Alzheimer's & Dementia

Genetics

Altered expression of insulin-degrading enzyme and regulator of calcineurin in the rat intracerebral streptozotocin model and human apolipoprotein E-ε4–associated Alzheimer's disease

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Abstract

Introduction: This study assesses insulin-degrading enzyme (IDE) and regulator of calcineurin 1 (RCAN1) as potential mediators of brain insulin deficiency and neurodegeneration in experimental and human Alzheimer's disease (AD).

Methods: Temporal lobes from Long Evans rats treated with intracerebral streptozotocin or vehicle and postmortem frontal lobes from humans with normal aging AD (Braak 0-2), moderate (Braak 3-4) AD, or advanced (Braak 5-6) AD were used to measure IDE and RCAN mRNA and protein.

Results: Intracerebral streptozotocin significantly increased IDE and RCAN mRNA and protein. In humans with apolipoprotein E (ApoE) ε3/ε4 or ε4/ε4 and AD, IDE was elevated at Braak 3-4, but at Braak 5-6, IDE expression was significantly reduced. RCAN1 mRNA was similarly reduced in ApoE ε4+ patients with moderate or severe AD, whereas RCAN1 protein declined with the severity of AD and ApoE ε4 dose.

Discussion: The findings suggest that IDE and RCAN1 differentially modulate brain insulin signaling in relation to AD severity and ApoE genotype.

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Keywords: Alzheimer's disease; Insulin deficiency; Insulin resistance; Streptozotocin; Insulin-degrading enzyme; RCAN1; Neurodegeneration

1. Introduction

Alzheimer's disease (AD) is the most common form of dementia-associated neurodegeneration [1,2], yet despite decades of intensive research based on the β-amyloid (Aβ) and pTau hypotheses, no effective treatments have emerged. On the other hand, growing evidence supports an alternative concept that cognitive impairment and neurodegeneration in AD are mediated by deficits in insulin and insulin-like growth factor (IGF-1) signaling and metabolic functions in the brain [3–15]. The fact that progressive brain insulin and IGF-1 resistances accompany declines in ligand expression in the brain suggests that metabolic abnormalities in AD mimic combined effects of types 1 and 2 diabetes [16–20]. The term "Type 3 diabetes" was coined [6,7] to reflect this fundamental concept, as well as emphasize the need for both trophic factor supplementation and insulin sensitizer treatment interventions. Further research in this field would improve understanding of how therapeutic strategies already developed for diabetes mellitus and other insulin resistance diseases could be repurposed for selectively targeting neurodegeneration. In addition, we must determine the underlying causes of insulin/IGF-linked brain

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metabolic dysfunction to develop rational strategies for prevention and treatment.

Two molecules (enzymes) that could have major roles in mediating brain insulin deficiency in AD are insulin-degrading enzyme (IDE) [21,22] and regulator of calcineurin 1 (RCAN1) [23–26]. IDE is a 110-kDa thiol zinc-metalloendopeptidase [27,28] encoded by a gene located on chromosome 10q23.33 [29,30] and expressed in many organs and tissues including the brain, liver, testis, kidney, heart, and skeletal muscle [31]. The surface membrane and the cytosolic, peroxisomal, and mitochondrial subcellular localizations of IDE [32–35] suggest its functions are diverse. Initially, IDE was described as an insulinase [36], but later, it was demonstrated to degrade other small polypeptides including atrial natriuretic peptide, transforming growth factor-α, amylin, bradykinin, kallidin, and Aβ [37–39].

The fact that IDE can degrade insulin, amylin, and Aβ suggests that AD may be linked to type 2 diabetes mellitus via chromosome 10 [40–42]. Correspondingly, the finding that Aβ plaque size inversely correlates with IDE expression and activity [35,43] suggests that IDE deficiency could mediate plaque buildup and possibly cognitive impairment in AD. On the other hand, the paradoxical findings of progressive brain insulin deficiency and insulin resistance in AD [19,44] suggest that increased rather than decreased IDE activity is the culprit. A third possible scenario is that increased IDE activity preferentially targets and degrades insulin rather than Aβ, driving brain insulin deficiency vis-à-vis Aβ accumulation, as it occurs in the intracerebral streptozotocin (i.c. STZ) model [45–49].

The *RCAN1* gene is located within the Down locus on chromosome 21 [50,51]. Alternative splicing of RCAN1 produces three protein isoforms, RCAN1.1 L, RCAN1.1S, and RCAN1.4 [51,52]. RCAN1 is expressed at high levels in the brain, spinal cord, kidney, liver, mammary gland, placenta, skeletal muscle, and heart [51,52], and its principal function is to inhibit calcineurin, a serine-threonine phosphatase [53,54]. RCAN1's potential role in neurodegeneration is through inhibition of calcineurin [55,56] and attendant-increased glycogen synthase kinase 3β (GSK-3β) activity [55,56], leading to hyperphosphorylation of tau and neurofibrillary tangle formation [57], activation of stress pathways, and increased neuronal apoptosis [51,57–59]. In Down syndrome and AD, hyperphosphorylated tau immunoreactive neurofibrillary tangles, dystrophic neurites, and cortical neuritic plaques correlate with severity of cognitive impairment and neurodegeneration [60–62].

Importantly, calcineurin may have an important role in mediating AD neurodegeneration via dysregulation of Ca^{2+} [63] and Ca^{2+}/calmodulin-dependent signaling [64] in relation to cognition and neuronal plasticity [65–67], Aβ synaptotoxicity and dendritic spine pathology [68,69], driver of neuroinflammation [70,71], excitotoxicity [72],

and suppression of synaptic mRNA transcripts [73]. Apart from its roles in protecting against the development and progression of various structural and functional AD-associated pathologies that correlate with cognitive impairment, RCAN1 causes hypoinsulinemia and has been linked to diabetes mellitus, pancreatic β cell dysfunction, and altered mitochondrial function [25,74,75]. Therefore, either increased or reduced levels of RCAN1 expression in the brain could have profound effects on AD pathogenesis and progression. In particular, similar to IDE, upregulated expression of RCAN1 could promote brain insulin deficiency in AD.

