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Central Resistin/TLR4 Impairs Adiponectin Signaling, Contributing to Insulin and FGF21 Resistance

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Adiponectin, an insulin-sensitizing hormone, and resistin, known to promote insulin resistance, constitute a potential link between obesity and type 2 diabetes. In addition, fibroblast growth factor (FGF)21 has effects similar to those of adiponectin in regulating glucose and lipid metabolism and insulin sensitivity. However, the interplay between adiponectin, FGF21, and resistin signaling pathways during the onset of insulin resistance is unknown. Here, we investigated whether central resistin promotes insulin resistance through the impairment of adiponectin and FGF21 signaling. We show that chronic intracerebroventricular resistin infusion downregulated both hypothalamic and hepatic APPL1, a key protein in adiponectin signaling, associated with decreased Akt-APPL1 interaction and an increased Akt association with its endogenous inhibitor tribbles homolog 3. Resistin treatment also decreased plasma adiponectin levels and reduced both hypothalamic and peripheral expression of adiponectin receptors. Additionally, we report that intracerebroventricular resistin increased plasma FGF21 levels and downregulated its receptor components in the hypothalamus and peripheral tissues, promoting FGF21 resistance. Interestingly, we also show that resistin effects were abolished in TLR4 knockout mice and in neuronal cells expressing TLR4 siRNAs. Our study reveals a novel mechanism of insulin resistance onset orchestrated by a central resistin-TLR4 pathway that impairs adiponectin signaling and promotes FGF21 resistance.

Obesity is closely linked to several metabolic disorders including insulin resistance and type 2 diabetes (T2D) (1–6). However, the mechanisms underlying obesity-associated insulin resistance are not fully understood. Recently, an emerging hypothesis suggests that the alteration of whole-body insulin sensitivity could have a starting point in the brain and precisely in the hypothalamus (7). Indeed, chronic high-fat feeding promotes hypothalamic resistance to leptin and insulin through an inflammatory-dependent mechanism leading to impaired energy homeostasis, obesity, and insulin resistance (4,7). Furthermore, compelling evidence indicates that changes in adipocyte-derived factors in obesity dramatically affect insulin sensitivity (4–6). Among these adipokines, resistin is described as a causal factor for obesity-induced insulin resistance and T2D. Indeed, the circulating levels of resistin are increased in obesity, promoting both inflammation and insulin resistance (8–12). In addition, serum resistin has been positively correlated with resistin cerebrospinal fluid levels, and increased cerebrospinal fluid resistin concentrations have been associated with T2D (13). Resistin is also expressed in the hypothalamus (14), and central resistin modulates food intake and glucose and lipid metabolism (15–19). Interestingly, we recently reported that central resistin, via hypothalamic Toll-like receptor (TLR)4, induces overall inflammation and impairment of insulin responsiveness in rat, presenting the first evidence for the crucial role of a hypothalamic resistin-TLR4 pathway in the onset of whole-body insulin resistance and inflammation (20).

Besides resistin, adiponectin has received a special focus because of its insulin-sensitizing and anti-inflammatory properties (21–24). In obesity, a decrease in plasma adiponectin is considered an important risk factor for insulin resistance (24). Conversely, exogenous adiponectin alleviates chronic inflammation, glucose intolerance, and insulin resistance in obese and diabetic rodents (24–26). Adiponectin exerts its effects through two adiponectin receptors,

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AdipoR1 and AdipoR2 (24,27). Adiponectin also activates liver kinase B1–AMPK signaling pathways, promoting insulin sensitivity (23,27). Furthermore, the adaptor protein containing pleckstrin homology domain and leucine zipper motif 1 (APPL1) has been implicated in the insulin-sensitizing effect of adiponectin (27,28). APPL1 is also involved in the insulin-mediated Akt signaling, suggesting a cross talk between adiponectin and insulin signaling at the level of the Akt pathway (27). Recent studies have reported that adiponectin is crucially involved in hypothalamic control of energy homeostasis (29,30). Additionally, central adiponectin improves glucose tolerance and reverses hypothalamic inflammation and insulin resistance in obese mice (31,32). These data demonstrate that the insulin-sensitizing properties of adiponectin are at least partially related to a neuroendocrine mechanism that involves central action of adiponectin.

Fibroblast growth factor (FGF)21, a metabolic hormone predominantly produced by liver, has many functional similarities to adiponectin in regulating glucose and lipid metabolism and insulin sensitivity (33–37). FGF21 acts through a cell-surface receptor composed of a canonical FGF receptor (FGFR) associated with a coreceptor, β-Klotho (KLB) (36–38). Pharmacologic FGF21 treatment counteracts obesity and its related metabolic disorders including dyslipidemia, glucose intolerance, and insulin resistance (39). In white adipose tissue (WAT), a main target of FGF21 actions, FGF21 stimulates glucoses uptake, modulates lipolysis, and enhances adiponectin expression and secretion (34–37,39,40). FGF21 also acts directly in the hypothalamus to modulate energy balance and insulin sensitivity (41,42). Recent studies have reported that serum FGF21 levels are elevated in obese rodents and humans (43–45). In obese mice, the expression levels of FGFRs were also markedly downregulated. This blunts the metabolic effects of FGF21, promoting an obesity-associated FGF21 resistance state (43,44).

Thus, obesity is associated with adiponectin and FGF21 resistances, leading to the impairment of insulin sensitivity. However, the mechanisms linking obesity to adiponectin/FGF21 signaling impairment are not yet elucidated. On the basis of our previous work demonstrating that central resistin overexposure induces striking alterations of whole-body insulin sensitivity, we hypothesized that resistin could be the missing link. Therefore, we investigated whether central resistin promotes insulin resistance through the impairment of adiponectin and FGF21 signaling. We show that chronic intracerebroventricular resistin infusion downregulated both hypothalamic and hepatic APPL1 expression associated with decreased Akt-APPL1 interaction and an increased Akt association with its inhibitor, tribbles homolog 3 (TRB3). Resistin treatment also reduced AdipoR1/2 expression in the hypothalamus and peripheral tissues. Interestingly, we report that central resistin, via TLR4, impairs FGF21 signaling through the downregulation of hypothalamic expression FGF21 and both hypothalamic and peripheral expression of its receptors, promoting FGF21 resistance.

RESEARCH DESIGN AND METHODS

Animals

Adult male Wistar rats, C57BL/6J mice (Janvier Laboratories), and TLR4 knockout (TLR4-KO) mice with the same genetic background (TAAM-UPS44; CNRS, Orleans, France) were housed individually in a temperature-controlled environment with a 12-h light/12-h dark cycle and unrestricted access to water and standard diet (SAFE, Augy, France). All experimental procedures were performed according to the institutional guidelines of laboratory animal care and approved by the governmental commission of animal research.

Chronic Intracerebroventricular Infusion of Resistin

Osmotic mini-pumps (models 2002 for rats and 1003D for mice; Alzet) were implanted under ketamine (150 mg/kg)/xylazine (5 mg/kg) anesthesia. Brain infusion cannulas were stereotaxically placed into the right lateral brain ventricle using the following coordinates: 0.8 mm anterior to bregma, 1.5 mm lateral to the midline, and 3.5 mm dorso-ventral for rats and 0.58 mm anterior to bregma, 1 mm lateral, and 2 mm dorso-ventral for mice. The rats were then infused with either vehicle or resistin (2 or 12 μL/day, respectively; pumping rate 0.5 μL/h) for 14 days. C57BL/6J and TLR4-KO mice were infused for 3 days with either vehicle or resistin (2 or 6 μL/day; pumping rate 0.2 μL/h).

Measurement of Blood Glucose and Plasma Hormone Levels

Blood glucose levels were measured immediately with a blood monitoring system (Accu-Chek, Roche). Plasma levels of resistin, insulin, leptin, adiponectin, and FGF21 were quantified with ELISA immunoassays according to the manufacturer's instructions (Millipore).

