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Insulin and adipokine signaling and their cross-regulation in postmortem human brain

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ABSTRACT

Aberrant insulin and adipokine signaling has been implicated in cognitive decline associated with both type 2 diabetes mellitus and neurodegenerative diseases. We established methods that reliably measure insulin, adiponectin and leptin signaling, and their crosstalk, in thawed postmortem mid-frontal cortical tissue from cognitively normal older subjects with a short postmortem interval. Insulin-evoked insulin receptor (IR) activation increases activated, tyrosine-phosphorylated IRβ on tyrosine residues 960, 1150, and 1151, insulin receptor substrate-1 recruitment to IRβ and phosphorylated RAC-α-serine/threonine-protein kinase. Adiponectin augments, but leptin inhibits, insulin signaling. Adiponectin activates adiponectin receptors to induce APPL1 binding to adiponectin receptor 1 and 2 and T-cadherin and downstream adenosine monophosphate–dependent protein kinase phosphorylation. Insulin inhibited adiponectin-induced signaling. In addition, leptin-induced leptin receptor (OB-R) signaling promotes Janus kinase 2 recruitment to OB-R and Janus kinase 2 and downstream signal transducer and activator of transcription 3 phosphorylation. Insulin enhanced leptin signaling. These data demonstrate insulin and adipokine signaling interactions in human brain. Future studies can use these methods to examine insulin, adiponectin, and leptin metabolic dysregulation in aging and disease states, such as type 2 diabetes and Alzheimer's disease–related dementias.

1. Introduction

Type 2 diabetes mellitus (T2D) is an established risk factor for cognitive decline and dementia in later life (Cheng et al., 2012; Gudala et al., 2013). The neurobiological mechanisms for this increased risk remain uncertain. Systemic insulin resistance with resultant hyperglycemia best characterizes T2D, most often occurring in the setting of overweight and obesity states. Obesity is associated with derangement in adipocyte release and action of adipokines, including adiponectin and leptin. In peripheral tissues, there is extensive crosstalk between insulin, adiponectin, and leptin signaling pathways (Kadowaki et al., 2006; Knights et al., 2014; Kwon and Pessin, 2013; Yadav et al., 2013). However, relatively little is known about the actions of these hormones in the brain (Bloemer et al., 2018; Jovanovic and Yeo, 2010; Thundyil et al., 2012). The contributions from changes and interactions between insulin and adipokine signaling to aging, T2D, and various dementias including Alzheimer's disease (AD) remain largely unknown.

Expression of the receptors for adiponectin and leptin have been shown in the central nervous system (Couce et al., 1997; Kubota et al., 2007; Takeuchi et al., 2000), and adiponectin and leptin are measurable in cerebrospinal fluid, indicating that these hormones are released from adipose tissue and may communicate directly within the brain (Ebinuma et al., 2007; Nam et al., 2001; Neumeier et al., 2007; Schwartz et al., 1996). Adiponectin and leptin are important regulators of insulin's actions (Clegg et al., 2006; Kadowaki et al., 2006; Kwon and Pessin, 2013; Paz-Filho et al., 2012). In neural cells and animal models, adiponectin potentiates insulin's effects through binding to its cognate receptors adiponectin receptor 1 (AdipoR1), adiponectin receptor 2 (AdipoR2), and T-cadherin, leading to recruitment of the signal transducer adaptor protein containing pleckstrin homology domain, phosphotyrosine-binding domain, and leucine zipper motif (APPL1) (Cheng et al.,

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2012; Deepa and Dong, 2009; Mao et al., 2006; Ruan and Dong, 2016). These initial signaling events activate adenosine monophosphate–dependent protein kinase (AMPK), peroxisome proliferator-activated receptor-α, p38 mitogen-activated protein kinase and probably other as-of-yet unknown signaling pathways (Hug et al., 2004; Thundyil et al., 2012; Yamauchi et al., 2007). Adiponectin has been shown to reduce inflammation and oxidative stress, thereby imparting cell protection (Esmaili et al., 2014; Park et al., 2008; Shrestha and Park, 2016). Reduction of adiponectin in obesity is associated with insulin resistance, dyslipidemia, and atherosclerosis in humans (Nigro et al., 2014). Nevertheless, the alterations to adiponectin and its receptors in human CNS during T2D or the pathogenesis of brain disorders and their contributions to brain insulin resistance, as in AD, remain elusive (Arnold et al., 2018; Talbot et al., 2012; Yarchoan and Arnold, 2014).

While adiponectin secretion is reduced in obese subjects, circulating leptin levels, by contrast, are increased (Considine et al., 1996; Maffei et al., 1995). Leptin suppresses appetite, promotes thermogenesis, enhances fatty acid oxidation, decreases glucose levels and reduces body weight and fat (Amitani et al., 2013; Paz-Filho et al., 2012). Although some epidemiological data suggest a negative influence of leptin on brain function (Whitmer et al., 2005), neuroprotective effects of leptin have also been observed in animals (Signore et al., 2008; Tezapsidis et al., 2009; Wan et al., 2015). There is evidence that glucose uptake by different tissues after leptin treatment is altered in a tissue-specific manner (Minokoshi et al., 1999). Given that most studies on the crosstalk between insulin and adipokines have been conducted in peripheral tissues of rodents (Berthou et al., 2011; Cusin et al., 1998; Pelleymounter et al., 1995; Pérez et al., 2004), it is essential to assess the interactions between insulin and adipokines directly in human brain tissues, especially in diseases that are poorly modeled in animals, such as AD.

In this study, we describe an ex vivo stimulation method to study insulin, adiponectin and leptin signaling, and their interactions, in human postmortem brain tissues. This method has been modified and advanced from an earlier method previously described by our group that was used successfully to study the signaling of various receptors (Hahn et al., 2006; Talbot et al., 2012; Wang et al., 2009, 2012, 2017). This method directly and reliably detected insulin, adiponectin and leptin signaling, as well as their interactions. Implementing this method in disease settings such as Alzheimer's disease–related dementias (ADRD) will help identify disease-relevant mechanisms as potential targets for effective interventions.

2. Methods

2.1. Study approval

The human brain study protocol using postmortem human brain tissues conformed to the tenets of the Declaration of Helsinki as reflected in a previous approval by the City College of New York and City University of New York Medical School human research committee. It has been determined that this project does not meet the definition of human subject research as defined by the federal regulations (45 CFR 46.102 (d) (f)) and therefore no further IRB review or approval is required.

The animal procedures comply with the National Institutes of Health *Guide for Care Use of Laboratory Animals* and were approved by the City College of New York Animal Care and Use Committee (IACUC)—Protocol#:987.1.

2.2. Description of subjects and postmortem intervals

This study was derived from the "Brain Insulin Resistance in Aging" (BIRA) study, funded by the National Institutes of Health (NIH; see Acknowledgements). Descriptive information on the brain specimens, subjects, and associated data studied are provided in the Table 1.

2.3. Validation tests of the ex vivo method in human postmortem tissue

Dose-response effects of insulin, adiponectin, and leptin were tested in approximately 10 mg of postmortem human dorsolateral prefrontal cortical (DLPFC) slices (100 μm × 100 μm × 3 mm) derived from 3 cognitively normal control subjects (2 females and 1 male) with mean age at death = 84.8 (±3.4) years and postmortem intervals (PMIs) of 7.4 (±1.2) hours. To assess the dose-dependency of the insulin effects, DLPFC slices were incubated with 0, 0.1, 1, and 10 nM insulin in oxygenated Krebs-Ringer at 37 °C for 30 minutes as described previously in our earlier studies (Talbot et al., 2012; Wang et al., 2012). The insulin signaling was determined by the levels of insulin receptor substrate-1 (IRS-1) recruited to IRβ and phosphorylation of IRβ and RAC-α-serine/threonine-protein kinase (AKT1). Similar conditions were used to test the adiponectin and leptin signaling by incubating the slices with 0, 1, 10, and 100 μg/mL recombinant human adiponectin and with 0, 1, 10, and 100 nM recombinant human leptin, respectively. The adiponectin signaling was assessed by the levels of APPL1-coupled AdipoR1, AdipoR2, and T-cadherin, as well as phosphorylation of AMPKα1/2. The leptin signaling was measured by the levels of Janus kinase 2 (JAK2)-associated leptin receptors, OB-Rs, and phosphorylation of JAK2 and signal transducer and activator of transcription 3 (STAT3).