The present work further evaluates the potential roles of IDE and RCAN1 as mediators of neurodegeneration in an established experimental rat model of i.c. STZ and in human brains with moderate or severe AD pathology. Intracerebral STZ has been widely used as a model of sporadic AD-type neurodegeneration because cognitive impairment is associated with Aβ and pTau accumulations, mitochondrial dysfunction, neuroinflammation, insulin and IGF deficiency and resistance, and impaired insulin/IGF-1 signaling through pathways that regulate energy metabolism, neuronal plasticity, cellular homeostasis, and neuronal survival [45,46,48,76–80]. STZ functions in part by killing insulin-producing cells, particularly β cells in pancreatic islets. However, i.c. STZ mainly has neurotoxic effects, reducing insulin gene expression [46] and insulin polypeptide levels [79] in the brain, although with prolonged survival and after development of neurodegeneration with cognitive impairment, peripheral insulin resistance, and diabetes eventually emerge [48]. The relevance of this model to AD is further exemplified by reversal of cognitive deficits and neurodegeneration by treatment with insulin sensitizers [45,47,48,81] or insulin analogs [79].

In human brains, greater severities, that is, higher histopathologic Braak stage scores of AD, are associated with decreased signaling through insulin and IGF-1 receptors, insulin receptor substrate, and downstream Akt pathways that mediate neuronal survival, plasticity, growth, metabolism, and cholinergic function and inhibit neuroinflammation, oxidative stress, and apoptosis [6,7,82]. Furthermore, the fact that apolipoprotein E (ApoE) ε4 allele, the strongest dose-dependent risk factor for late-onset sporadic AD [83–86], also promotes insulin resistance supports the concept that impairments in brain insulin signaling mediate AD-type neurodegeneration. Correspondingly, brain insulin resistance and its associated deficits in cognition can be partially reversed by consistent implementation of healthy lifestyle choices [87–90] or treatment with insulin sensitizers [91], that is, approaches that are currently used to treat peripheral insulin resistance disease states. In addition, intranasal insulin has been used to normalize brain insulin levels and improve cognition [92–97] and glucose metabolism [98] in AD. Similarly, treatment with incretins could be used to stimulate endogenous production and release of insulin in the brain [19,99].

Table 1
Primer pairs used for qRT-PCR analysis of human and rat IDE and RCAN genes

Primers	Direction	Sequence	Position	Amplicon
Human				
IDE	Forward	TACCTCCGCTTGCTGATGAC	2110	106
IDE	Reverse	GGAGCTGAGGTATGAAGGCC	2215	
RCAN1	Forward	AGGCTCCAGCTGCATAAGAC	258	111
RCAN1	Reverse	CTGCTTGTCTGGATTTGGCG	368	
Rat				
IDE	Forward	CTGTGCCCCTTGTTTGATGC	523	139
IDE	Reverse	TGAAGGGGTGCTTGGGATTC	661	
RCAN1-2-3	Forward	AACTTCAGCAACCCCCTGTC	231	80
RCAN1-2-3	Reverse	AGTTTCATCTCCTTCCCCAGG	310	

Abbreviations: IDE, Insulin-degrading enzyme; RCAN1, regulator of calcineurin 1; qRT-PCR, quantitative reverse transcriptase polymerase chain reaction.

The concept that brain insulin/IGF resistance and deficiency are at the core of AD neurodegeneration and link multiple facets of disease in both the i.c. STZ model and human cases of AD highlights the need to determine the underlying mechanisms of dysregulated signaling and metabolism in the brain. Understanding the dynamic alterations in brain insulin/IGF-1 signaling that mediate AD progression [6,19] is critical because abnormalities present in the early stages, for example, insulin deficiency, may be superseded by additional alterations in signaling due to insulin resistance [6,7,82,100–102] and impairments in central nervous system expression of related gut hormones [103–105] that required different treatment strategies. Our overarching hypothesis is that alterations in RCAN1 and IDE expression are integrally related to the stages and mediators of AD neurodegeneration. In these regards, we hypothesize that ApoE genotype has modifying effects on both IDE and RCAN1 expressions and thereby drive brain insulin deficiency and resistance with AD progression.

2. Methods

2.1. Experimental model

A model of sporadic AD was produced in 8-week-old male and female Long Evans rats (6/group) by intracerebral (i.c.) administration of 0.9 mg/kg STZ (0.2 mg/rat) under ketamine/xylocaine anesthesia [5,46]. Controls were administered as i.c. saline. The rats were sacrificed 6 weeks later, and the temporal lobes were snap frozen and stored at $-80^{\circ}C$ for molecular and biochemical studies. The use of experimental animals was approved by the Institutional Animal Care and Use Committee at the Lifespan Rhode Island Hospital and in accordance with guidelines established by the National Institutes of Health.

2.2. Human subjects

Postmortem fresh frozen human frontal lobe tissue samples from Brodmann Area 8/9 were obtained from the Duke Kathleen Price Bryan Brain Bank and Biorepository (Durham, NC). The banked brains are processed according to a standardized protocol that ensures storage of high-quality fresh frozen tissue for molecular and biochemical analyses and rendering of accurate histopathological diagnoses. In addition, the brain banking protocol includes genotyping for ApoE [ε3/ε3, ε3/ε4, or ε4/ε4] (https://neurology. duke.edu/research/research-centers/joseph-and-kathleen-br yan-alzheimers-disease-research-center/brain-bank). However, because the banked brains are deidentified, clinical information other than standard demographics is not available. For this study, we obtained 72 brain samples from men and women. The cases were grouped for analysis based on their Braak stages of AD and Apo E genotypes as follows: Braak 0-2 = normal aging, Braak 3-4 = moderate AD, Braak 5-6 = severe or advanced AD. Permission to use deidentified postmortem human brain tissue for this research was granted by the Lifespan Hospitals Institutional Review Board.

2.3. Quantitative reverse transcriptase polymerase chain reaction assays

Total RNA was extracted from 100-mg samples of fresh frozen temporal lobe using the RNeasy Mini Kit (Qiagen, Hilden, Germany). RNA was reverse transcribed using the AMV First Strand cDNA Synthesis Kit (Roche, Indianapolis, IL). The cDNA templates were used to measure RCAN1 and IDE by quantitative reverse transcriptase polymerase chain reaction (qRT-PCR) in a Roche LightCycler 480 System [106]. Primer pairs were designed using MacVector 12 software (Cary, NC) (Table 1). Relative mRNA abundance was calculated using the $2^{\Delta C_t}$ method, with results normalized to actin or hypoxanthine phosphoribosyltransferase 1.