Hormones and Chemicals

Human resistin was a generous gift of Protein Laboratories Rehovot Ltd. (Rehovot, Israel). Cell culture reagents were from Invitrogen. Insulin and chemicals were from Sigma-Aldrich. Immunobilon-FL transfer membranes were from Millipore. Fluorescent secondary antibodies were from Life Technologies.

Cell Culture and Stimulation

SH-SY5Y human neuroblastoma cells and adult mouse hypothalamic (mHypo) cells were grown in DMEM and RPMI supplemented with 10% FCS, 2 mmol/L L-glutamine, 100 units/mL penicillin, and 100 μg/mL streptomycin in 5% CO_2 atmosphere at 37°C. Serum-starved cells were incubated for 16 h in serum-free DMEM in the presence or absence of resistin (200 ng/mL), insulin (100 nmol/L), and insulin (100 nmol/L) + adiponectin (250 ng/mL). Cells were then stimulated with insulin (100 nmol/L), adiponectin (250 ng/mL), or FGF21 (100 nmol/L) for 10 min to evaluate the impact of chronic treatments on SH-SY5Y cell sensitivity of both insulin and adiponectin.

Western Blot Analysis

Protein lysates from mice and rats tissues and SH-SY5Y cells were analyzed by Western blot as previously described

(20). Immunoblots were incubated overnight at 4°C with primary antibodies directed toward the following: (phospho-Ser^{473})-Akt, Akt, phospho-ERK1/2 (extracellular signal–related kinase 1/2), Erk1/2, (phospho-Ser^{307})-IRS-1 (insulin receptor substrate 1), IRS-1, phospho-JNK (c-Jun N-terminal kinase), JNK, APPL1, TRB3, β-tubulin (these antibodies are from Cell Signaling Technology), insulin receptor (IR), AdipoR1/2, APPL2, TLR4, or PTP1B (these antibodies are from Santa Cruz Biotechnology).

Immunoprecipitation

Protein lysates of rat tissues were incubated with antibodies against Akt overnight at 4°C. The immune complexes were precipitated with protein A and protein G coupled to sepharose after 2-h incubation at 4°C and subjected to SDS-PAGE and immunoblotted with anti-APPL1, anti-TRB3, and anti-Akt antibodies.

In Vitro FGFR21 Stimulation on Liver Membranes

The in vitro phosphorylation of FGFR1 receptor was performed as previously described (25).

In Vitro Adiponectin Stimulation on Liver Membranes

In vitro adiponectin-dependent activation of liver AdipoR2 was evaluated by analyzing the AdipoR2-APPL1 coimmunoprecipitation using crude liver membranes after adiponectin stimulation.

RNA Extraction and Quantitative RT-PCR

Total RNA from mice and rats tissues and SH-SY5Y cells was isolated using TRIzol reagent. RNA (1 μg) was reverse transcribed, and the cDNAs were submitted to quantitative real-time PCR analysis using a SYBR Green QPCR system (Applied Biosystems) with specific primers (Supplementary Table 1). A ratio of target mRNA–to–18S amplification was calculated.

Small Interfering RNA Silencing

A pool of specific small interfering RNA (siRNA) duplexes targeting human TLR4 and APPL1 genes and control siRNA were purchased from Ambion. Transfection of siRNAs was performed using lipofectamine 2000 transfection reagent (Invitrogen). Downregulation of APPL1 and TLR4 protein synthesis was monitored by Western blot.

Data Analysis and Statistics

Data are shown as means ± SEM. Student *t* test was applied for comparisons between two groups, whereas the ANOVA test was used to compare three or more sets of data, followed by Fisher post hoc test (Statview Software program). $P < 0.05$ was considered statistically significant.

RESULTS

Adiponectin Improves Insulin Signaling in Human Neuronal SH-SY5Y Cells and mHypo Cells

We examined the impact of adiponectin on the insulin-dependent phosphorylation of Akt and ERK1/2. We showed that acute incubation with insulin or adiponectin significantly increased both Akt and ERK1/2 phosphorylation in SH-SY5Y and mHypo cells (Fig. 1*A* and *D*). A notable synergistic effect of insulin and adiponectin was observed on Akt phosphorylation but not ERK1/2 phosphorylation (Fig. 1*A* and *D*). Cell overexposure to insulin reduced acute insulin-dependent Akt and ERK1/2 phosphorylation, though the latter was affected to a lesser degree (Fig. 1*B* and *D*). When cells were overexposed to both insulin and adiponectin, the insulin-dependent Akt and ERK1/2 phosphorylation was completely restored (Fig. 1*C* and *D*). Next, we investigated the mechanisms underlying an adiponectin-protective effect against insulin resistance. We showed that adiponectin significantly attenuated the insulin-dependent downregulation of IR at both mRNA and protein levels (Fig. 2*A*). Additionally, chronic exposure of SH-SY5Y cells to insulin significantly increased the Ser^{307} phosphorylation of IRS-1 (Fig. 2*B*), JNK phosphorylation (Fig. 2*C*), and PTP1B expression (Fig. 2*D*), whereas the concomitant overexposure of cells to insulin and adiponectin markedly reduced JNK and IRS-1^{Ser307} phosphorylation and reduced PTP1B expression to levels comparable with those of control cells (Fig. 2*B–D*).

APPL1 Mediates the Insulin-Sensitizing Effect of Adiponectin in SH-SY5Y Cells

To assess the potential role of APPL1 in the insulin-sensitizing effect of adiponectin, we generated APPL1-depleted SH-SY5Y cells by siRNA-mediated knockdown (Fig. 3*A*). Cells were pretreated with insulin or insulin + adiponectin prior to insulin or adiponectin stimulation in the absence or presence of APPL1 siRNA (Fig. 3*B*). In control cells, both insulin and adiponectin induced Akt phosphorylation (Fig. 3*B*). Insulin pretreatment reduced Akt phosphorylation in response to insulin and adiponectin. However, the insulin- and adiponectin-dependent phosphorylation of Akt was almost completely restored when cells were pretreated with both hormones, an effect that was completely abolished in APPL1-suppressed cells (Fig. 3*B*).

To further evaluate the role of APPL1, we investigated its impact on IR expression and JNK and IRS-1^{Ser307} phosphorylation. We showed that APPL1 siRNA did not affect IRS-1^{Ser307} or JNK phosphorylation or IR expression (Fig. 3*C*). Insulin overexposure reduced IR expression and increased the phosphorylation of JNK and IRS-1^{Ser307} (Fig. 3*C*). Insulin pretreatment effects were significantly reduced by simultaneous adiponectin pretreatment or in cells expressing APPL1 siRNA. (Fig. 3*C*).