PMI effects on insulin signaling and its regulation by adipokines, adiponectin, and leptin were measured by the responses to 1 nM insulin alone and in combination with 10 μg/mL adiponectin or 10 nM leptin in the postmortem prefrontal cortex of 2.5-month-old male Sprague-Dawley rats (Taconic Farms) and conducted similarly to the test described previously (Talbot et al., 2012). The prefrontal

Table 1
Description of subjects and postmortem intervals

Demographics	BIRA subset for ex vivo stimulation	
	Without MCI or dementia and diabetes	With MCI or dementia or diabetes
Number of cases (n)	15	63
Experimental methods study	included	excluded
History of diabetes (n, %)	0 (0.00%)	39 (61.90%)
Age at death, y (mean, STD)	87.02 (6.21)	87.23 (6.01)
Postmortem intervals, hours (mean, STD)	5.26 (2.46)	4.74 (2.05)
Sex, women (n, %)	7 (42.86%)	27 (42.86%)
White non-Latino (n, %)	15 (100.00%)	57 (90.48%)
Education, y (mean, STD)	19.07 (3.37)	18.16 (3.36)

The 15 cases included in this study are part of the subset of 78 cases from the Brain Insulin Resistance in Aging (BIRA) study in which ex vivo stimulation data are available. In the total sample used in the BIRA study (n = 150), cases were autopsied subjects (75 with diabetes and 75 without), randomly selected from large ongoing longitudinal clinical-pathologic study of aging and dementia, the Religious Orders Study (ROS). The research volunteers from ROS are community-dwelling, older (nuns, priests, and brothers) from across the United States, without known dementia at study entry, who agree to annual clinical evaluations, including detailed neuropsychological testing, and brain donation at time of death. The follow-up rate in ROS is 95%, and the autopsy rate exceeds 90% (Bennett et al., 2018). Of the 150 BIRA subjects, a total of 78 subjects had PMI <12 hours and, among these, 15 subjects for this study were specifically selected among those with normal cognition and without diabetes, and without significant neurodegenerative or other neuropathology (De Jager et al., 2018). The 15 subjects in this experimental study did not differ with the others (n = 63) on age at death (t (76) = 0.12, $p = 0.902$), PMI (t (76) = −0.85, $p = 0.400$), education (t (76) = −0.94, $p = 0.350$), sex and race (both fisher exact, $p > 0.99$).

cortex is defined as the medial frontal pole cortex (pregenual) that contains limbic and infralimbic regions. In compliance with the NIH Guide for the Care and Use of Laboratory Animals, the animals were sacrificed by quick CO_2 asphyxiation. To simulate human postmortem conditions, brains were removed quickly on ice at death (PMI, 0 hour) or after reminded in the intact body at 4 °C for 4, 8, 12 or 16 hours. In other animals, the intact body was kept at 25 °C for 4 hours before brain extraction. On removal, brains were frozen and stored at −80 °C for 4 weeks until the experimental days.

2.4. Brain slice preparation

Fresh-frozen human DLPFC tissue was gradually thawed from −80 °C to −20 °C by placing the tissues in −20 °C freezer for approximately 10 minutes; about 20 mg section was removed from the ice bed and was cut into 100 μm × 100 μm × 3 mm slices using a chilled McIlwain tissue chopper. Slices equaling about 20 mg of tissue were suspended in 1 mL ice-cold oxygenated Krebs-Ringer solution (K-R) containing 25 mM HEPES (pH 7.4), 118 mM NaCl, 4.8 mM KCl, 1.3 mM $CaCl_2$, 1.2 mM KH_2PO_4, 1.2 mM $MgSO_4$, 25 mM $NaHCO_3$, 10 mM glucose, 100 μM ascorbate, EDTA-free protease inhibitor cocktail (Roche, 04693159001), and 0.01 U/mL soybean trypsin inhibitor and centrifuged briefly. After 2 additional washes with 1 mL ice-cold K-R, brain slices were suspended in 1 mL K-R.

2.5. Assessment of Ca^{2+} influx as the indices of mitochondrial function and cell death

Because the level of voltage-gated Ca^{2+} channel activity indicates the physiological integrity of the cells, depolarization-induced Ca^{2+} influx in the postmortem rat prefrontal cortex slices was used to assess the PMI effects on cell integrity. The PMI effects on depolarization-induced Ca^{2+} influx and insulin signaling were then compared with ascertain whether postmortem tissues retain sufficient mitochondrial function and responsiveness to insulin stimulation. As described previously with slight modification (Wang et al., 2010, 2012), approximately 5 mg of thawed postmortem rat prefrontal cortical slices (100-μm × 100-μm × 3-mm) were incubated at 37 °C for 5 min in oxygenated K-R that have 1.3 mM Ca^{2+} containing 5 μCi $^{45}Ca^{2+}$ (10 Ci/mmol) followed by incubation with vehicle, or 65 mM K^+ (made with isomolar replacement of Na^+) for 1 minute in a total incubation volume of 0.5 mL. The reaction was terminated by the addition of 0.5 mL of ice-cold Ca^{2+}-free K-R containing 0.5 mM EGTA and centrifugation at 4 °C. After 2 additional washes, $^{45}Ca^{2+}$ contents in brain slices were solubilized in 1% Triton-X100 and assessed using scintillation spectrometry (Beckman). Background $^{45}Ca^{2+}$ was estimated using lysed brain slices. Basal Ca^{2+} influx was defined as the $^{45}Ca^{2+}$ levels in the Krebs-Ringer incubated brain slices subtracting background $^{45}Ca^{2+}$ count. Depolarization-induced Ca^{2+} influx was defined as the $^{45}Ca^{2+}$ contents in the 65 mM K^+-incubated brain slices subtracting background $^{45}Ca^{2+}$ count. The percentage increase in Ca^{2+} influx was calculated as percentage [(K^+-depolarization treated-Krebs-Ringer)/Krebs-Ringer].

2.6. Ex vivo insulin stimulation

Approximately 20 mg of thawed postmortem human DLPFC slices were incubated for 30 minutes at 37 °C with vehicle (K-R) alone or with vehicle plus recombinant human insulin (Invitrogen 12,585-014), recombinant human adiponectin (#4901, Biovision) or recombinant human leptin (CYT-338, Prospec). The incubation mixture (total volume, 0.5 mL) was aerated every 10 minutes with 95% O_2 and 5% CO_2 for 1 minute. Signaling was terminated with 1.5 mL ice-cold Ca^{2+}-free K-R. The tissue was collected by brief centrifugation and homogenized in 250 μL ice-cold immunoprecipitation buffer (25 mM HEPES, pH 7.5; 200 mM NaCl; 1 mM EDTA; protease inhibitor cocktail (Roche), 5 mM NaF; 1 mM sodium vanadate; 0.5 mM β-glycerophosphate; and 0.02% 2-mercaptoethanol). Homogenates were centrifuged at 1000 g for 5 minutes at 4 °C, and the resultant supernatant (postmitochondrial fraction) was sonicated for 10 seconds on ice. Proteins were then solubilized in immunoprecipitation buffer containing 0.5% digitonin, 0.2% sodium cholate, and 0.5% NP-40 for 60 minutes at 4 °C with end-to-end rotation. The resultant lysates were then cleared by centrifugation at 50,000 g for 5 minutes and diluted with 750 μL immunoprecipitation buffer. Protein concentrations were measured using the Bradford method (Bio-Rad).

2.7. Immunoprecipitation

Insulin, adiponectin, and leptin signaling were each assessed separately by isolating the proteins of interest, 200 μg of tissue lysates were immunoprecipitated overnight at 4 °C with 1.0–1.5 μg of antibodies to IRβ and AKT1 (insulin signaling), APPL1 and AMPKα1/2 (adiponectin signaling), and JAK2 and STAT3 (leptin signaling) covalently immobilized onto protein A/G–agarose beads (Pierce Thermo). The antibodies used are shown in Table 2. Immunoprecipitates were incubated with 75 μL antigen elution buffer pH 2.8 (Pierce Thermo) and 2% SDS for 2 minutes at room temperature, centrifuged to remove antibody–protein A/G–agarose complexes, and neutralized immediately with 10 μL of 1.5 M Tris buffer (pH 8.8) followed by addition of 65 μL 2× PAGE sample buffer and boiled for 5 minutes. Supernatants were probed for any residual target proteins, which showed that greater than 90% of the target proteins were immunoprecipitated in all subjects (Fig. 1).