2.4. Preparation of protein homogenates

Brain tissue samples were homogenized in buffer containing protease and phosphatase inhibitors using a TissueLyser II (Qiagen, Germantown, MD). Supernatants obtained after centrifugation (14000 × g for 10 min at $4^{\circ}C$) were used in enzyme-linked immunosorbent assays (ELISAs).

2.5. Direct binding duplex ELISAs

Direct binding duplex ELISAs measured immunoreactivity to RCAN1 and IDE, followed by large acidic ribosomal protein (RPLPO) [107] to normalize expression of target proteins. RCAN1 and IDE were detected with horseradish peroxidase–conjugated secondary antibody and Amplex UltraRed (Invitrogen, Carlsbad, CA), and RPLPO was subsequently detected with biotinylated anti-RPLPO (Proteintech Group Inc, Chicago, IL) and streptavidin-conjugated alkaline phosphatase with 4-methylumbelliferyl phosphate (4-MUP). Fluorescence intensities (Amplex Red: Ex565/Em595; 4-MUP: Ex360/Em450) were measured in a SpectraMax M5 (Molecular Devices, Sunnyvale, CA). The calculated specific protein-to-RPLPO ratios were used for intergroup statistical comparisons.

2.6. Statistics

Data corresponding to group means ± standard deviation are tabulated. Intergroup comparisons were made using Student *t* tests or analysis of variance with post hoc Tukey tests using GraphPad Prism 7 software (San Diego, CA).

2.7. Sources of reagents

Mouse or rabbit polyclonal antibodies to RCAN1 and IDE were purchased from Abcam (Cambridge, MA). Rabbit polyclonal antibody to RPLPO (RPL23 16086-1-AP) was from Proteintech Inc (Chicago, IL). The Amplex UltraRed fluorophore horseradish peroxidase and 4-MUP alkaline phosphatase substrates were obtained from Invitrogen (Carlsbad, CA).

3. Results

3.1. Effects of i.c. STZ on IDE and RCAN1 expression

The mean mRNA level of IDE measured by qRT-PCR analysis and normalized to hypoxanthine phosphoribosyltransferase was significantly higher in i.c. STZ temporal lobe samples relative to control (Fig. 1A). Duplex ELISA also demonstrated upregulation of IDE in i.c. STZ brains with a more striking difference from control compared with the effects on mRNA (Fig. 2A). In addition, RCAN1 mRNA (Fig. 1B) and protein (Fig. 2B) expression were also significantly elevated in the i.c. STZ temporal lobe samples relative to control.

3.2. Subject population

All brains included in this study had been banked using an established protocol including dissections and snap freezing of fresh frozen pieces from specific regions. Formalin-fixed paraffin-embedded histological sections were used to render diagnoses and stage severity of AD. Among the 72 cases studied, 38 were genotyped as ApoE ε3/ε3, 25 as ApoE
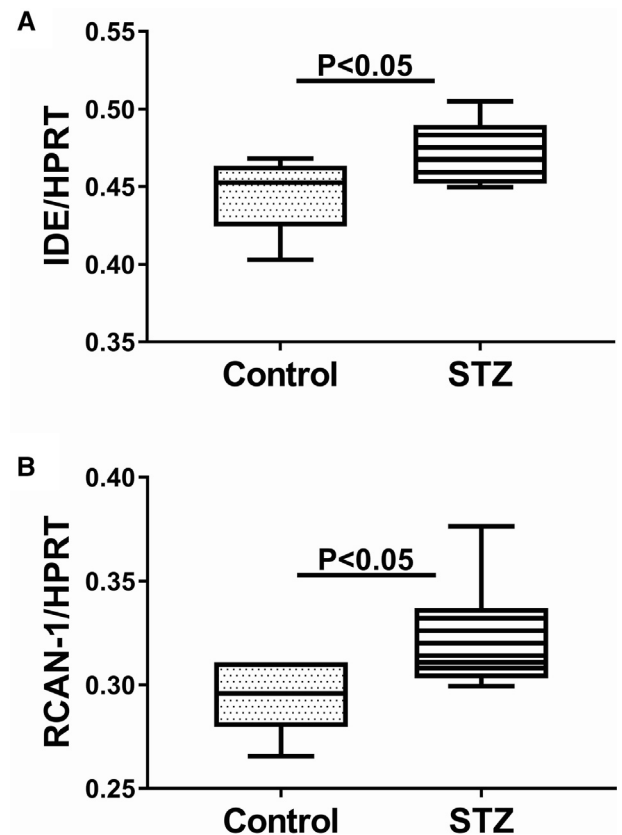

Fig. 1. Increased temporal lobe mRNA expression of IDE and RCAN1 in i.c. STZ-treated rats. Long Evans rats were administered single i.c. injections of STZ or vehicle at 4 weeks of age (N = 6/group). The rats were sacrificed 4 weeks later to measure temporal lobe mRNA expression of (A) IDE and (B) RCAN1 by qRT-PCR analysis, with results normalized to HPRT measured simultaneously in the same samples. Graphs depict box plots with mean levels of expression (horizontal bars), 95% confidence interval limits (upper and lower boundaries of the boxes) and ranges (upper and lower stems). Student *t* tests were used for intergroup comparisons. Abbreviations: IDE, Insulin-degrading enzyme; RCAN1, regulator of calcineurin 1; qRT-PCR, quantitative reverse transcriptase polymerase chain reaction; HPRT, hypoxanthine phosphoribosyltransferase; STZ, streptozotocin.

ε3/ε4, and 9 as ApoE ε4/ε4 (Table 2). Analysis of variance with the Tukey post hoc test (repeated measures with 5% false discovery correction) demonstrated no significant differences in mean age among the subgroups, except for ApoE ε3/ε4, Braak 0-2 which were significantly younger than the AD group but not the ApoE ε3/ε3 controls. Owing to the small number of Braak 0-2, ApoE ε3/ε4 cases, control data from the ApoE ε3/ε3 and ApoE ε3/ε4 cases were combined for statistical comparisons with those of the AD groups.