Resistin Negatively Regulates Adiponectin Signaling Through the Downregulation of APPL1 and AdipoR1 in SH-SH5Y and mHypo Cells

To test the hypothesis of whether the promoting effects of resistin on insulin resistance are mediated by the alteration of adiponectin action, we investigated the cross talk between resistin and adiponectin signaling. For this purpose, the effect of chronic exposure to resistin on adiponectin signaling was studied in SH-SY5Y and mHypo cells. In nonpretreated cells, adiponectin increased Akt and ERK1/2 phosphorylations (Fig. 4*A* and *B*). Pretreatment with

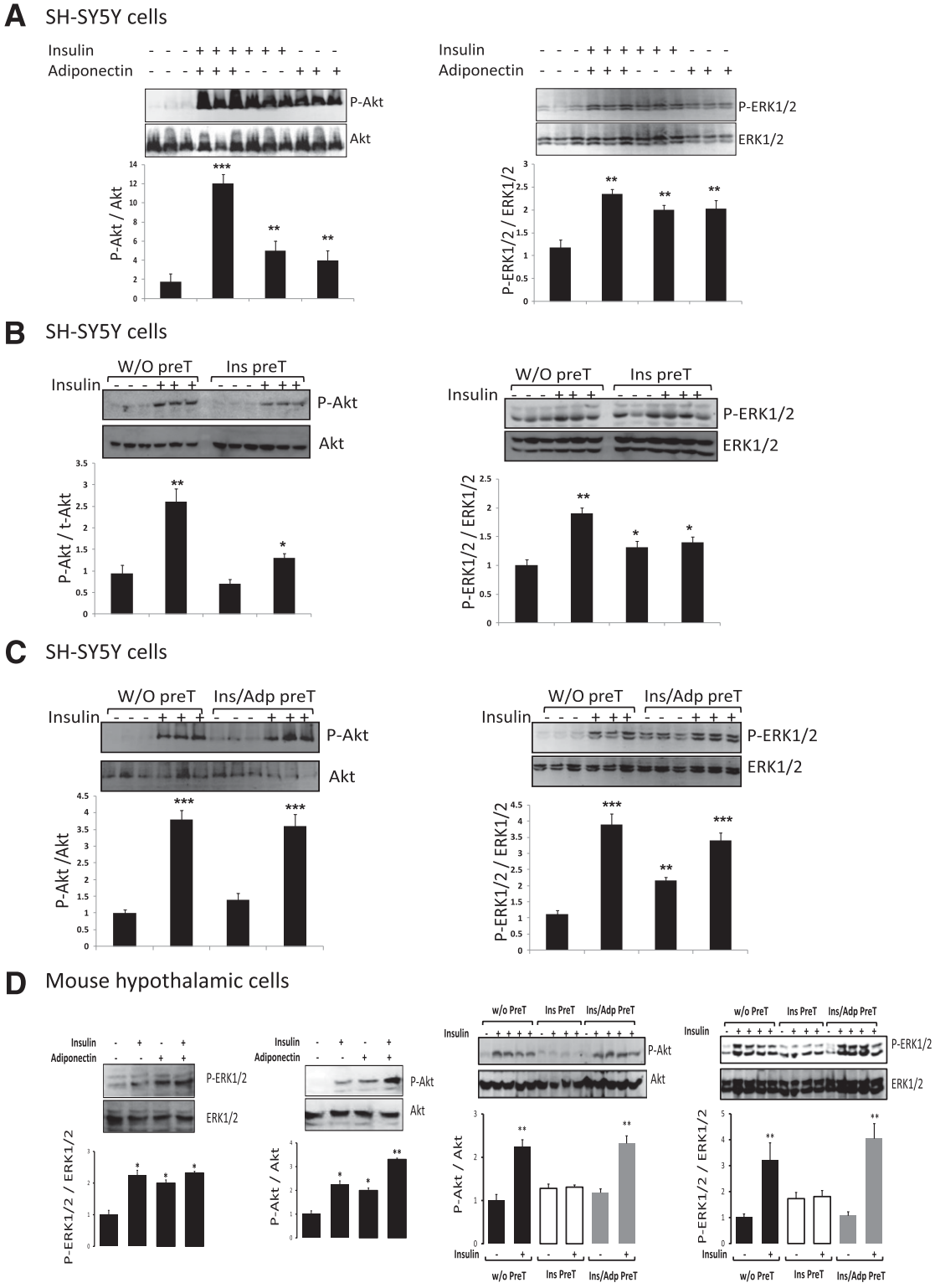

Figure 1—Adiponectin improves insulin-dependent phosphorylation of Akt and ERK1/2 in human SH-SY5Y neuroblastoma cells. *A* and *D*: SH-SY5Y and mHypo cells were serum starved overnight and treated for 10 min with or without insulin (100 nmol/L), adiponectin (250 ng/mL), and insulin + adiponectin. Phosphorylation of Akt and ERK1/2 was detected by Western blot analysis with the specific antibodies indicated. Proteins on the blots were quantified by densitometry. Bar graphs represent the ratios of p-Akt to Akt or p-ERK1/2 to ERK1/2. Serum-starved SH-SY5Y and mHypo cells were pretreated for 16 h without (W/O preT) or with (Ins preT) (100 nmol/L) insulin (*B* and *D*) or insulin (100 nmol/L) + adiponectin (250 ng/mL) (Ins/Adp preT) (*C* and *D*) and then stimulated with insulin (100 nmol/L) for 10 min. p-Akt and p-ERK1/2 and their protein levels in cell lysates were detected by Western blot analysis with specific antibodies as indicated. Proteins on the blots were quantified by densitometry. Bar graphs represent the ratios of p-Akt to Akt or p-ERK1/2 to ERK1/2. All data are expressed as means ± SEM (n = 3–6 /group). *P < 0.05, **P < 0.01, ***P < 0.001 compared with control.