2.8. Western blotting

Solubilized immunoprecipitates (IPs) derived from 100 μg brain slice lysates were size fractionated on 7.5% or 10% SDS-PAGE, and then electrophoretically transferred to nitrocellulose membranes. Membranes were washed with PBS, blocked for 2 hours at room temperature with 10% nonfat milk in PBS containing 0.1% Tween-20 (PBST), and washed 3× in 0.1% PBST (2 minutes each). To assess protein activation, membranes loaded with the aforementioned IPs were incubated overnight at 4 °C with antibodies (Table 3) to $pY^{1150/1151}$IRβ, pY^{960}IRβ, IRS-1, pS^{473}AKT1, pT^{308}AKT1, AdipoR1, AdipoR2, T-cadherin, $pT^{183/172}$AMPKα1/2, APPL1, $pY^{1007/1008}$JAK2, OB-R, or pY^{705}STAT3. To assess total protein levels as well as IRS-1 recruited to IRβ, membranes were stripped, washed, and blocked with 10% nonfat milk and reprobed overnight at 4 °C with antibodies (Table 3) to IRβ, IRS-1, AKT1, AdipoR2, APPL1, JAK2, or STAT3. After initial probing and again after reprobing, membranes were washed 3× in 0.1% PBST (2 minutes each), incubated for 1 hour with 1:5000

Table 2
Antibodies used for immunoprecipitation

Antigen	Antibody (Ab)	Ab type	Ab/200 μg lysate
AKT1	Santa Cruz 5298	Mouse mAb	1.2 μg
AMPKα1/2	Santa Cruz 74461	Mouse mAb	1.0 μg
APPL1	Santa Cruz 67402	Rabbit pAb	1.2 μg
JAK2	Santa Cruz 294	Rabbit pAb	1.2 μg
IRβ	Santa Cruz 20739	Rabbit pAb	1.0 μg
STAT3	Santa Cruz 7179	Rabbit pAb	1.0 μg

Key: AMPK, adenosine monophosphate–dependent protein kinase; APPL1, adaptor protein containing pleckstrin homology domain phosphotyrosine-binding domain and leucine zipper motif; JAK2, Janus kinase 2; IR, insulin receptor; STAT3, signal transducer and activator of transcription 3.

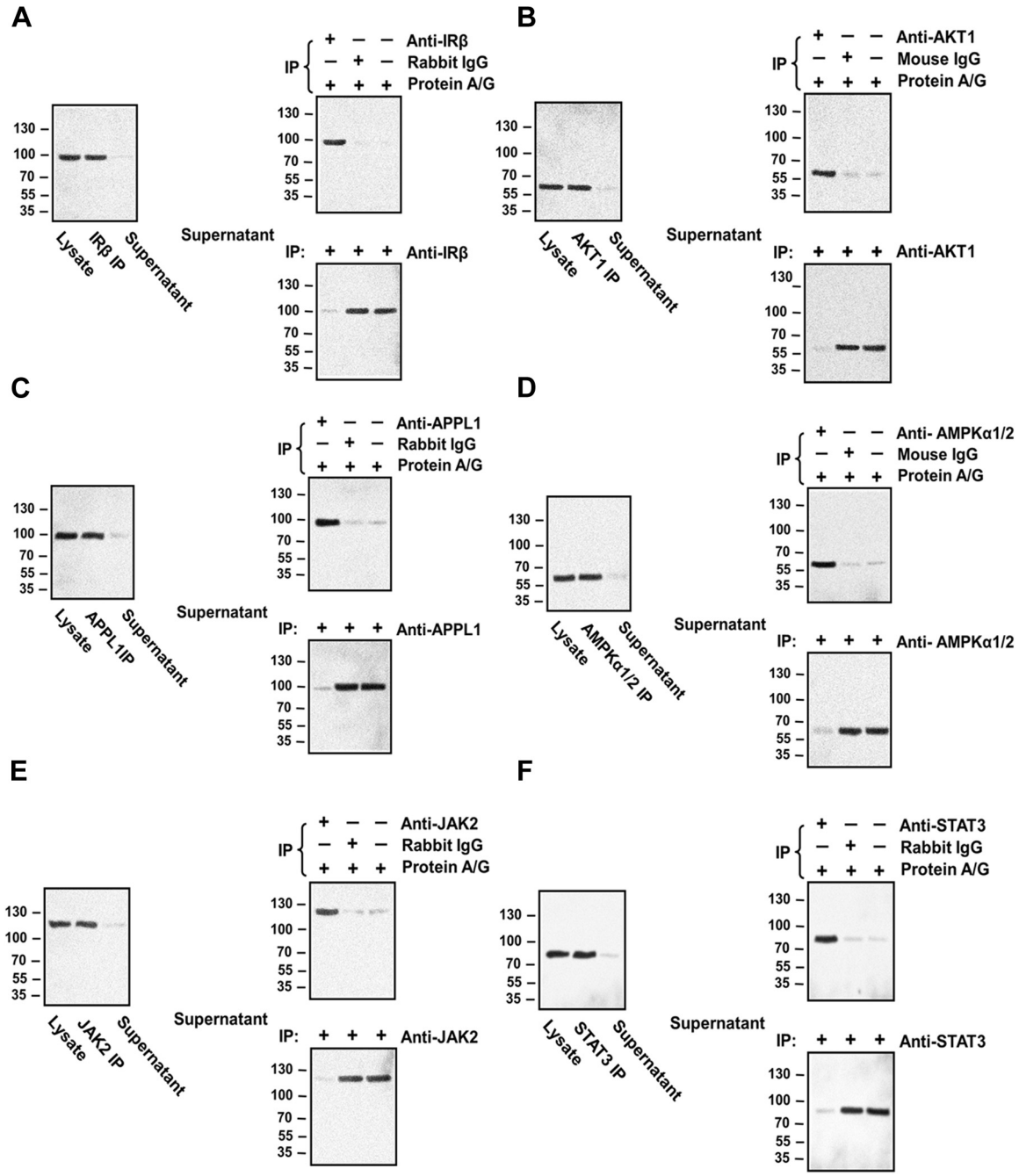

Fig. 1. Representative western blots verifying completeness and specificity of immunoprecipitation for the frontal cortical proteins studied in postmortem human brains: IRβ (A), AKT1 (B), APPL1 (C), AMPKα1/2 (D), JAK2 (E), and STAT3 (F). Three postmortem human frontal cortices from control subjects were chosen for testing. Results shown are the representative of 3 samples. The lanes in each blot show relative amount of antigen in the tissue lysate, the immunoprecipitate (IP) of that lysate, and the remaining supernatant. The solubilized lysate was used to assess the specificity of immunoprecipitation using indicated antibody, IgG, and protein A/G beads. Furthermore, the resultant supernatant after immunoprecipitation was IP with specific antibodies. Results show at least 90% of each antigen was immunoprecipitated and each antibody was specific to the antigen indicated. N = 3. Abbreviations: AMPK, adenosine monophosphate–dependent protein kinase; APPL1, adaptor protein containing pleckstrin homology domain phosphotyrosine-binding domain and leucine zipper motif; JAK2, Janus kinase 2; IR, insulin receptor; STAT3, signal transducer and activator of transcription 3.

dilution of species-appropriate, HRP-conjugated secondary antibodies, and washed 3× in 0.1% PBST baths (2 minutes each). Immunoreactivity was visualized by reaction in ECL Plus chemiluminescent reagent for exactly 5 minutes, followed by exposure of the blot to X-ray film. Bands at the relevant molecular masses were quantified using a GS-800 calibrated densitometer (Bio-Rad Laboratories). Ratios of phosphorylated to total levels of each antigen were calculated.

3. Results

3.1. The completeness and specificity of immunoprecipitation

The completeness and specificity of immunoprecipitation for each antigen protein of interest in the insulin, adiponectin, and leptin signaling cascades were assessed. In Fig. 1, each antigen was demonstrated in representative western blots to verify that

Table 3
Antibodies used for western blotting

Antigen	Antibody (Ab)	Ab type	Ab dilution
AdipoR1	Santa Cruz 518,030	Mouse mAb	1:1000
AdipoR2	Santa Cruz 514045	Mouse mAb	1:1000
AKT1-3	Santa Cruz 8312	Rabbit pAb	1:500
pS^{473}AKT1-3	Santa Cruz 514032	Mouse mAb	1:750
pT^{308}AKT1-3	Santa Cruz 271964	Mouse mAb	1:750
AMPKα1/2	Santa Cruz 74461	Mouse mAb	1:750
$pT^{183/172}$AMPKα1/2	Abcam 23875	Rabbit pAb	1:1000
APPL1	Santa Cruz 271,909	Mouse mAb	1:750
IRβ	Santa Cruz 81465	Mouse mAb	1:500
pY^{960}IRβ	Invitrogen 44-800G	Rabbit pAb	1:1000
$pY^{1150/1151}$IRβ	Santa Cruz 81500	Mouse mAb	1:1000
IRS-1	Santa Cruz 8038	Mouse mAb	1:750
JAK2	Santa Cruz 390,539	Mouse mAb	1:750
$pY^{1007/1008}$JAK2	Cell Signaling #3776	Rabbit mAb	1:1000
OB-R	Santa Cruz 8391	Mouse mAb	1:750
STAT3	Santa Cruz 8019	Mouse mAb	1:750
pY^{705}STAT3	Santa Cruz 8059	Mouse mAb	1:750
T-cadherin	Santa Cruz 166875	Mouse mAb	1:750