3.3. AD Braak Stage and ApoE genotype effects on IDE expression

Among cases with an ApoE ε3/ε3 genotype, the mean level of IDE mRNA was highest at Braak stage 3-4, whereas with advanced AD pathology (Braak 5-6), the mean level of
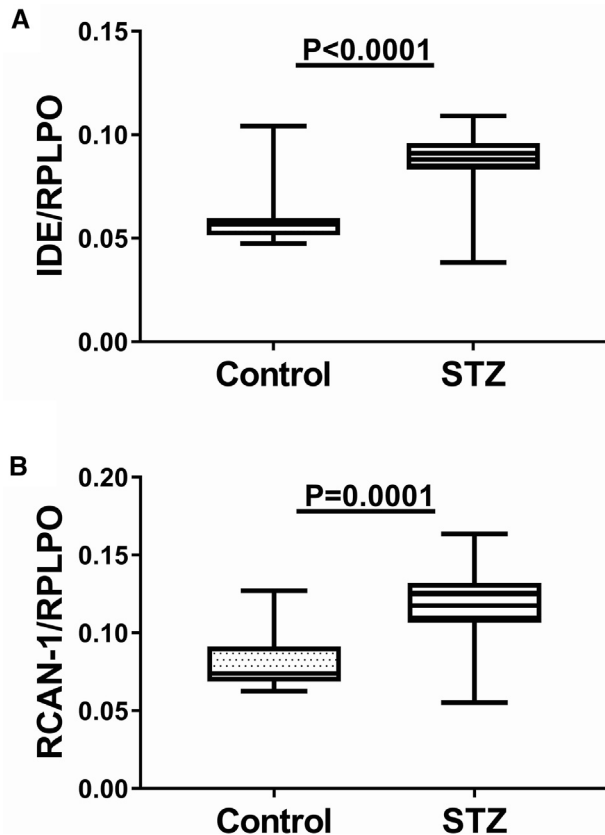
Fig. 2. Increased temporal lobe protein expression of IDE and RCAN1 in i.c. STZ-treated rats. Long Evans rats were given single i.c. injections of STZ or vehicle at 4 weeks of age (N = 6/group). Four weeks later, the rats were sacrificed to measure temporal lobe protein expression of (A) IDE and (B) RCAN1 by duplex direct-binding ELISAs with results normalized to RPLPO measured sequentially in the same samples. Graphs depict box plots with mean levels of expression (horizontal bars), 95% confidence interval limits (upper and lower boundaries of the boxes), and ranges (upper and lower stems). Student *t* tests were used for intergroup comparisons. Abbreviations: IDE, Insulin-degrading enzyme; RCAN1, regulator of calcineurin 1; STZ, streptozotocin; ELISA, enzyme-linked immunosorbent assays; RPLPO, ribosomal protein.

Table 2
Human subjects

Stage	Apolipoprotein E ε3/ε3			Apolipoprotein E ε3/ε4			Apolipoprotein E ε4/ε4		
	#	Age	M/F	#	Age	M/F	#	Age	M/F
Braak 0-2	18	78.33 ± 11.16	9 M; 9 F	3	72.33 ± 14.15	2 M; 1 F	0		
Braak 3-4	14	81.57 ± 6.81	6 M; 8 F	15	84.13 ± 4.02	9 M; 6 F	3	84.03 ± 3.60	2 M; 1 F
Braak 5-6	6	84.01 ± 2.45	3 M; 3 F	7	83.71 ± 5.47	2 M 5 F	6	81.11 ± 4.47	3 M; 3 F

NOTE. Postmortem banked fresh frozen frontal lobe brain samples were used for qRT-PCR analysis and ELISA measurements of IDE and RCAN1. ANOVA with post hoc Tukey tests (multiple comparisons with 5% false discovery correction) revealed that the Braak 0-2, Apo E ε3/ε4 group was significantly younger than the AD groups ($P < .05$).

Abbreviations: AD, Alzheimer's disease; M, male; F, female; qRT-PCR, quantitative reverse transcriptase polymerase chain reaction; ELISA, enzyme-linked immunosorbent assays; IDE, insulin-degrading enzyme; ANOVA, analysis of variance; RCAN1, regulator of calcineurin 1.

3.4. Effects of AD Braak stage and ApoE genotype on RCAN expression

Among the ApoE ε3/ε3 cases, the mean levels of RCAN1 mRNA were similar, that is, invariant with respect to AD Braak stage (Fig. 5A). In contrast, ApoE ε4 genotype had significant inhibitory effects on RCAN1 expression both at AD Braak 3-4 (Fig. 5B) and Braak 5-6 (Fig. 5C). It is noteworthy that significant reductions RCAN1 mRNA were observed for ApoE ε3/ε4 and ε4/ε4 relative to ApoE ε3/ε3 in brains with either moderate or severe AD; however, an ApoE ε4 dose effect was not detected. In general, the effects of AD grade and ApoE genotype on RCAN1 protein expression were concordant with their effects on the mRNA. In cases with an ApoE ε3/ε3 genotype, RCAN1 protein expression progressively and significantly declined with AD Braak stage severity (Fig. 6A). In cases with an ApoE ε3/ε4 genotype, frontal lobe RCAN1 protein expression also progressively and significantly declined with AD Braak stage severity, and the levels in all AD subgroups were significantly lower than those in control (Fig. 6B). At AD Braak stage 5-6, the mean levels of RCAN1 protein were significantly and similarly reduced relative to control for all ApoE genotypes (Fig. 6C).

4. Discussion

The progressive declines in brain expression of genes and proteins required for insulin signaling as well as evidence for increased insulin resistance with severity of AD led to the concept that AD is a form of brain diabetes mellitus, that is, type 3 diabetes [6,7]. Deficiencies in insulin supply could be mediated by reduced expression of mRNA transcripts in the brain [6,7,46,48,104] or impaired transport of insulin from the peripheral circulation [108–110]; the main source of insulin in the brain is