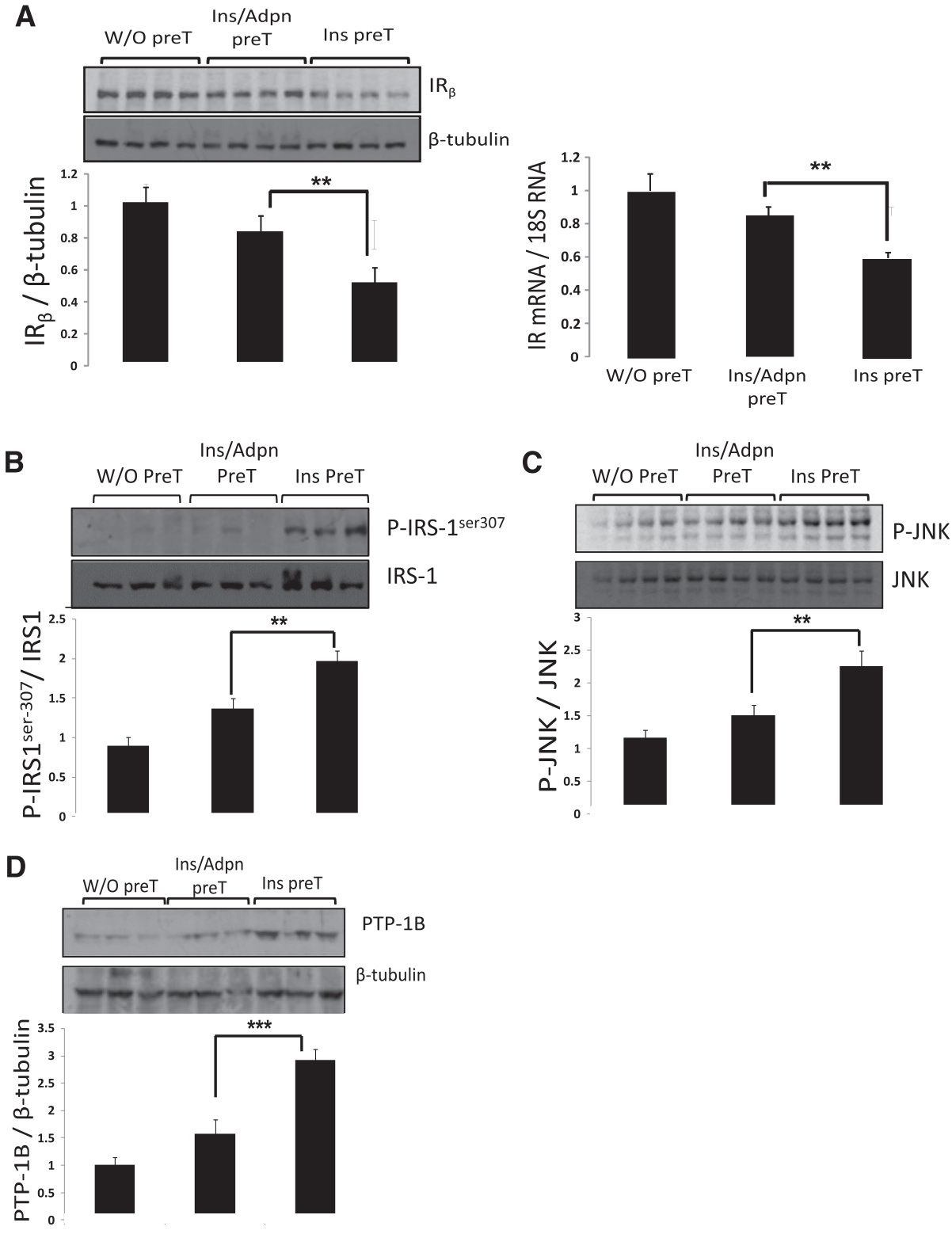

Figure 2—Adiponectin improves insulin signaling in SH-SY5Y cells through the upregulation of IR, downregulation of PTP1B, and inhibition of JNK activation and IRS-1^{Ser307} phosphorylation. SH-SY5Y cells were serum starved overnight and treated for 16 h without (W/O preT) or with (Ins preT) (100 nmol/L) insulin or insulin (100 nmol/L) + adiponectin (250 ng/mL) (Ins/Adpn preT). Expression levels of IR were evaluated by Western blot (*A*) and SYBR Green Real-Time RT-PCR (*B*). The phosphorylation of IRS-1^{Ser307} (*C*) and JNK (*D*) and the protein expression of PTP1B (*E*) were detected by Western blot analysis using specific antibodies as indicated. Proteins on the blots were quantified by densitometry. All data are expressed as means ± SEM (n = 3–6/group). P values were calculated using the Student t test (**$P < 0.01$, ***$P < 0.001$).

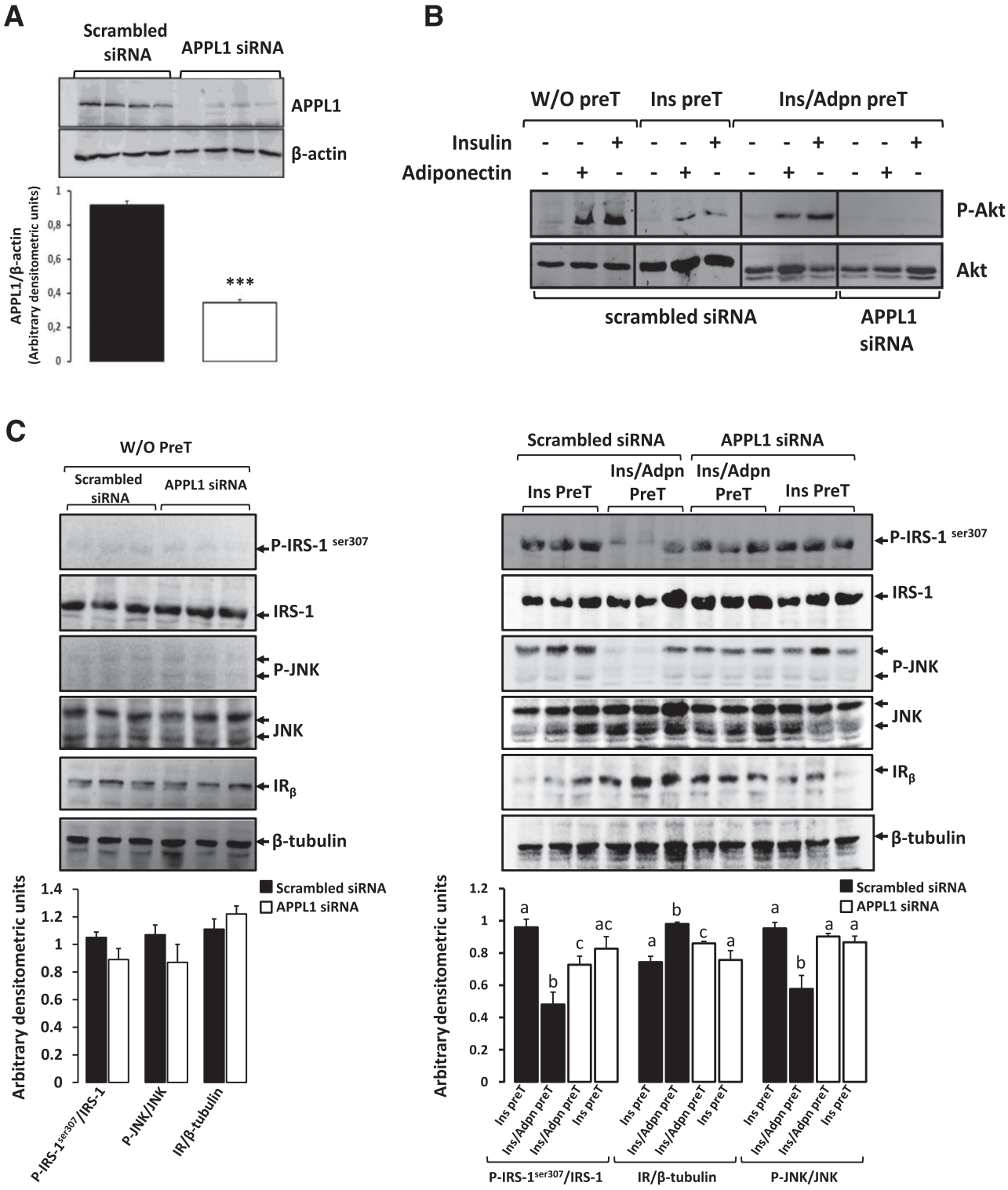

Figure 3—APPL1 mediates the insulin-sensitizing effect of adiponectin in human SH-SY5Y neuroblastoma cells. SH-SY5Y cells were treated with control siRNA or specific siRNA duplexes targeting APPL1. The protein lysates were then subjected to Western blot analysis. *A*: Immunoblot analysis of APPL1 content in control and small interfering APPL1-treated cells. The results are expressed as the ratio of APPL1 to β-tubulin. *B*: Immunoblot analysis of the phosphorylation of AKT by insulin (100 nmol/L for 10 min) and adiponectin (250 ng/mL for 10 min) in control and APPL1-supressed SH-SY5Y, treated for 16 h without (W/O preT) or with insulin (Ins preT) (100 nmol/L) or insulin (100 nmol/L) + adiponectin (250 ng/mL) (Ins/Adpn preT). *C*: Immunoblot analysis of the protein expression of IR and the phosphorylation of JNK and IRS-1^{Ser307} in control and APPL1-suppressed SH-SY5Y cells treated for 16 h without or with (100 nmol/L) insulin or insulin (100 nmol/L) + adiponectin (250 ng/mL). Bar graphs represent the ratios of IR to β-tubulin, p-JNK (p-JNK54/46) to total JNK, and p–IRS-1^{Ser307} to total IRS-1. All results are means ± SEM (n = 3–6/group). ***P < 0.001 compared with control cells. ^{a,b,c}Significant differences by ANOVA and Fisher post hoc test at P < 0.05.