Key: AdipoR1, adiponectin receptor 1; AdipoR2, adiponectin receptor 2; AMPK, adenosine monophosphate–dependent protein kinase; APPL1, adaptor protein containing pleckstrin homology domain phosphotyrosine-binding domain and leucine zipper motif; OB-R, leptin receptor; JAK2, Janus kinase 2; IR, insulin receptor; STAT3, signal transducer and activator of transcription 3; pY, phosphorylated; IRS-1, insulin receptor substrate-1.

immunoprecipitation with anti-IRβ (A), -AKT1 (B), -APPL1 (C), -AMPKα1/2 (D), -JAK2 (E), and -STAT3 (F) was complete and specific. The completeness of immunoprecipitation was shown by the comparable amount of antigen in the tissue lysate and the IP of that lysate with negligible antigen observed in the remaining supernatant (left panel in each group). As the supernatants of the IP show, greater than 90% of each antigen was immunoprecipitated. The specificities of the antibodies for IRβ (A), -AKT1 (B), -APPL1 (C), -AMPKα1/2 (D), -JAK2 (E), and -STAT3 (F) were demonstrated by the data showing that nonimmune rabbit IgG and protein A/G beads brought down negligible amounts of IRβ, AKT1, APPL1, AMPKα1/2, JAK2, and STAT3 (right upper panel in each group). The fact that the remaining supernatant from nonimmune rabbit IgG and protein A/G beads recovered comparable amounts of IRβ and AKT1 with the anti-IRβ and anti-AKT1 IPs further confirms the completeness of these immunoprecipitations (right lower panel in each group).

3.2. Insulin, adiponectin, and leptin dose-dependently activate their cognate receptors in human postmortem brain tissues

To test the effects of insulin in activating insulin receptor, thawed postmortem DLPFC slices from 3 cognitively normal elderly subjects were incubated ex vivo with 0.1–10 nM of human recombinant insulin for 30 minutes. Lysate prepared from the Krebs-Ringer and insulin-incubated DLPFC slices showed that 0.1–10 nM insulin induced clear concentration-dependent activation of insulin receptor signaling. Increasing insulin concentrations resulted in increasing phosphorylation of the tyrosine residues: Y^{1150}, Y^{1151}, and Y^{960} in the IRβ leading to recruitment of IRS-1 (Fig. 2) and activation of the downstream signaling molecule, AKT1, indicated by an increase in phosphorylation of the serine (pS^{473}) and threonine (pT^{308}) residues in AKT1. These results suggest that stimulation with 1 nM of insulin is adequate for evaluating IR responses in the follow-up studies (Fig. 2).

The effects of adiponectin on activating adiponectin receptor signaling were tested in DLPFC slices by ex vivo incubation with 1–100 μg/mL human recombinant adiponectin. In response to varying concentrations of adiponectin, association of APPL1 with AdipoR1, AdipoR2, and T-cadherin was increased in a concentration-dependent manner. The adiponectin dose-response curve for the increased APPL1 and adiponectin receptor complexes parallels that of activation of the downstream AMPKα1/2 indicated by increasing $pT^{183/172}$AMPKα1/2 (Fig. 3). These data collectively indicate 10 μg/mL of adiponectin, a concentration well within physiological levels, is suitable for the assessment of adiponectin-elicited effects in the later investigations.

The effects of leptin on leptin receptor (OB-R) signaling were assessed in DLPFC slices by ex vivo incubation with 1–100 nM recombinant human leptin. Exposure to 1–100 nM of leptin led to dose-dependent increases in coupling of long- and short-form OB-Rs with JAK2 and activation of JAK2 and STAT3 as indicated by the increases in $pY^{1007/1008}$JAK2 and pY^{505}STAT3, respectively (Fig. 4). These data indicate that 10 nM of leptin is appropriate can be used to probe the biological effects of leptin.

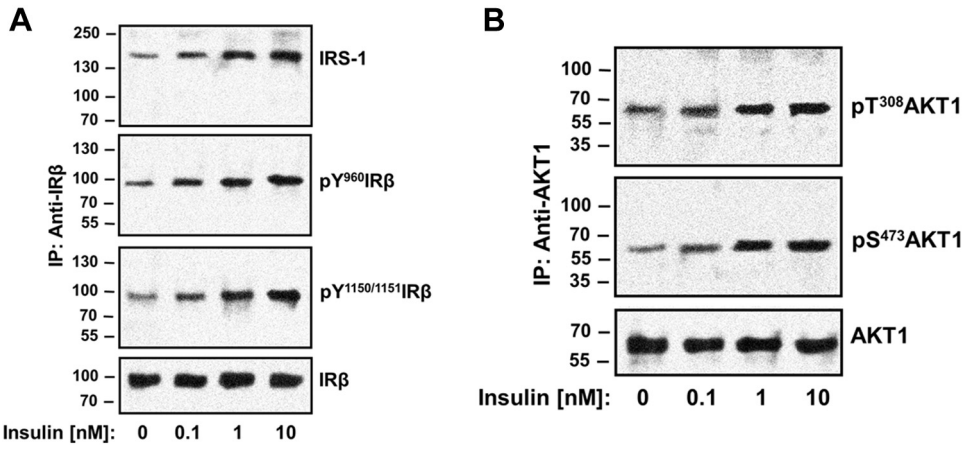

Fig. 2. Dose-dependency of insulin signaling in postmortem human frontal cortices. Frontal cortical slices from neurologically normal subjects with postmortem interval <12 hours were used to determine insulin signaling induced by 0.1–10 nM recombinant human insulin. After incubation with varying concentrations of insulin for 30 minutes at 37 °C, brain slices were collected, solubilized, and immunoprecipitated with immobilized anti-IRβ or -AKT1 antibodies. The contents in resultant immunoprecipitates were analyzed by western blotting. Insulin dose-dependently increased phosphorylation of tyrosine residues: 1150/1151 and 960 of the insulin receptor β subunit (IRβ): $pY^{1150/1151}$ and pY^{960} as well as recruitment of insulin receptor substrate-1 (IRS-1) to IRβ (A) and phosphorylation of AKT1 at the $serine^{473}$ (by mTOR2) and $threonine^{308}$ (by PDK1) (B). The blots were stripped and reprobed with anti-IRβ or -AKT1 to ascertain comparable IRβ and AKT1 were immunoprecipitated, respectively. These data indicate that postmortem brain tissues with short postmortem interval are responsive to insulin can be reliably used to assess changes in insulin receptor activities and signaling. N = 3. Abbreviations: IR, insulin receptor; pY, tyrosine-phosphorylated.

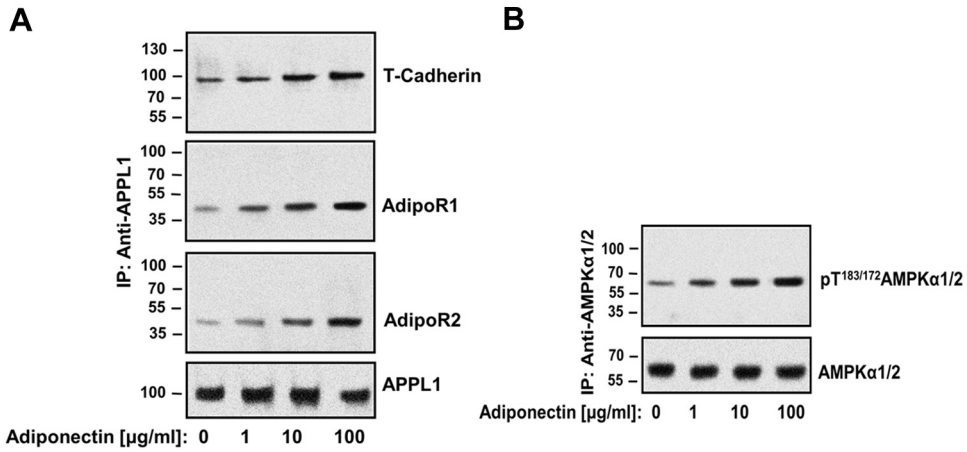

Fig. 3. Dose-dependency of adiponectin signaling in postmortem human frontal cortices. Frontal cortical slices from neurologically normal subjects with postmortem interval <12 hours were used to determine insulin signaling induced by 1–100 μg/mL recombinant human adiponectin. After incubation with varying concentrations of adiponectin for 30 minutes at 37 °C, brain slices were collected, solubilized, and immunoprecipitated with immobilized anti-APPL1 or–AMPKα1/2 antibodies. The contents in resultant immunoprecipitates were analyzed by western blotting. Adiponectin dose-dependently increased association of the adiponectin receptor subtypes: AdipoR1, AdipoR2, and T-cadherin with signaling adaptor protein, APPL1 (A) as well as in the phosphorylation of AMPKα1/2 at the threonine[183] and threonine[172] of the AMPK (B). These data indicate that postmortem brain tissues with short postmortem interval are responsive to insulin can be reliably used to assess changes in adiponectin receptor signaling. N = 3. Abbreviations: AdipoR1, adiponectin receptor 1; AdipoR2, adiponectin receptor 2; APPL1, adaptor protein containing pleckstrin homology domain phosphotyrosine-binding domain and leucine zipper motif; AMPK, adenosine monophosphate–dependent protein kinase.