IDE was significantly reduced relative to control (Braak stage 0-2) and AD (Braak stage 3-4) (Fig. 3A). Focusing on the effects of ApoE ε4 dose, at Braak Stages 3-4, IDE mRNA levels were significantly and similarly reduced in brains from patients with an ApoE ε3/ε4 or ε4/ε4 genotype (Fig. 3B). IDE mRNA levels were significantly reduced in brains with Braak 5-6 stage AD, irrespective of ApoE genotype (Fig. 3C). However, a modest but significant ApoE ε4 dose effect further reduced IDE expression in brains from subjects with ApoE ε4/ε4 compared with ApoE ε3/ε3 (Fig. 3C). In contrast to the mRNA levels, IDE protein expression was almost invariant with respect to AD Braak stage and ApoE genotype (Fig. 4). The only exception was that IDE protein expression was significantly elevated at AD Braak stage 3-4 from patients with ApoE ε4/ε4 compared with the other ApoE genotypes (Fig. 4B).
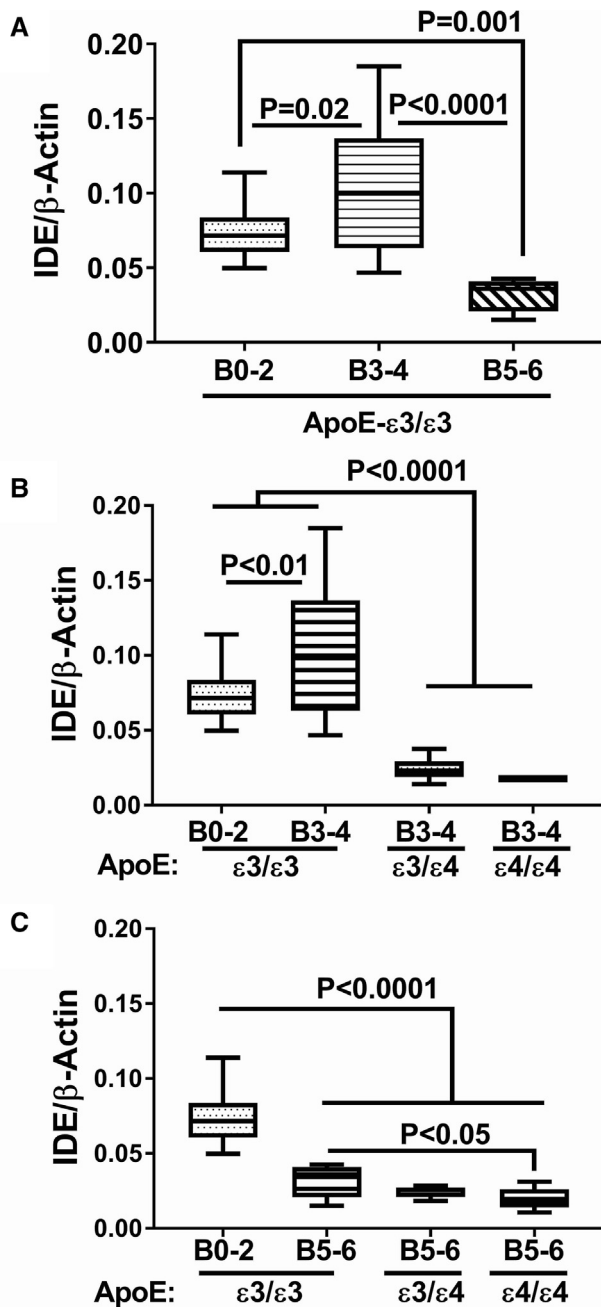
Fig. 3. Effects of AD Braak stage and ApoE genotype on IDE gene expression in human postmortem brains. Fresh frozen postmortem human frontal lobe tissue samples from cases with Braak stage [B] 0-2 (normal aging), B3-4 (moderate AD), or B5-6 (severe AD) were used to measure IDE mRNA expression by qRT-PCR analysis with results normalized to β-actin mRNA measured simultaneously in the same samples. Statistical comparisons assessed changes in IDE mRNA expression in relation to: (A) AD severity in cases with ApoE ε3/ε3 genotype, (B) ApoE genotype in cases with moderate AD (B3-4), and (C) ApoE genotype in cases with severe AD. Graphs depict box plots with mean levels of expression (horizontal bars), 95% confidence interval limits (upper and lower boundaries of the boxes), and ranges (upper and lower stems). One-way ANOVA with post hoc repeated measures Tukey tests were used for intergroup comparisons. Abbreviations: AD, Alzheimer's disease; ANOVA, analysis of variance; ApoE, apolipoprotein E; IDE, insulin-degrading enzyme; qRT-PCR, quantitative reverse transcriptase polymerase chain reaction.

thought to be from the peripheral circulation [97]. In addition, insulin resistance marked by reduced receptor responsiveness also contributes to neurodegeneration due to the associated impairments in downstream signaling through multifaceted PI3K-Akt pathways. Insulin resistance can be driven by insulin deficiency due to trophic factor withdrawal–mediated injury or killing of insulin responsive (receptor-bearing) cells or failure of systems that mediate insulin uptake. Regardless of the mechanism, insufficient supply of insulin and impaired insulin receptor responsiveness compromise glucose utilization, which is needed for energy metabolism, neuronal plasticity, cognition, cell survival, mitochondrial function [16,19], and inhibition of neuroinflammation and oxidative stress, all of which are linked to AD neurodegeneration, that is, aberrant phosphorylation of tau and Aβ accumulation in the brain [16,19]. These concepts are supported by findings in AD that working memory and cognition are improved by the administration of intranasal insulin [92,93,95,111–113] and suggest that one of the core avenues for preventing or reducing severity of neurodegeneration is to increase understanding of the causes of brain insulin deficiency.

Potential roles for aberrant IDE and RCAN1 expression as mediators of brain insulin deficiency and ultimately insulin resistance in AD have been suggested due to their known roles in Down syndrome–associated neurodegeneration [114,115] and diabetes mellitus [116]. However, studies in humans and experimental animals have led to inconsistent and inconclusive findings. Herein, we have readdressed this question by simultaneously examining cerebral mRNA and protein levels of IDE and RCAN1 in an established i.c. STZ rat model of sporadic AD [46,48,76,81,117] and in histopathologically well-characterized human postmortem brains with normal aging, moderately severe AD (Braak 3-4), or advanced AD (Braak 5-6) pathology from patients with an ApoE ε3/ε3, ε3/ε4, or ε4/ε4 genotype.

In a previous study, we confirmed that the i.c. STZ model was associated with increased temporal lobe levels of pTau, ubiquitin, Aβ, and SNAP-25 and reduced expression of synaptophysin as in AD [6,7,82,104]. In addition, i.c. STZ significantly reduced the mean levels of Akt, p70S6K, mechanistic target of rapamycin, and phosphorylated glycogen synthase kinase 3β [11,17,18,48,118], which would compromise both insulin and IGF-1 signaling through neuronal and glial metabolic pathways, as well as account for the observed increases in brain levels of pTau and Aβ in the i.c. STZ model.