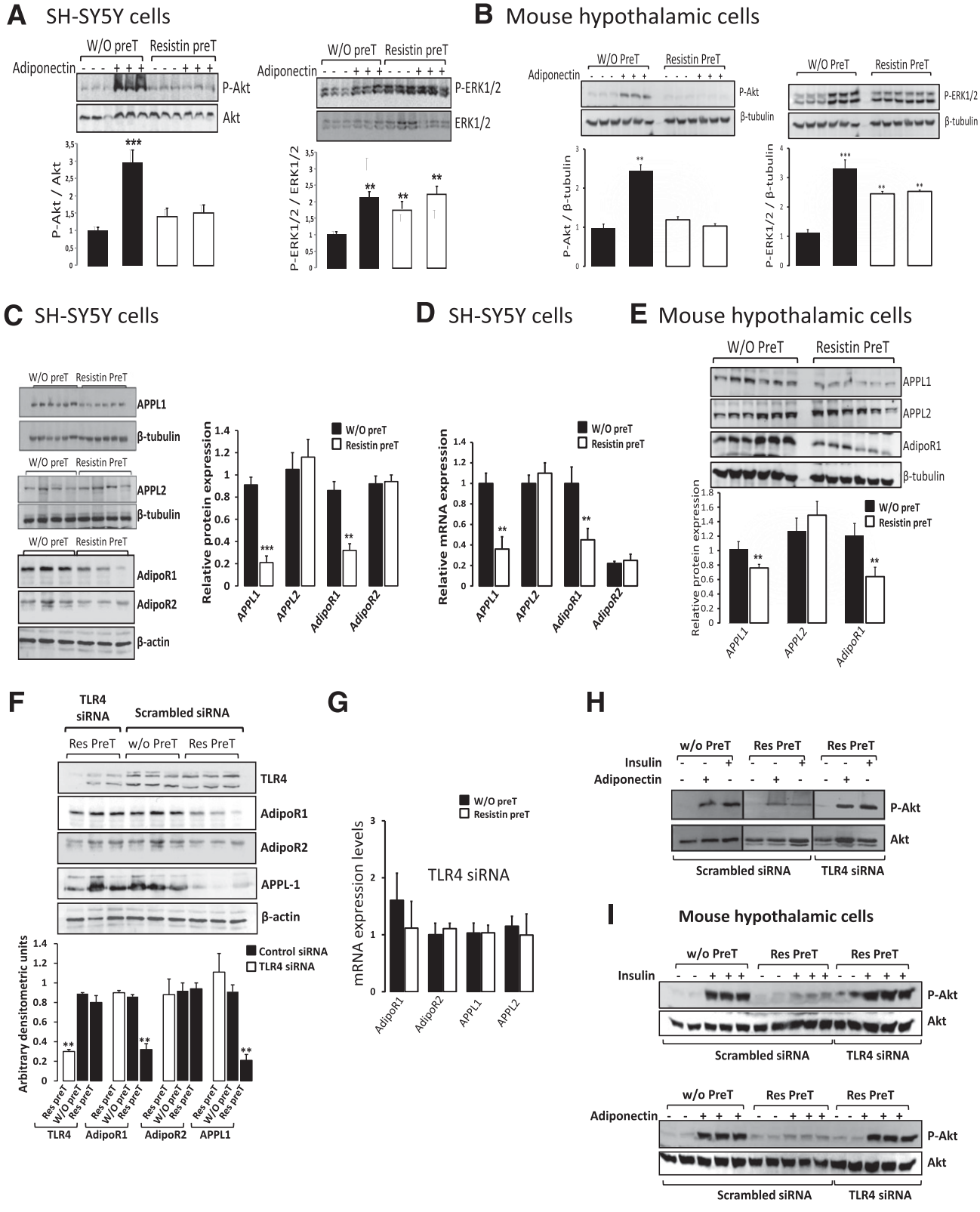

Figure 4—Resistin negatively regulates adiponectin signaling through the downregulation of both APPL1 and AdipoR1 in SH-SH5Y and mHypo cells. SH-SY5Y and mHypo cells were serum starved overnight and treated for 16 h with (Resistin preT) or without (W/O preT) resistin (200 ng/mL). *A* and *B*: Control and resistin-treated cells were stimulated for 10 min with or without adiponectin (250 ng/mL). Phosphorylation of Akt and ERK1/2 was detected by Western blot analysis with the specific antibodies indicated. Proteins on the blots were quantified by densitometry. Bar graphs represent the ratios of p-Akt to Akt or p-ERK1/2 to ERK1/2. *C* and *E*: Immunoblot analysis of the protein expression of APPL1, APPL2, AdipoR1, and AdipoR2 in control and resistin-treated cells. Results are expressed as ratio of APPL1 to β-tubulin, APPL2 to β-tubulin, AdipoR1 to β-actin, and AdipoR2 to β-actin. *D*: mRNA levels of APPL1, APPL2, AdipoR1, and AdipoR2 in control and resistin-treated SH-SY5Y (200 ng/mL for 16 h). Results were normalized to 18S RNA. *F–G*: SH-SY5Y cells were treated with control siRNA or specific

resistin abolished adiponectin-dependent phosphorylation of Akt and increased the basal phosphorylation of ERK1/2. Resistin treatment also downregulated APPL1 and AdipoR1 expression, whereas APPL2 and AdipoR2 expression was not affected (Fig. 4*C* and *D*). Furthermore, we showed that TLR4 knockdown (Fig. 4*D*) abrogated the resistin-dependent downregulation of AdipoR1 and APPL1 (Fig. 4*E* and *F*) and restored both insulin- and adiponectin-dependent Akt phosphorylation (Fig. 4*G* and *H*).

Central Resistin Infusion Promotes the Downregulation of APPL1 and AdipoR1/2 in Rats and Mice

Next, we examined whether resistin could affect APPL1, AdipoR1, and AdipoR2 expression. We showed that Wistar rats chronically (14 days) infused by intracerebroventricular resistin exhibited lower hypothalamic expression of APPL1, AdipoR1, and AdipoR2, whereas APPL2 levels remained unchanged (Fig. 5*A*). The protein levels of APPL1 were also reduced in the liver but not in the muscle or WAT of resistin-treated rats (Fig. 5*B*). Resistin treatment also reduced AdipoR1/2 expression in liver, muscle, and WAT (Fig. 5*B*).

The effect of intracerebroventricular resistin infusion for 3 days was also tested in wild-type and TLR4-KO mice. Intracerebroventricular resistin reduced hypothalamic expression of APPL1 and AdipoR1/2 in wild-type but not in TLR4-KO mice (Fig. 5*C* and *E*). In both strains, APPL2 remained unchanged. In peripheral tissues, APPL1/2 and AdipoR1/2 remained unchanged in resistin-treated mice (Fig. 5*D*).

Central Resistin Infusion Impairs the Interaction Between Akt and Its Binding Partners, APPL1 and TRB3, Altering the AdipoR2/APPL1 Signaling

We investigated whether intracerebroventricular resistin infusion affected Akt, APPL1, and TRB3 interplay in the hypothalamus and liver of rats. In the hypothalamus, we revealed the association of Akt with both APPL1 and TRB3 under the basal condition (Fig. 6*A*). In resistin-treated rats, Akt-APPL1 association was reduced, while Akt-TRB3 interaction was enhanced (Fig. 6*A*). Furthermore, in control rats, insulin treatment increased Akt-APPL1 coimmunoprecipitation and reduced Akt-TRB3 interaction (Fig. 6*A*). Conversely, in resistin-treated rats, the effect of insulin was abolished (Fig. 6*A*). In the liver, intracerebroventricular resistin abolished the insulin-dependent association of Akt with APPL1 and increased Akt-TRB3 coimmunoprecipitation (Fig. 6*B*). Next, we investigated the impact of intracerebroventricular resistin infusion on adiponectin-dependent association of AdipoR2 with APPL1 in rat liver membranes. We showed that resistin treatment reduced the coimmunoprecipitation of AdipoR2 and APPL1 in response to adiponectin stimulation (Fig. 6*C*).

Central Resistin Infusion Alters FGF21, FGFR, and KLB Expressions in the Hypothalamus and Peripheral Tissues

Next, we investigated whether central resistin-TLR4 pathways could contribute to FGF21 resistance. Thus, we assessed in mice and rats the impact of intracerebroventricular resistin infusion on FGF21, FGFR, and KLB expression in the hypothalamus and peripheral tissues. We showed that resistin decreased hypothalamic FGF21 expression in rats and mice. Hypothalamic expression of FGFR1/KLB was also significantly reduced by resistin treatment (Fig. 7*A* and *B*). In mice, intracerebroventricular resistin also decreased hypothalamic expression of FGFR2 and FGFR3 (Fig. 7*B*). Similarly, in SH-SY5Y cells, resistin overexposure reduced the expression levels of FGFR1, FGFR2, and KLB (Fig. 7*K*).

