance since, although leptin inhibits tyrosine phosphorylation of the insulin receptor substrate 1 (IRS-1) protein and interactions between IRS-1 and growth factor receptor-bound protein 2 (GRB2), its positive effects include stimulation of PI3K, which promotes metabolism, cell survival, neuronal plasticity, and other critical neuronal and glial brain functions [65].
Ghrelin is an endogenous ligand of the growth hormone secretagogue receptor 1a that regulates growth hormone secretion and energy balance. Ghrelin is expressed in the brain, including hypothalamus and besides its physiological role in glucose and lipid metabolism, ghrelin has functional roles in memory, reward, sleep, and neurogenesis [66], which are known to be disrupted in AD [67]. Therefore, reductions in ghrelin, either in the CNS or periphery, could reflect a pathophysiological state that mediates neurobehavioral features of neurodegeneration. Furthermore, evidence suggests that ghrelin has an important role in neuroprotection and may help mediate cognitive dysfunction and neurodegeneration [68]. This study showed no significant alteration of ghrelin expression in relation to AD in APOE e3/e3 or e3/e4 cases. However, ghrelin was significantly reduced in both B3–4 (moderate) and B5–6 (severe) APOE e4/e4 AD. This observation is novel and could reflect significantly impaired neuroprotective mechanisms and metabolic dysfunction linked to APOE e4 dose.

Glucagon increases gluconeogenesis and decreases glycolysis, raising plasma glucose in response to insulin-induced hypoglycemia and likely plays an important role in initiating and maintaining hyperglycemic conditions in diabetes mellitus. Only the APOE e4/e4 AD had significantly reduced frontal lobe glucagon expression. This response could not be attributed to GLP-1 suppression since GLP-1 expression was also downregulated in B3–4 and B5–6 APOE e4/e4. One potential explanation for the finding is that GLP-1/glucagon regulation may be uncoupled. A chronic brain diabetic state (Type 3 diabetes) [16, 25, 69] together with impaired capacity for glucose and lipid utilization could potentially lead to brain starvation.

The adipokine, resistin, promotes insulin resistance [64] and activates pro-inflammatory cytokines [70]. Correspondingly, central deficiency of resistin in APOE e3/e4 B3–4 relative to all other groups except APOE e3/e4 controls suggests that interactive effects of a single APOE4 allele and early or intermediate-stage AD contribute to brain insulin resistance. In contrast, reductions in brain resistin expression with increasing severity of AD among APOE e3/e3 cases, as well as the lower levels in more advanced stages of disease in APOE e3/e4 or APOE e4/e4 cases may reflect compensatory or adaptive responses, reducing neuroinflammation and increasing insulin sensitivity to support brain energy balance.

Visfatin is also an adipokine but, contrary to the effects of resistin, it increases insulin sensitivity [64], but like resistin, it activates pro-inflammatory cytokines [70]. Visfatin mediates its stimulatory effects on insulin responsiveness via activation of the insulin receptor with attendant lowering of blood glucose [70]. Visfatin, also known as ‘nicotinamide phosphoribosyltransferase’ (NAmPRTase or Nampt), is the rate-limiting enzyme in the nicotinamide adenine dinucleotide (NAD+) salvage pathway that converts nicotinamide to nicotinamide mononucleotide and enables NAD+ biosynthesis [70]. Visfatin-mediated increases in NAD+ have anti-aging and neuroprotective actions [72–78]. Although there were no significant effects of AD on visfatin expression among APOE e3/e3 cases, in both APOE e3/e4 and APOE e4/e4, the significant linear trend effects reflect increasing levels with AD severity. Therefore, in addition to the interactive effects of APOE e4 dose and AD grade, the results suggest that compensatory mechanisms may help preserve glucose utilization and energy metabolism vis-à-vis brain insulin resistance and deficiency.

PAI-1 is a serine protease inhibitor of tissue plasminogen activator, urokinase, Protein C, and matriptase-3/TMPRSS7, and functionally inhibits fibrinolysis [79]. Elevated levels of PAI-1 are strongly associated with insulin resistance, including in diabetes mellitus [80, 81], and have been deemed independent risk factors for type 2 diabetes [81]. Elevated PAI-1 is associated with increased risk for thrombotic cardiovascular [82] and cerebrovascular [79] diseases and ischemic stroke [83]. In this study we observed significantly increased PAI-1 expression in AD relative to control in APOE e3/e3 cases, but similarly elevated PAI-1 in B0–1, B3–4, and B5–6 APOE e3/e4, indicating independent effects of AD severity and APOE e4. The similarly elevated PAI-1 levels in B5–6 APOE e4/e4 compared with B3–4 or B5–6 APOE e3/e3 and APOE e3/e4 cases indicates that in this instance, APOE e4 dosage did not additionally affect PAI-1 expression, and instead, APOE and AD independent upregulated PAI-1. Mechanistically, pro-inflammatory cytokine activation has been linked to increased PAI-1 expression [80], and in earlier reports we showed increased pro-inflammatory and reduced anti-inflammatory cytokine/chemokine levels in AD [40, 42].
Conclusions

This study provides strong evidence that insulin-related endocrine pathways are dysregulated in AD, and in relation to APOE4, particularly APOE ε4/ε4. Dominant APOE4-independent abnormalities included reductions in insulin, incretin (GIP-1), leptin, and resistin, and increases in PAI-1. Major interactive effects of APOE ε4/ε4 and AD included reductions in insulin, C-peptide, GIP-1, GLP-1, leptin, ghrelin, glucagon, and resistin, and increases in PAI-1. Leptin expression was broadly inhibited in relation to AD severity and APOE4, independent of ε4 dose. Altogether, these findings provide exciting new information about the complexity of insulin-linked metabolic dysfunction in AD and the additive adverse impact of APOE ε4, particularly ε4/ε4. One limitation of this study is that the case series is small and some subgroups had few or no cases. However, the research was entirely human-based and therefore fully dependent upon available tissue. Nonetheless, the data and interpretations support the concepts that: 1) AD pathogenesis and progression are linked to insulin deficiency together with insulin resistance, i.e., Type 3 diabetes [16, 17, 84]; 2) APOE4, particularly ε4/ε4, interacts with AD to significantly alter expression of insulin network polypeptides that have roles in energy metabolism and neuroinflammation; and 3) broadly reduced leptin expression, along with insulin deficiency, may be a critical mediator of brain hypometabolism in AD. Since it appears that multiple neuroendocrine abnormalities contribute to insulin deficiency and insulin resistance, eventual remediation of metabolic derangements in AD will likely require multi-pronged treatment approaches.

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CONFLICT OF INTEREST

The authors have no conflict of interest to report.

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