Our studies demonstrated moderately but significantly higher levels of IDE and RCAN1 mRNA and more pronounced increases in the corresponding proteins in temporal lobe tissue from the i.c. STZ-treated relative to control rats. The finding of increased RCAN1 expression is consistent with previous observations in human AD brain neurons and Down syndrome brains [23,25,26,119,120] and supports the concept that upregulated RCAN1 expression can have a role in neurodegeneration.
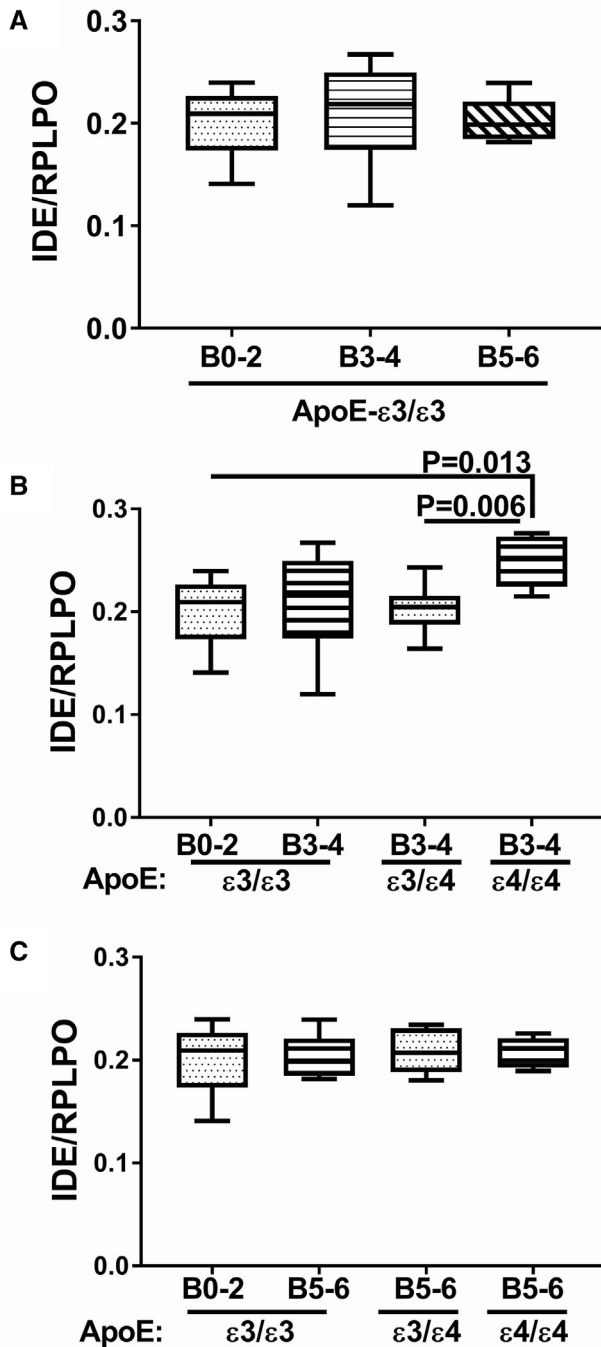
Fig. 4. Effects of AD Braak stage and ApoE genotype on IDE protein expression in human postmortem brains. Fresh frozen postmortem human frontal lobe tissue samples from cases with Braak stage (B) 0-2 (normal aging), B3-4 (moderate AD), or B5-6 (severe AD) were used to measure IDE protein expression by duplex ELISA with results normalized to RPLPO measured simultaneously in the same samples. Graphs depict box plots with mean levels of expression (horizontal bars), 95% confidence interval limits (upper and lower boundaries of the boxes), and ranges (upper and lower stems). Statistical comparisons assessed changes in IDE protein expression in relation to: (A) AD severity in cases with ApoE $\varepsilon 3/\varepsilon 3$ genotype, (B) ApoE genotype in cases with moderate AD (B3-4), and (C) ApoE genotype in cases with severe AD. One-way ANOVA with post hoc repeated measures Tukey tests were used for intergroup comparisons. Abbreviations: AD, Alzheimer's disease; ANOVA, analysis of variance; ApoE, apolipoprotein E; IDE, Insulin-degrading enzyme; RPLPO, ribosomal protein; ELISA, enzyme-linked immunosorbent assays; RPLPO, ribosomal protein.
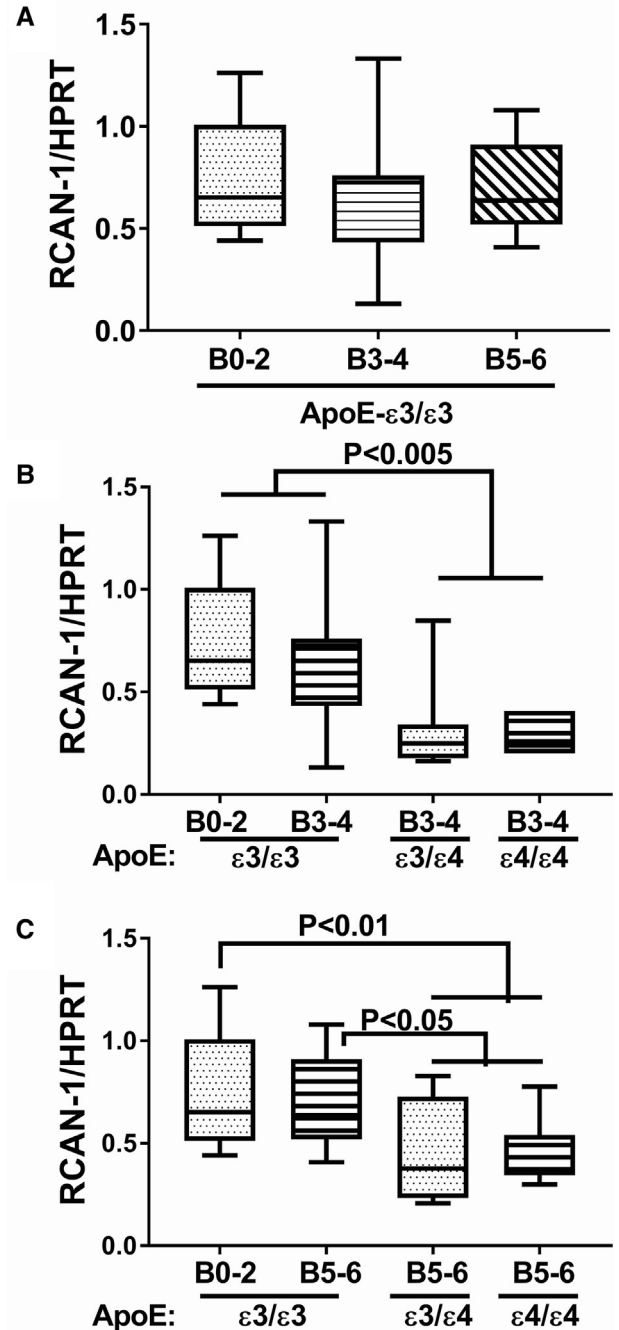
Fig. 5. Effects of AD Braak stage and ApoE genotype on RCAN1 gene expression in human postmortem brains. Fresh frozen postmortem human frontal lobe tissue samples from cases with Braak stage (B) 0-2 (normal aging), B3-4 (moderate AD), or B5-6 (severe AD) were used to measure RCAN1 mRNA expression by qRT-PCR analysis with results normalized to HPRT