3.3. Insulin signaling and its regulation by adipokines are demonstrated in brain tissue with short PMI in a simulating postmortem rat model

Ex vivo tests of the PMI effects on insulin signaling and its regulation by the adipokines were conducted in 4 individual sets of postmortem rat prefrontal cortex slices with PMIs ranging from 0 (snap-frozen) to 16 hours. Insulin induced clear and reliable activation of the IR when the PMIs were under 12 hours (Fig. 5A). Within 12-hour PMIs, adiponectin potentiated but leptin suppressed this insulin-evoked IR activation. The significantly elevated basal activity and diminished responsiveness to insulin and adipokines led to dramatic reduction in insulin signaling and its regulation by adipokines at the 16-hour time point.

Because the biological activities of insulin and adipokines depend heavily on intact mitochondria, we tested the effects of different PMIs on cell mitochondrial integrity in the postmortem rat prefrontal cortex slices using depolarization-induced $^{45}Ca^{2+}$ influx that is highly sensitive to mitochondrial changes. In response to 30-second 65 mM K^+-evoked depolarization, $^{45}Ca^{2+}$ influx via the voltage-gated Ca channels was markedly higher than in the non-stimulated Krebs-Ringer condition (Fig. 5B). However, the non-stimulated $^{45}Ca^{2+}$ levels started to increase in the 8-hour PMI samples and further doubled at 16 hours, indicating reduced cell mitochondrial integrity. Simultaneously, we also observed a reduction in K^+-evoked depolarization-induced $^{45}Ca^{2+}$ influx when PMI reached 12 hours and, more prominently, at 16 hours. These data therefore suggest that postmortem human brain tissues obtained with PMIs within 12 hours have sufficient cell mitochondrial integrity for the study of insulin, adiponectin, and leptin signaling (Fig. 5A and B).

3.4. Insulin, adiponectin, and leptin signaling and their regulation in postmortem frontal cortices from cognitively normal human subjects

Insulin signaling and its regulation by adiponectin and leptin were assessed by ex vivo incubation of human DLPFC slices from 15 autopsied subjects (mean age at death = 87.02 ± 1.60 years; 8 women) without neurodegenerative or other CNS diseases, or

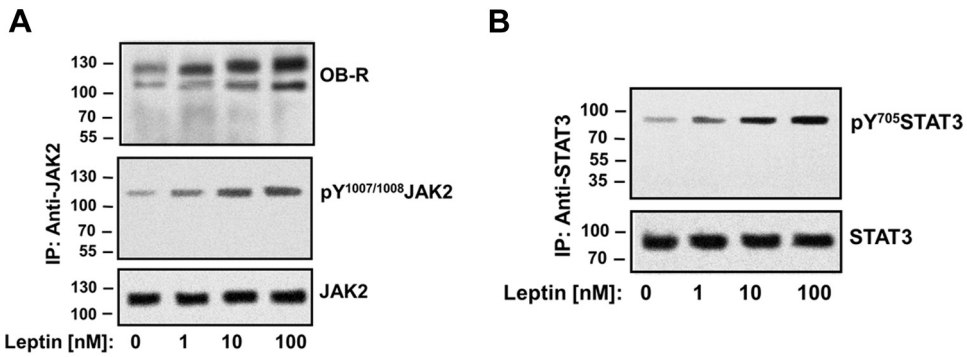

Fig. 4. Dose-dependency of leptin signaling in postmortem human frontal cortices. Frontal cortical slices from neurologically normal subjects with postmortem interval <12 hours were used to determine insulin signaling induced by 1–100 nM recombinant human leptin. After incubation with varying concentrations of leptin for 30 minutes at 37 °C, brain slices were collected, solubilized, and immunoprecipitated with immobilized anti-JAK2 or -STAT3 antibodies. The contents in resultant immunoprecipitates were analyzed by Western blotting. Adiponectin dose-dependently increased association of OB-Rs with and activation (tyrosine phosphorylation) of JAK2, a nonreceptor tyrosine kinase (A) as well as activation (tyrosine phosphorylation) of STAT3 (B). These data indicate that postmortem brain tissues with short postmortem interval are responsive to insulin can be reliably used to assess changes in leptin receptor signaling. N = 3. Abbreviations: OB-R, leptin receptor; JAK2, Janus kinase 2; STAT3, signal transducer and activator of transcription 3.

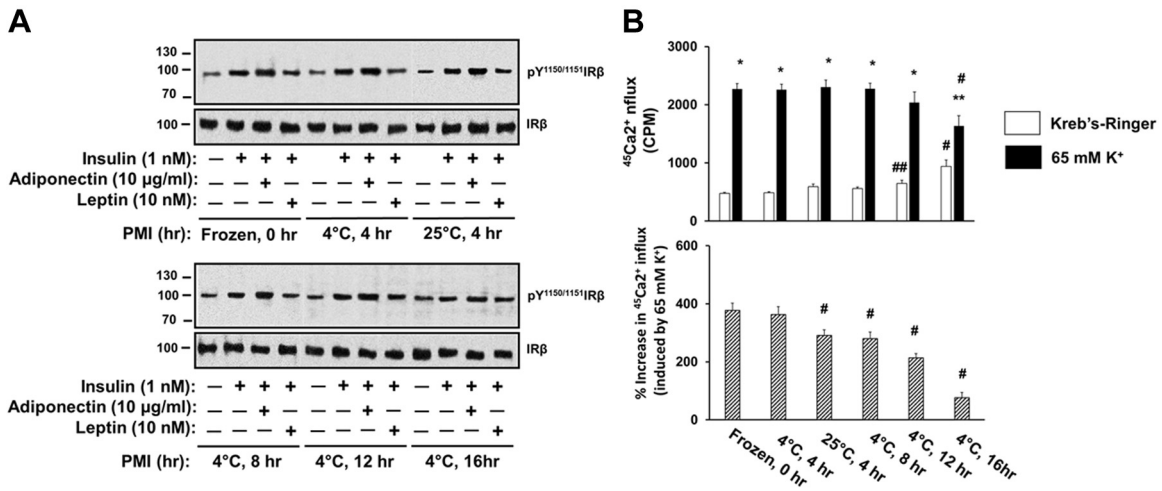

Fig. 5. Effects of postmortem delays on insulin signaling and adipokine regulation as well as basal and K^+-depolarization–evoked calcium influx using postmortem rat frontal cortices. (A). Sample blots on the rat frontal cortices showing that PMIs up to 12 hours had no significant effects on basal levels of IRβ, IRβ activation as indicated by the $pY^{1150/1151}$ levels evoked by 1 nM insulin, or effects of adiponectin (10 μg/mL) and leptin (10 nM). An elevated basal IRβ and reduced IRβ activation as well as adiponectin and leptin effects was observed at 16 hours PMI. (B) PMI effects on mitochondrial function and cell viability were tested by assessing basal and 65 mM K^+-evoked Ca influx in 5 μCi $^{45}Ca^{2+}$ (10 Ci/mmol)-incubated rat prefrontal cortical slices followed by incubation with Krebs-Ringer, or 65 mM K^+ (made with isomolar replacement of Na^+) for 1 minute. The Ca uptake was indicated by the levels of $^{45}Ca^{2+}$ retained in the brain slices assessed by scintillation spectrometry. N = 4 per PMI. $^*p < 0.01$, $^{**}p < 0.05$ compared with respective nonstimulated Krebs-Ringer baseline for each PMI. $^{\#}p < 0.01$, $^{\#\#}p < 0.05$ compared with basal $^{45}Ca^{2+}$ level insulin stimulation alone. Abbreviations: IR, insulin receptor; PMI, postmortem interval.

diabetes mellitus. We used 1 nM insulin alone or together with 10 μg/mL adiponectin or 10 nM leptin for 30 minutes at 37 °C in oxygenated Krebs-Ringer. We then analyzed the lysates derived from these slices for insulin signaling by anti-IRβ and anti-AKT1 immunoprecipitation followed by western blotting. In accordance with our earlier work (Talbot et al., 2012), 1 nM insulin activated insulin signaling evidenced as a 4.4- to 4.7-fold increase in $pY^{1150/1151}$IRβ, pY^{960}IRβ, and IRS-1 bound IRβ (Fig. 6A). Insulin-induced insulin signaling was also supported by a 4.2-fold increase in activated AKT1 ($pS^{473}$and pT^{308}) (Fig. 6B). Coincubation of insulin with adiponectin resulted in 20.4 ± 3.4–28.2 ± 3.0% potentiation in insulin-evoked increases in $pY^{1150/1151}$IRβ, pY^{960}IRβ, and IRS-1 bound IRβ (Figure 6A) and 25.8 ± 3.1–28.1 ± 3.1% potentiation in insulin-induced increases in pS^{473}- and pT^{308}-AKT1 (Fig. 6B). By contrast, coincubation with leptin decreased insulin-evoked increases in $pY^{1150/1151}$IRβ, pY^{960}IRβ, and IRS-1 bound IRβ by 17.6 ± 1.7–18.1 ± 2.5% (Fig. 6A) and insulin-induced increases in pS^{473}- and pT^{308}-AKT1 by 15.2 ± 2.2–17.9 ± 2.7% (Fig. 6B).