In peripheral tissues, intracerebroventricular resistin infusion increased the expression levels of FGF21 in rat liver and WAT but not in muscle (Fig. 7*D*). In addition, FGFR1 was downregulated in both WAT and muscle but not in liver (Fig. 7*D*). The expression levels of KLB and FGFR4 were attenuated in the WAT of resistin-treated rats but not in muscle and liver (Fig. 7*D*). Interestingly, intracerebroventricular resistin reduced WAT adiponectin expression (Fig. 7*E*) and plasma adiponectin levels (Fig. 7*F*). In contrast, the circulating levels of FGF21 and glucose were increased in resistin-treated rats (Fig. 7*G* and *J*), whereas plasma insulin and resistin levels were not affected (Fig. 7*H* and *I*). In mice, intracerebroventricular resistin infusion increased plasma levels of FGF21 but not those of insulin or resistin and also did not affect FGFRs or KLB expression at the peripheral level (Supplementary Fig. 1).

Next, we examined the role of TLR4 in resistin-dependent regulation of FGF21, FGFR, and KLB gene expressions. TLR4 repression in SH-SY5Y cells abrogated the effect of resistin on FGFR2 and KLB expression (Fig. 7*K*). In addition, the resistin effect on FGFR1 expression was attenuated in TLR4-suppressed cells (Fig. 7*K*). The resistin-dependent downregulation of FGF21, FGFR1, and KLB was abolished in the hypothalamus of TLR4-KO mice (Fig. 7*C*). We also showed that resistin overexposure reduced FGF21-dependent phosphorylation of FGFR1 and ERK1/2 in SH-SY5Y (Fig. 7*L*) and mHypo (Fig. 7*M*) cells. This was associated with a significant decrease of protein expression of FGFR1 (Fig. 7*L* and *M*). Additionally, rat chronic intracerebroventricular resistin infusion significantly reduced

siRNA duplexes targeting TLR4 and then stimulated for 16 h with or without resistin (Res) (200 ng/mL). Expression levels of AdipoR1, AdipoR2, APPL1, and TLR4 were evaluated by Western blot (*F*) and SYBR Green real-time RT-PCR (*G*) in control and TLR4-suppressed SH-SY5Y cells. Phosphorylation of Akt and ERK1/2 in response to acute (10 min) stimulation with insulin or adiponectin was detected by Western blot analysis in control and resistin-treated SH-SY5Y (*H*) and mHypo (*I*) cells using specific antibodies as indicated. All results are means ± SEM (n = 3–6/group). $^{**}P < 0.01$, $^{***}P < 0.001$ compared with control cells without any pretreatment.

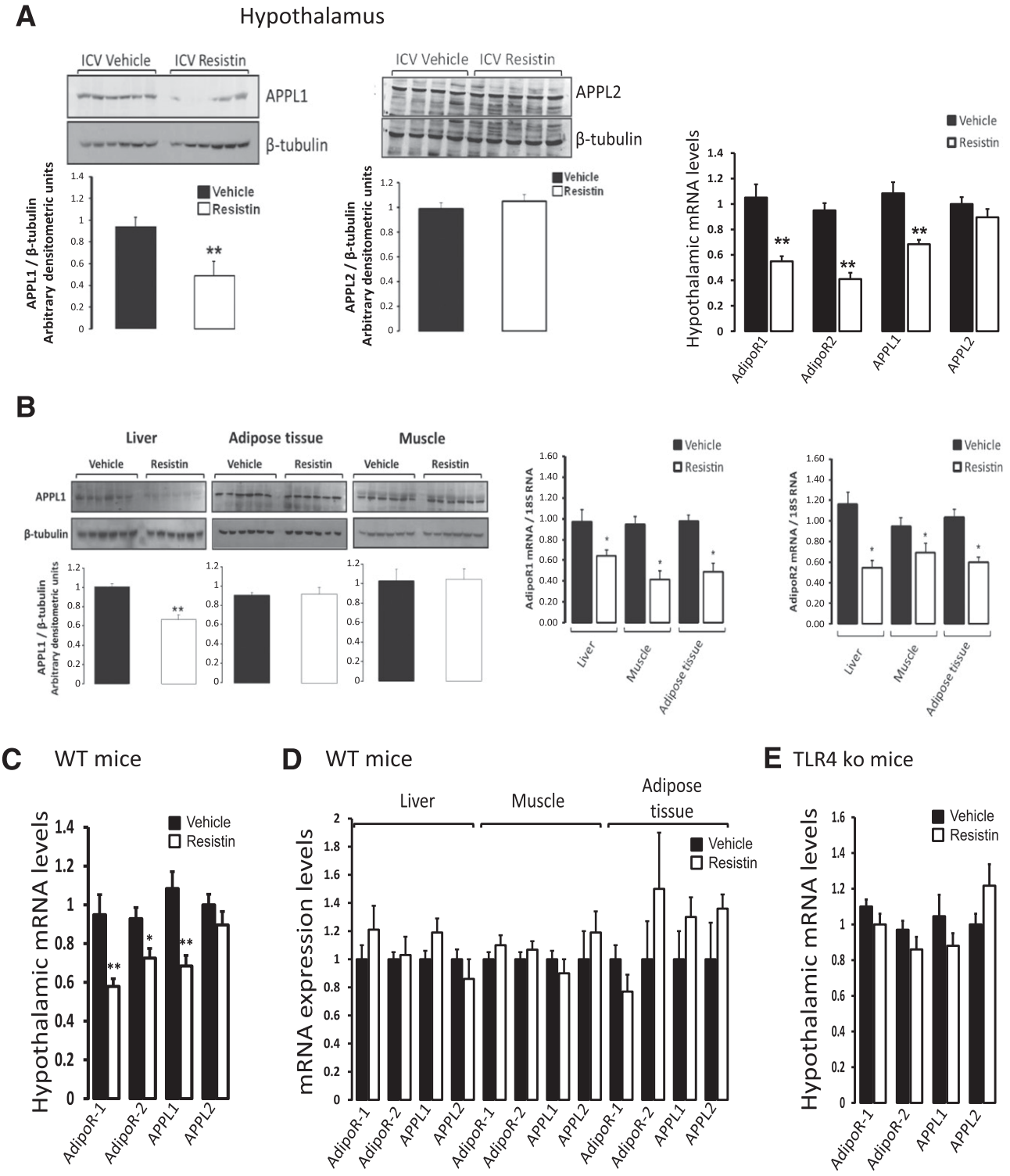

Figure 5—Central resistin infusion promotes the downregulation of APPL1 and AdipoR1/2 in rats and mice. Male Wistar rats intracerebroventricularly (ICV) received vehicle or resistin (2 μg/12 μL/day; pumping rate 0.5 μL/h) during a period of 2 weeks. At the end of the infusion period, the expression levels of APPL1, APPL2, AdipoR1, and AdipoR2 were evaluated by Western blot and SYBR Green Real-Time RT-PCR in the hypothalamus (*A*) and peripheral insulin-sensitive tissues (*B*). *C–E*: Male wild-type (WT) C57BL/6J mice or TLR4-KO mice with the same genetic background were intracerebroventricularly injected with vehicle or resistin (2 μg/6 μL/day; pumping rate 0.25 μL/h) during a period of 3 days. At the end of the infusion period, mRNA expression of APPL1, APPL2, AdipoR1, and AdipoR2 was evaluated by SYBR Green Real-Time RT-PCR in the hypothalamus (*C*) and peripheral insulin-sensitive tissues (*D*) of WT mice and in the hypothalamus of TLR-KO mice (*E*). All data are expressed as means ± SEM (n = 3–6 /group). P values were calculated using the Student t test. $^*P < 0.05$, $^{**}P < 0.01$ compared with vehicle.