mRNA measured simultaneously in the same samples. Graphs depict box plots with mean levels of expression (horizontal bars), 95% confidence interval limits (upper and lower boundaries of the boxes), and ranges (upper and lower stems). Statistical comparisons assessed changes in RCAN-1 mRNA expression in relation to: (A) AD severity in cases with ApoE $\varepsilon 3/\varepsilon 3$ genotype, (B) ApoE genotype in cases with moderate AD (B3-4), and (C) ApoE genotype in cases with severe AD. One-way ANOVA with post hoc repeated measures Tukey tests were used for intergroup comparisons. Abbreviations: AD, Alzheimer's disease; ANOVA, analysis of variance; ApoE, apolipoprotein E; qRT-PCR, quantitative reverse transcriptase polymerase chain reaction; RCAN1, regulator of calcineurin 1; HPRT, hypoxanthine phosphoribosyltransferase.
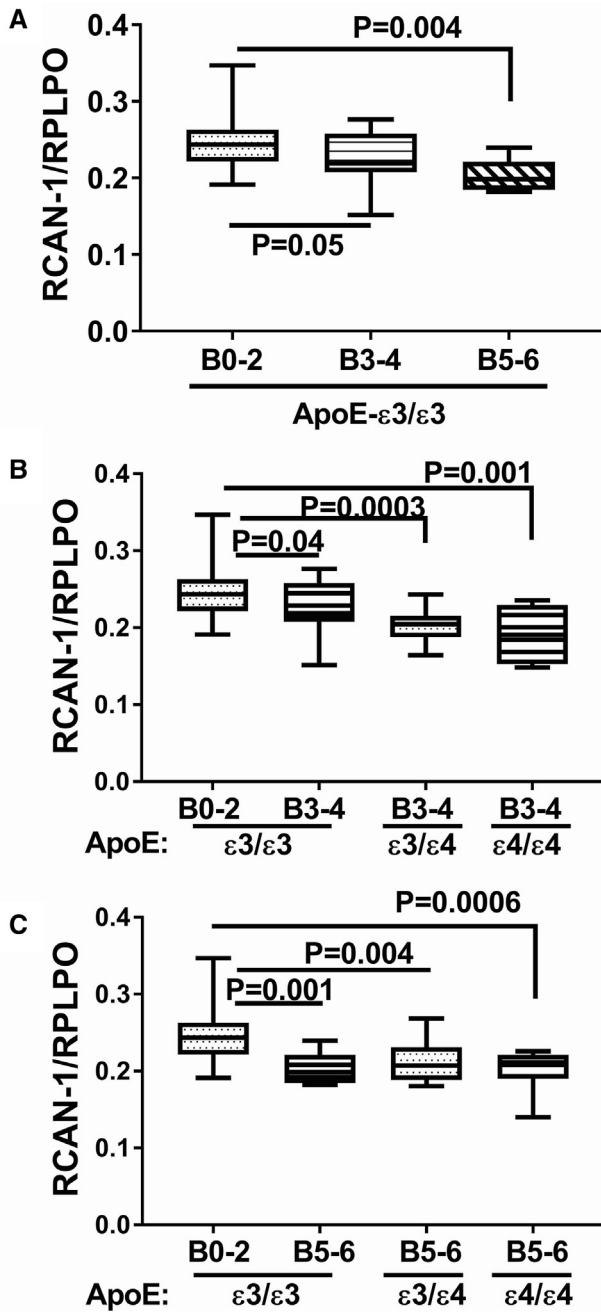
Fig. 6. Effects of AD Braak stage and ApoE genotype on RCAN1 protein expression in human postmortem brains. Fresh frozen postmortem human frontal lobe tissue samples from cases with Braak stage (B) 0-2 (normal aging), B3-4 (moderate AD), or B5-6 (severe AD) were used to measure RCAN-1 protein expression by duplex ELISA with results normalized to RPLPO measured simultaneously in the same samples. Statistical comparisons assessed changes in RCAN-1 protein expression in relation to: (A) AD severity in cases with ApoE ε3/ε3 genotype, (B) ApoE genotype in cases with moderate AD (B3-4), and (C) ApoE genotype in cases with severe AD. Graphs depict box plots with mean levels of expression (horizontal bars), 95% confidence interval limits (upper and lower boundaries of the boxes), and ranges (upper and lower stems). One-way ANOVA with post hoc repeated measures Tukey tests were used for intergroup comparisons. Abbreviations: AD, Alzheimer's disease; ANOVA, analysis of variance; ApoE, apolipoprotein E; ELISA, enzyme-linked immunosorbent assays; IDE, insulin-degrading enzyme; RCAN1, regulator of calcineurin 1; HPRT, hypoxanthine phosphoribosyltransferase; RPLPO, ribosomal protein.