To assess adiponectin signaling and its regulation by insulin and leptin, postmortem human DLPFC slices were incubated with 10 μg/mL exogenous adiponectin alone or together with 1 nM insulin or 10 nM leptin for 30 minutes at 37 °C in oxygenated Krebs-Ringer. The lysates derived were analyzed for adiponectin signaling by the levels of APPL1-associated adiponectin receptors AdipoR1, AdiopR2, and T-cadherin as well as the activated downstream AMPKα1/2 in the anti-APPL1 and anti-AMPKα1/2 IPs, respectively, using western blots. Adiponectin (10 μg/mL) increased APPL1–AdipoR1, –AdiopR2, and –T-cadherin complexes by 4.6- to 4.7-fold (Fig. 7A). Adiponectin at 10 μg/mL also induced a 4.7-fold increase in activated ($pT^{183/172}$) AMPKα1/2 (Fig. 7B). Coincubation of insulin but not leptin with adiponectin resulted in 18.6 ± 2.8–19.6 ± 3.5% reduction in adiponectin-evoked increases in the levels of the APPL1–AdipoR1, –AdipoR2, and –T-cadherin complexes (Fig. 7A) and a 20.2 ± 4.4% decrease in the adiponectin-evoked increases in $pT^{183/172}$AMPKα1/2 (Fig. 7B).

To assess the leptin signaling and its regulation by insulin and adiponectin, postmortem human DLPFC slices were incubated with 10 nM exogenous leptin individually or together with 1 nM insulin or 10 μg/mL adiponectin for 30 minutes at 37 °C in oxygenated Krebs-Ringer. The lysates derived from these slices were then analyzed for leptin signaling by the levels of activated ($pY^{1007/1008}$)JAK2 and JAK2-associated leptin receptors, OB-Rs as well as the activated (pY^{705}) downstream STAT3 in the anti-JAK2 and anti-STAT3 IPs, respectively, using western blots. Leptin (10 nM) increased $pY^{1007/1008}$JAK2 and JAK2-coupled long and short form of OB-R by 4.2- to 4.3-folds (Fig. 8A). In accord, 10 nM leptin at 10 nM also induced a 4.3-fold increase in pY^{705}STAT3 (Fig. 8B). Coincubation of leptin with insulin but not adiponectin increased leptin signaling as indicated by 21.8 ± 2.2%, 24.6 ± 2.9%, and 20.5 ± 3.1% increases in leptin-evoked increases in the levels of $pY^{1007/1008}$JAK2, JAK2-coupled OB-R long and short form, respectively (Fig. 8A). In support, insulin also increased leptin-induced pY^{705}STAT3 by 22.1 ± 2.5% (Fig. 8B).

4. Discussion

In this study, we experimentally demonstrated insulin, adiponectin, and leptin signaling in the postmortem human brain. We further showed that adiponectin enhances, and leptin inhibits, insulin signaling in human brain. This finding is similar to what has been shown in peripheral tissues (Kadowaki et al., 2006; Knights et al., 2014; Kwon and Pessin, 2013; Yadav et al., 2013; Yates et al., 2012). Less established is how insulin conversely regulates adiponectin and leptin signaling pathways, although there is evidence that insulin increases adiponectin and leptin synthesis and secretion from adipose cells (Blümer et al., 2008; Hajri et al., 2010; Marques-Oliveira et al., 2018; Tsubai et al., 2016). Here we show that insulin inhibits adiponectin and enhances leptin signaling, suggesting an autoregulatory feedback loop within this hormonal brain network. To our knowledge, this has not previously been directly examined in either brain or peripheral tissues in human or other systems.

In accord with our previous work (Talbot et al., 2012; Wang et al., 2012, 2017), ex vivo insulin induced clear, reliable, dose-dependent activation of the insulin signaling pathway. Increasing insulin doses promoted tyrosine phosphorylation (pY) of the IRβ kinase regulatory domain (Y1150, Y1151) and IRS-1 docking site (Y960) and recruitment of IRS-1 to IRβ, as well as activation of downstream signaling molecule, pS^{473}- and pT^{308}-AKT1. To assess the effect of