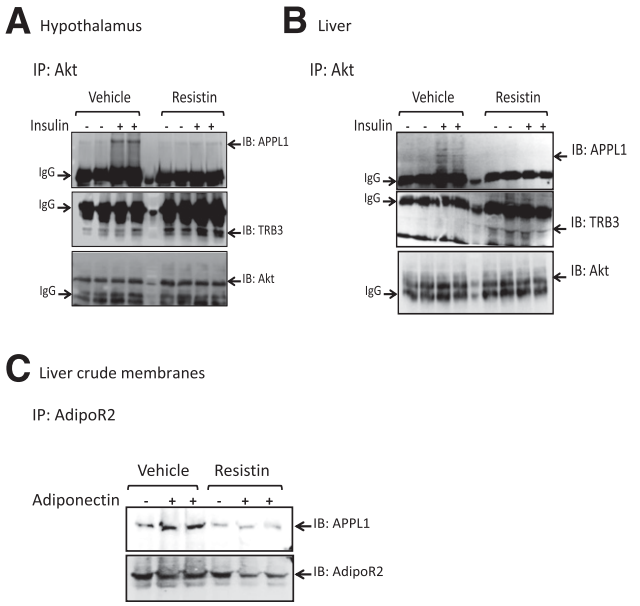

Figure 6—Central resistin infusion impairs the interaction between Akt and its binding partners, APPL1 and TRB3, as well as AdipoR2/APPL1 association. Male Wistar rats were treated with or without resistin (2 μg/12 μL/day i.c.v.; pumping rate 0.5 μL/h) for 14 days. At the end of the infusion period, vehicle and resistin-treated rats received human insulin (1 units/kg body wt i.p.) or saline bolus 30 min before euthanasia. Protein lysates from hypothalamus and liver were subjected to immunoprecipitation (IP)/immunoblot (IB) analysis to detect the interaction between Akt, APPL1, and TRB3 in the hypothalamus (*A*) and liver (*B*) using specific antibodies as indicated. *C*: Representative immunoblot of APPL1 and AdipoR2 coimmunoprecipitation in rat liver crude membranes.

FGF21-dependent phosphorylation of FGFR1 in vitro in liver crude membranes (Fig. 7*N*).

DISCUSSION

Resistin is a critical factor in obesity-mediated insulin resistance. The molecular mechanisms mediating its effects are not fully characterized, particularly at the neuronal level. This study reveals novel mechanisms by which central resistin induces whole-body insulin resistance through the impairment of adiponectin signaling and by promoting FGF21 resistance. We show that chronic intracerebroventricular resistin infusion downregulated hypothalamic and hepatic APPL1 expression, reducing Akt-APPL1 interaction and favoring Akt association with its inhibitor, TRB3. Resistin treatment also reduced hypothalamic and peripheral expression of AdipoR1/2. Importantly, we report that central resistin, via TLR4, promoted FGF21 resistance through the downregulation of FGF21 hypothalamic expression and both hypothalamic and peripheral expression of its receptor components.

Adiponectin increased insulin sensitivity in peripheral tissues (21,22,24). At the central level, the insulin-sensitizing effect of adiponectin remains poorly documented. To investigate whether adiponectin regulates neuronal insulin signaling, we examined, in SH-SY5Y and mHypo cells, the impact of adiponectin on Akt and ERK1/2. First, we showed that adiponectin significantly increased Akt and ERK1/2 phosphorylation. We reported a synergistic effect of adiponectin and insulin regarding Akt phosphorylation suggesting a cross talk between these two hormones. This is in good agreement with recent studies showing that adiponectin and insulin signaling cascades converge on Akt through the adapter protein APPL1, known to regulate both insulin- and adiponectin-dependent Akt activation (27). Furthermore, we showed that the overexposure of cells to insulin impaired Akt and ERK1/2 phosphorylation in response to insulin. Interestingly, when cells were overexposed to insulin and adiponectin, the insulin-dependent Akt and ERK1/2 phosphorylations were totally restored. This agreed with previous studies demonstrating an adiponectin-dependent improvement of both hypothalamic and peripheral insulin sensitivity (27,31). Importantly, the insulin-sensitizing effects of adiponectin were abolished in APPL1-suppressed SH-SY5Y cells, presenting the first evidence for the critical role of APPL1 in neuronal adiponectin/insulin cross talk and in mediating the insulin-sensitizing effect of adiponectin. We investigated the mechanisms underlying an adiponectin effect on insulin signaling. Previous studies have reported that hyperinsulinemia-induced downregulation of IR is considered an important mechanism promoting insulin resistance (46,47). Consistent with these studies, we show that insulin overexposure markedly reduces IR expression in SH-SY5Y cells. When cells were overexposed to both insulin and adiponectin, the insulin-dependent downregulation of IR was fully reversed. Adiponectin pretreatment also abolished the insulin-dependent upregulation of PTP1B, known as a promoter of insulin resistance (48). Interestingly, adiponectin treatment decreased insulin-dependent phosphorylation of JNK and IRS-1^{Ser307} phosphorylation known to impair insulin signaling (49,50). Our findings demonstrate that in the insulin resistance state, adiponectin improves neuronal insulin responsiveness by restoring insulin receptor expression, suppressing PTP1B expression, and inhibiting both JNK and IRS-1^{Ser307} phosphorylations.

Obesity is associated with hypoadiponectinemia, considered an important risk factor for insulin resistance (24). In contrast, plasma levels of resistin are increased in obese insulin resistant rodents and humans (8,10,11). Unequivocally, much evidence causally links resistin to insulin resistance (8,10,11,19,20). The underlying mechanisms remain poorly characterized, particularly at the neuronal level. Therefore, we assessed whether resistin could indirectly promote insulin resistance through the impairment of neuronal adiponectin signaling. We show that resistin overexposure dramatically reduced the adiponectin-dependent phosphorylation of Akt and ERK1/2 in SH-SY5Y and mHypo cells. This could be attributed to the resistin-dependent downregulation of AdipoR1. We provide the first evidence for a resistin-dependent downregulation of APPL1. To assess whether resistin