Mechanistically, increased RCAN1 promotes GSK-3β activity [55,56], which increases tau phosphorylation [57]. Mitochondrial dysfunction, oxidative stress, and neuroinflammation promoted by i.c. STZ [25,74,75] could also have increased RCAN1 expression and thereby further activate GSK-3β–driven pathogenic processes, including tau phosphorylation.

The significantly elevated temporal lobe expression levels of IDE mRNA and protein observed in our i.c. STZ rat model discordant with previous findings in closely related in vivo models [79,116], STZ-treated astrocyte cultures [121], transgenic AD mouse models [122–124], and human AD gene linkage studies [125]. On the other hand, the responses were concordant with findings in human AD brains [126,127], genetic models of AD [128], and experimental i.c. STZ [129] or toxin [130] exposure models, and they were partially concordant with experimental models of insulin resistance and diabetes mellitus [131]. Variability in IDE responses could be linked to the genotypic or phenotypic features of the models or the specific experimental conditions used to generate the models. For example, genetic models that caused high levels of Aβ or that used high doses of i.c. STZ (4 mg/rat) with shorter intervals to study (2 weeks) than in the present study (0.3-0.4 mg/rat and 6 weeks) reported inhibition of IDE. Based on the experimental conditions used herein, we conclude that brain insulin deficiency and resistance in the i.c. STZ could be mediated by upregulated expression of RCAN1 and IDE.

Besides neurons, microglia and astrocytes are potential sources of RCAN1 and IDE [126,132,133] expression. IDE and RCAN1 expression have been detected in activated microglia and astrocytes by immunohistochemical staining and analysis of cultured cells [49,132–134]. In human AD and the i.c. STZ model, increased activation of microglia and astrocytes is at the core of the neuroinflammation, which is linked to many aspects of neurodegeneration [19]. In this study, the finding that the temporal lobes of i.c. STZ-treated rats had constitutively increased levels of RCAN1 and IDE expression may suggest that these responses could serve as etiopathic cofactors of brain insulin deficiency. We propose that oxidative stress driven by progressive neuroinflammatory and gliotic pathologies may have a role in activating RCAN1 and IDE, thereby causing brain insulin deficiency in the setting of progressive insulin resistance in AD, Down syndrome, and i.c. STZ models, that is, type 3 diabetes.

In contrast to the i.c. STZ model, the human brain studies demonstrated reduced RCAN1 expression in AD at both mRNA and protein levels, with the major inhibitory effects associated with ApoE-ε4 dose rather than severity of AD. These findings contrast with the i.c. STZ results and with earlier studies demonstrating higher levels of RCAN1 in AD, Down syndrome, and Down syndrome + AD relative to normal control brains [25,119,120]. However, the outcome differences could be attributed in part to the facts that in one study, the investigators used in situ approaches to assess neuronal labeling [119], yet RCAN1 is expressed in

various cell types including activated microglia and astrocytes [119,135,136], and in another article, the analysis was focused on Down syndrome and Down syndrome + AD [120] rather than sporadic AD or AD associated with the ApoE ε4 allele. The reductions in RCAN1 expression demonstrated by both qRT-PCR analysis and ELISAs strengthen the conclusion that RCAN1 expression declines with severity of AD but more strikingly with ApoE ε4 dose. Conceivably, the disparate observations that the i.c. STZ model and human AD could reflect could be explained by differences in disease durations and the greater complexity of AD versus i.c. STZ-mediated neurodegeneration. To address the question of timing, that is, disease duration, future studies should assess RCAN1 expression in very early stages of AD to determine if the levels initially increase but subsequently decline, perhaps as a compensatory mechanism to preserve brain metabolic homeostasis. Correspondingly, elevated RCAN1 expression has been detected in peripheral blood lymphocytes of asymptomatic healthy young carriers of ApoE-ε4 [137].

In the present study of human brains, IDE mRNA was upregulated in moderate AD (Braak 3-4) among patients with ApoE ε3/ε3 genotype, whereas IDE protein expression was significantly elevated only in patients with Braak 3-4 AD and an ApoE ε4/ε4 genotype. Importantly, the findings suggest that IDE upregulated expression in AD occurs at intermediate stages of neurodegeneration and at least partly linked to ApoE ε4. IDE's upregulation in the earlier rather than late stages of AD could account for insulin depletion in the brain. On the other hand, its downregulation in the later stages of AD could be tied to accumulations of Aβ with disease progression, particularly in ApoE ε4 carriers or homozygotes. In previous studies, variability in findings pertaining to alterations in IDE expression [127], with some studies reporting increased IDE in AD brains [128,138] and others showing reduced [122,123,139] or selective cellular increases [126] in IDE, could have been due to heterogeneity in AD stage and ApoE ε4 genotypes. Although genetic linkage studies failed to demonstrate significant or generalizable associations between IDE and AD [138], several polymorphisms have been shown to either increase or decrease AD risk [139–141].

5. Conclusions

The findings in this study illustrate that in RCAN1 and IDE, mRNA and protein expressions are both altered in the i.c. STZ model of sporadic AD and in humans with AD, particularly when associated with ApoE ε4. In the i.c. STZ model, which exhibits brain pathology and neurobehavioral deficits that correspond to the early or intermediate stages of AD, both RCAN and IDE expression were upregulated at mRNA and protein levels. These results suggest that several key molecular, biochemical, and structural abnormalities including tau phosphorylation, reduced brain insulin levels, and oxidative stress/neuroinflammation in the early stages of AD could be mediated in part by increased expression of RCAN and/or

IDE, similar to the findings in Down syndrome. Corresponding with results obtained with the i.c. STZ model, increased IDE expression was detected in brains with moderate (Braak 3-4) but not advanced (Braak 5-6) AD, suggesting that IDE plays a role in insulin deficiency and attendant insulin resistance in the early and intermediate stages of AD. However, in contrast, human brains with moderate or advanced AD mainly exhibited reductions in RCAN and IDE expression, which may represent compensatory homeostatic responses mobilized to maintain brain function.

RESEARCH IN CONTEXT

1. Systematic review: The authors searched national and international biomedical literature databases, including PubMed and Google Scholar to perform a systematic review of the current state of knowledge related to the main scientific questions. The database searches were from 1990 to 2019.

2. Interpretation: The authors have objectively interpreted their results and declare that their findings contribute to the accumulated knowledge base because:

   a. The work covers new territory about potential mechanisms of brain insulin deficiency in Alzheimer's disease (AD).

   b. In an established rat model of sporadic AD in which intracerebral streptozotocin produces all typical features of AD, including brain insulin deficiency and insulin resistance, temporal lobe levels of both insulin degrading enzyme (IDE) and regulator of calcineurin-1 (RCAN1) were significantly elevated with neurodegeneration.

   c. In humans, altered expression of IDE and RCAN1 shifted with apolipoprotein E (ApoE-ε4) genotype dose and severity of neurodegeneration, with higher levels of IDE correlating with earlier stages of disease when brain insulin levels decline.

3. Future directions: Future research directions should mechanistically assess the roles IDE and RCAN1 play in neurodegeneration by determining if their inhibition favorably alters the course of neurodegeneration.

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