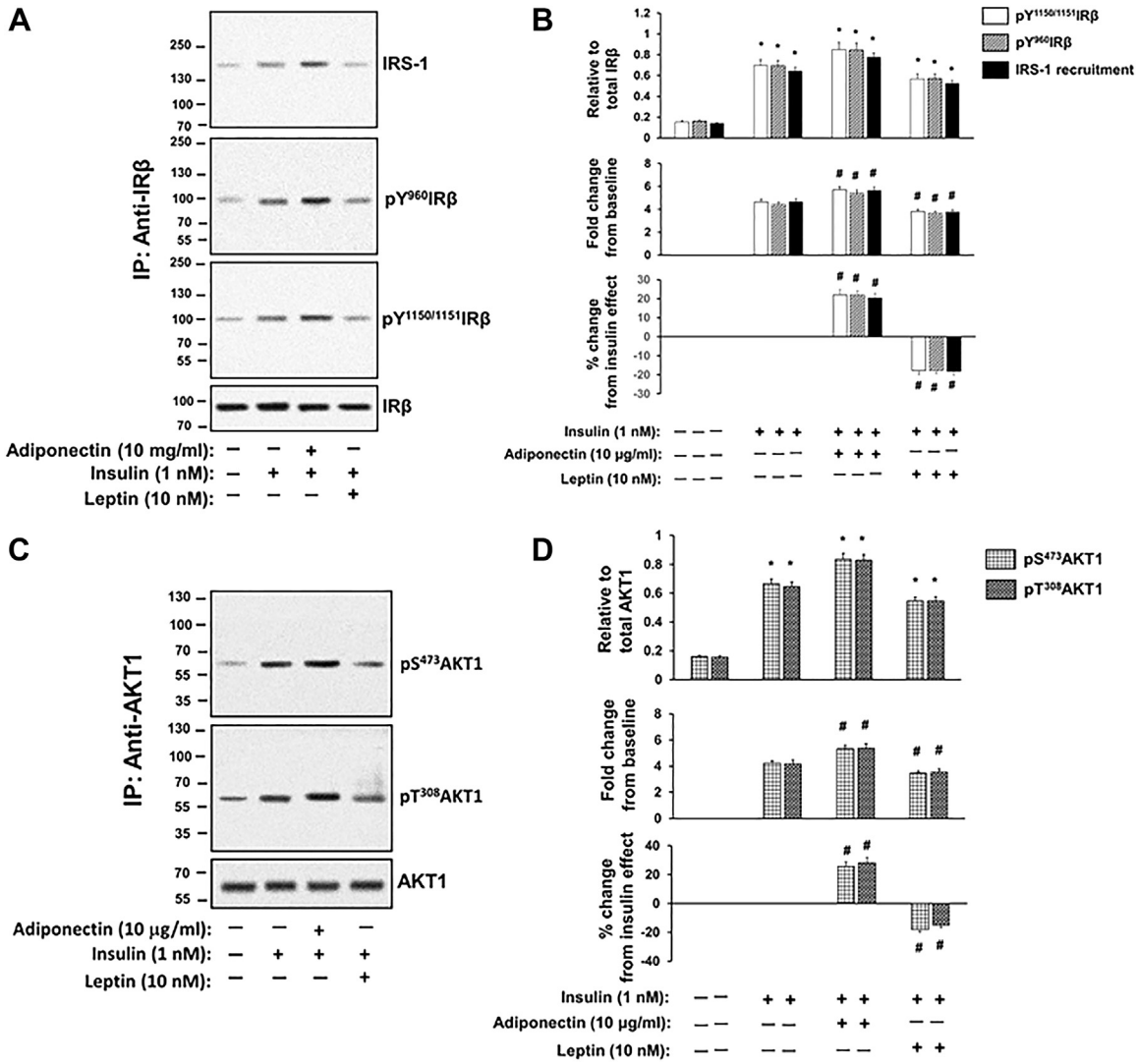

Fig. 6. Direct demonstration of adiponectin enhances and leptin suppresses insulin receptor activation and signaling in postmortem human brain. Frontal cortical slices from neurologically normal subjects with postmortem interval <12 hours were used to assess insulin signaling and its regulation by adipokines: adiponectin and leptin at the near physiological concentrations. Incubation with insulin alone for 30 minutes at 37 °C leads to phosphorylation of tyrosine residues: 1150/1151 and 960 of the insulin receptor β subunit (IRβ): $pY^{1150/1151}$ and pY^{960} as well as recruitment of insulin receptor substrate-1 (IRS-1) to IRβ (A, B) and phosphorylation of AKT1 at the $serine^{473}$ (by mTOR2) and $threonine^{308}$ (by PDK1) (C, D). Addition of adiponectin (10 μg/mL) and leptin (10 nM) together with insulin enhance and suppress insulin signaling, respectively (A, B, C, D). These data indicate that postmortem brain tissues can be reliably used to assess changes in insulin receptor activities and regulation in pathological conditions. N = 15. *$p < 0.01$ compared with nonstimulated Krebs-Ringer baseline. #$p < 0.01$ compared with insulin stimulation alone. Abbreviations: pY, tyrosine-phosphorylated.

adiponectin and leptin on insulin responses, we selected the 1 nM concentration, close to physiological levels of brain insulin (Baskin et al., 1987; Le Roith et al., 1983).

Although adiponectin acts through AdipoR1 and AdipoR2 in brain to promote neurogenesis and modulate synaptic activity in animals (Song et al., 2015; Yau et al., 2014; Zhang et al., 2017), adiponectin-mediated signaling mechanisms have not been directly demonstrated. APPL1, first identified as the intracellular binding partner of AdipoR1 and AdipoR2 using a yeast hybrid strategy, directly binds to the intracellular domains of AdipoR1 and AdipoR2 via its C-terminal phosphotyrosine-binding and coiled coil domains to mediate adiponectin signaling, including activation of AMPK (Liu et al., 2002; Mao et al., 2006; Mitsuuchi et al., 1999). In support of these observations, we showed that exogenous human recombinant adiponectin increases the levels of APPL1-coupled AdipoR1 and AdipoR2 as well as activated AMPKα1/2 ($pT^{183/172}$AMPKα1/2). We also made a novel observation that T-cadherin is able to bind to APPL1. Although there is evidence that T-cadherin is present in human brain (Takeuchi et al., 2000) and potentially mediates various metabolic effects (Chung et al., 2011; Fava et al., 2011; Fujishima et al., 2017; Org et al., 2009), little is known of T-cadherin signaling. T-cadherin has also been shown to be a receptor for hexameric and high-molecular-weight forms of adiponectin, which are essential for revascularization (Hug et al., 2004; Parker-Duffen et al., 2013). Because adiponectin in CNS exists predominantly as the low-molecular-weight version (Kusminski et al., 2007), our novel finding that exogenous recombinant human adiponectin induces T-cadherin and APPL1 association suggests that T-cadherin can bind exogenous adiponectin and signal in the brain. However, its downstream effector system(s) remain unknown.

In CNS, leptin was shown to modulate synaptic plasticity (Harvey et al., 2006), neuronal excitability (Harvey, 2007; Moult et al., 2009), firing frequency of dopaminergic neurons in ventral tegmental area (Trinko et al., 2011), and dendritic morphology in hippocampus (O'Malley et al., 2007). These data support the notion that leptin, acting through its cognate receptors, regulates a wide

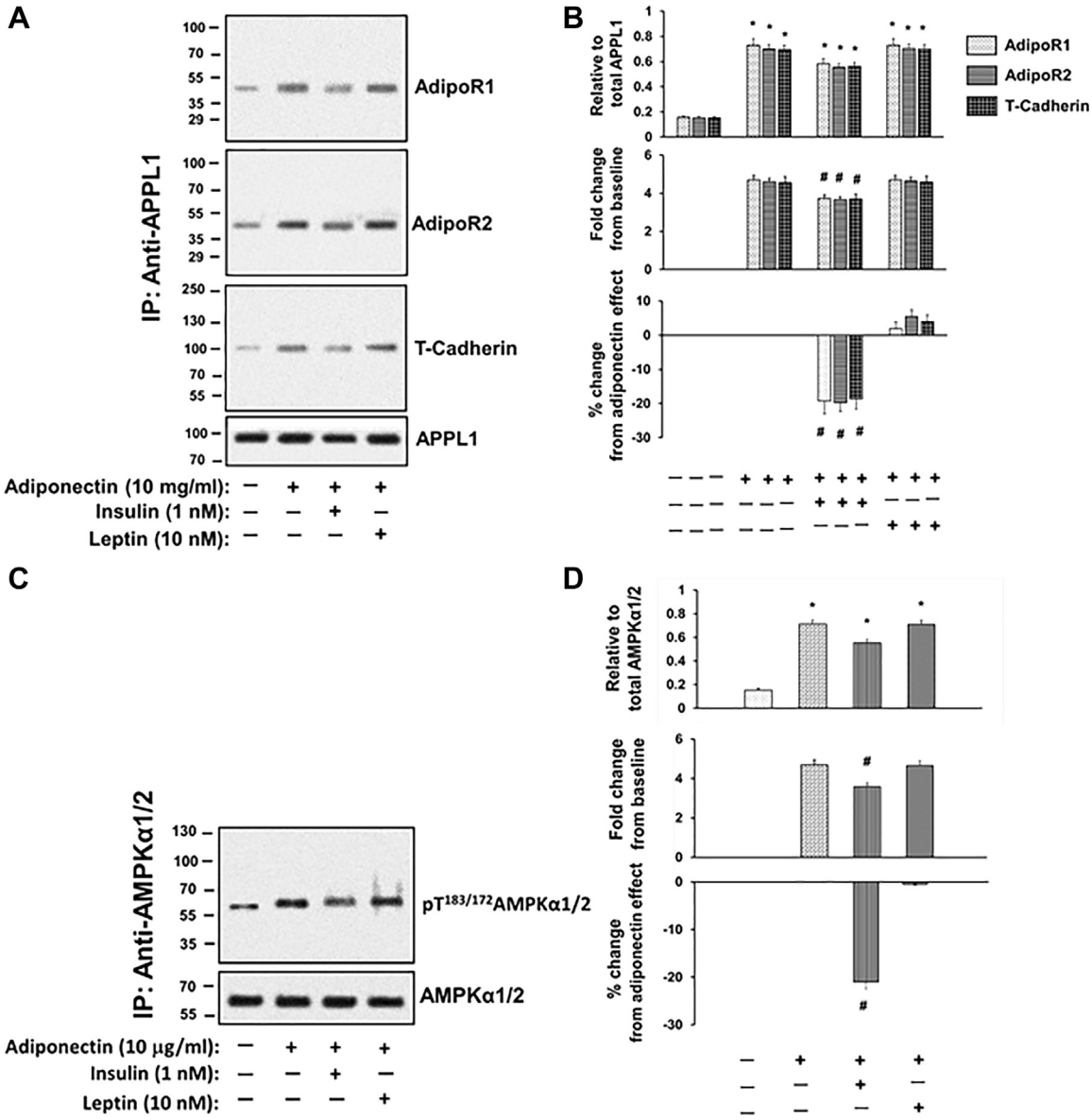

Fig. 7. Direct demonstration of insulin suppresses adiponectin signaling in postmortem human brain. Frontal cortical slices from neurologically normal subjects with postmortem interval <12 hours were used to assess signaling of the adiponectin receptor and its regulation by insulin and leptin at the near physiological concentrations. Incubation with adiponectin (10 μg/mL) alone for 30 minutes at 37 °C promotes adiponectin signaling as illustrated by increases in association of the adiponectin receptor subtypes: AdipoR1, AdipoR2, and T-cadherin with signaling adaptor protein, APPL1 (A, B) as well as in the phosphorylation of AMPKα1/2 at the threonine^{183} and threonine^{172} of the AMPK (C, D). Addition of insulin (1 nM) together with adiponectin reduces adiponectin signaling as indicated by lower AdipoR1, AdipoR2 and T-cadherin levels as well as pT$^{183/172}$ AMPKα1/2 than adiponectin alone (A, B, C, D). In contrast to insulin, leptin (10 nM) is without an effect on adiponectin signaling. These data indicate phosphorylation of AMPKα1/2 in the postmortem brain tissues can be reliably used to assess changes in adiponectin signaling cascade and regulation in pathological conditions. N = 15. $^{*}p < 0.01$ compared with nonstimulated Krebs-Ringer baseline. $^{\#}p < 0.01$ compared with insulin stimulation alone. Abbreviations: AdipoR1, adiponectin receptor 1; AdipoR2, adiponectin receptor 2; APPL1, adaptor protein containing pleckstrin homology domain phosphotyrosine-binding domain and leucine zipper motif; AMPK, adenosine monophosphate–dependent protein kinase.

range of brain functions. The leptin receptor is a member of the class I cytokine-receptor family, with 6 isoforms derived from alternative mRNA splicing and/or proteolytic processing (Frühbeck, 2006). While sharing common extracellular and transmembrane domains, a, c, d, and f OB-R isoforms have relatively short cytoplasmic domains, referred to as "short" isoforms. With ~300 more amino acids in the intracellular domain than the short isoforms, OB-Rb is referred to as the "long" isoform. In our present study, we show both long and short forms are present in the human frontal cortex. Furthermore, previous reports indicate that binding of leptin to its receptor OB-R results in rapid activation of intracellular JAK2 that is associated with OB-Rb leading to tyrosine phosphorylation of downstream JAK2 and STAT3 in cells (Banks et al., 2000; Ghilardi and Skoda, 1997; Guo et al., 2008; Kita et al., 2003). In accord, we showed that leptin increases tyrosine phosphorylation of JAK2 (pY$^{1007/1008}$) and STAT3 (pY^{705}). More intriguingly, we made another novel observation that leptin stimulation elicits the recruitment of JAK2 to both long and short forms of OB-R, although the level of recruitment is less for OB-Ra compared with OB-Rb. The fact that insulin similarly reduces the leptin-induced JAK2 recruitment to both forms of OB-R suggests that this is a physiological action of leptin, at least in human cortex.

In summary, we have demonstrated that under ex vivo oxygenated and normothermic conditions, the postmortem human brain within 10 hours PMI, remains responsive to insulin, adiponectin, and leptin. This finding is consistent with various earlier

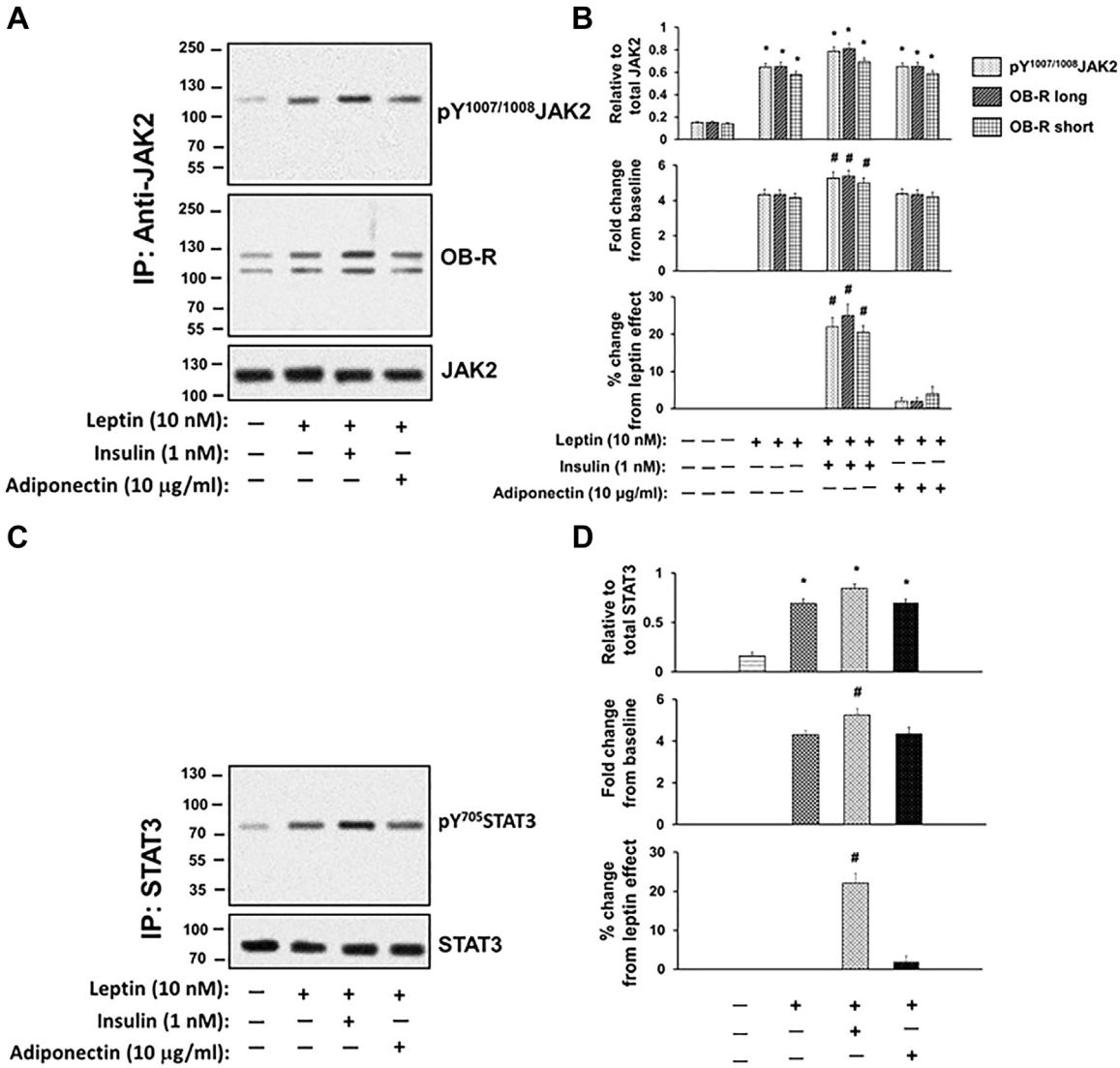

Fig. 8. Direct demonstration of insulin potentiates leptin signaling in postmortem human brain. Frontal cortical slices from neurologically normal subjects with postmortem interval <12 hours were used to assess signaling of the leptin receptor (OB-R) and its regulation by insulin and adiponectin at the near physiological concentrations. Incubation with leptin (10 nM) alone for 30 minutes at 37 °C increases association of OB-Rs with and activation (tyrosine phosphorylation) of JAK2, a non-receptor tyrosine kinase (A, B) as well as activation (tyrosine phosphorylation) of STAT3 (C, D). Addition of insulin (1 nM) but not adiponectin (10 μg/mL) together with leptin increases leptin signaling as indicated by a higher OB-R, $pY^{1007/1008}$JAK2 and pY^{705}STAT3 levels than leptin alone (A, B, C, D). These data indicate that JAK2 association with OB-Rs and activation of JAK2 in the postmortem brain tissues can be reliably measured and used to assess changes in leptin signaling pathway and regulation in pathological conditions. N = 15. $^{*}p < 0.01$ compared with non-stimulated Krebs-Ringer baseline. $^{\#}p < 0.01$ compared with insulin stimulation alone. Abbreviations: JAK2, Janus kinase 2; STAT3, signal transducer and activator of transcription 3; pY, tyrosine-phosphorylated.

reports by us and others that postmortem brains can withstand some ischemic insults and retain some functions and ability to signal (Hahn et al., 2006; Talbot et al., 2012; Verwer et al., 2002; Vrselja et al., 2019; Wang et al., 2003, 2012, 2017; Wang and Friedman, 1994). Although these findings indicate that postmortem brains may be used to extract functional data and assess the impacts of disease, there are many limitations in studies using postmortem tissues. Most notably, molecules are vulnerable to postmortem changes and can lose integrity and functionality. However, we used tissues in as optimal condition as possible, by selecting tissue with postmortem intervals of less than 12 hours and without confounding diseases such as tumors or traumatic brain injury. We also used rigorous scientific methods including vigilant monitoring of storage temperature and detailed procedures for tissue preservation so that the integrity of the receptors and signaling molecules minimally compromised. Despite its limitations, the present work illuminated crosstalk and signaling details between leptin, adiponectin, and insulin receptor signaling in the human brain. The present findings are an important first step in interrogating human brain changes of leptin, adiponectin, and insulin receptor signaling pathways, as well as their crosstalk, in aging and age-related diseases such as ADRD. The loci of dysregulation that we identified in these fundamental metabolic pathways in the brain offer critical insights into pathophysiologic mechanisms of age-related neurologic diseases. These points of metabolic dysfunction may lead to new interventions so urgently needed for ADRD and other age-related diseases of the brain.

Disclosure

The authors have declared that no conflict of interest exists.

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