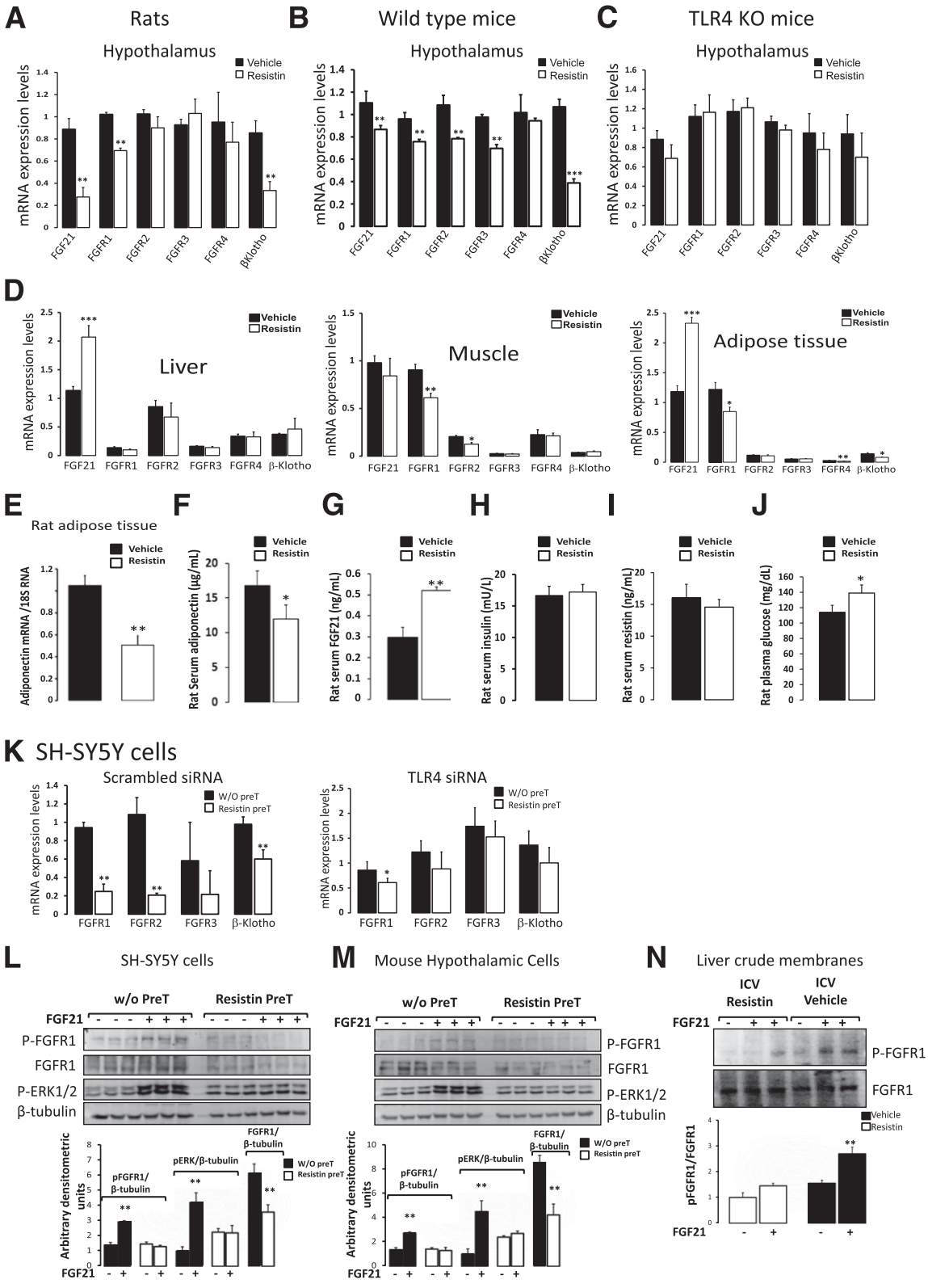

Figure 7—Effect of chronic resistin treatment on FGF21, FGFRs, KLB, and adiponectin expressions. Male Wistar rats received vehicle or resistin (2 μg/12 μL/day i.c.v.; pumping rate 0.5 μL/h) during a period of 2 weeks. Male wild-type (WT) C57BL/6J mice or TLR4-KO mice with the same genetic background were injected intracerebroventricularly with vehicle or resistin (2 μg/6 μL/day; pumping rate 0.25 μL/h) during a period of 3 days. Serum-deprived SH-SY5Y and mHypo cells were treated for 16 h with or without resistin (200 ng/mL). At the end of the treatment period, relative mRNA expression levels of FGF21, FGFR1, FGFR2, FGFR3, FGFR4, and KLB were evaluated by SYBR Green Real-Time RT-PCR in the hypothalamus of rats (*A*), wild-type mice (*B*), and TLR4-KO mice (*C*) and in peripheral insulin-sensitive tissues of rats (*D*). mRNA levels of adiponectin in WAT (*E*) and plasma levels of adiponectin (*F*), FGF21 (*G*), insulin (*H*), resistin (*I*), and glucose (*J*) in vehicle- and resistin-treated rats. mRNA expression of FGF21, FGFRs, and KLB in control and TLR4-suppressed SH-SY5Y

action on adiponectin signaling is mediated by TLR4, we investigated the impact of resistin overexposure on adiponectin signaling in TLR4-depleted SH-SY5Y and mHypo cells. Consistent with our recent report demonstrating that resistin, through hypothalamic TLR4, impaired hypothalamic insulin signaling (20), we showed that silencing TLR4 abrogated the resistin-dependent downregulation of AdipoR1 and APPL1 and restored insulin- and adiponectin-dependent Akt phosphorylation. This reveals a new signaling pathway through which resistin promotes insulin resistance by inducing a TLR4-dependent impairment of adiponectin signaling in neurons.

We next validated resistin/adiponectin cross talk in vivo by evaluating the impact of intracerebroventricular resistin infusion on the hypothalamic and peripheral expression of APPL1 and adiponectin receptors. We showed that chronic intracerebroventricular resistin infusion reduced the expression levels of AdipoR1/2 in the hypothalamus, liver, muscle, and WAT of rats. Importantly, resistin treatment induced a marked downregulation of APPL1 in the hypothalamus and liver. We also showed that intracerebroventricular resistin reduced liver adiponectin responsiveness of rats as evidenced by the impairment of AdipoR1-APPL1 interaction. Similarly, we reported in mice that intracerebroventricular resistin infusion for 3 days reduced the expression levels of AdipoR1/2 and APPL1 in the hypothalamus but not in peripheral tissues. The apparent discrepancy of results between mice and rats may be explained by genotype disparities as well as by the differences in the duration of resistin treatment. Furthermore, we demonstrated in TLR4-KO mice that intracerebroventricular resistin infusion did not impair APPL1 or AdipoR1/2 hypothalamic expression, suggesting a critical role of TLR4 in mediating resistin effects on adiponectin signaling in vivo. These results suggest that central resistin/TLR4 could promote adiponectin resistance through the downregulation of AdipoR1/2 and APPL1 at both hypothalamic and peripheral levels, leading to the impairment of adiponectin signaling and the abrogation of the insulin-sensitizing effects of adiponectin, at least in the liver. Our results present a new mechanistic explanation of previous findings highlighting that altered expression of APPL1 and adiponectin receptors in obesity reduces adiponectin sensitivity, leading to insulin resistance and T2D (23,24,27).

APPL1 is an important player in the insulin-mediated Akt signaling. APPL1 interacts with Akt and blocks Akt association with its inhibitor TRB3, promoting Akt activation (27). On the basis of these observations, we investigated whether intracerebroventricular resistin infusion could impair AKT, APPL1, and TRB3 interplay. Interestingly, coimmunoprecipitation analysis showed that under basal conditions, intracerebroventricular resistin reduced APPL1-Akt interaction and enhanced Akt-TRB3 association. Importantly, the insulin-dependent association of APPL1 with Akt was abolished, whereas the AKT-TRB3 interaction was enhanced in resistin-treated animals. These findings highlight a novel mechanism of central resistin action toward insulin-dependent Akt phosphorylation through the blockade of Akt-APPL1 interaction and the promotion of Akt-TRB3 association.

Another major finding of the current study is the characterization of the underlying mechanisms, that resistin most likely induced insulin resistance through the induction of FGF21 resistance in the hypothalamus and peripheral tissues. Indeed, FGF21 has several beneficial effects on insulin sensitivity and glucose and lipid metabolism (33–37). Subsequent studies reported that serum FGF21 levels are elevated in obese rodents and humans (43–45). Additionally, the expression levels of FGF21 receptors were downregulated in the liver and WAT of obese mice, leading to metabolic defects of FGF21, suggesting an obesity-associated FGF21 resistance (43,44). However, the mechanisms whereby obesity induces FGF21 resistance remained unknown. Consistent with these observations, we investigated the impact of chronic intracerebroventricular resistin on the expression levels of FGF21 and its receptor components in the hypothalamus and peripheral tissues. Importantly, we found that intracerebroventricular resistin infusion significantly decreased the expression levels of FGF21, FGFR1, and KLB in the hypothalamus of mice and rats. Similarly, resistin-treated SH-SY5Y and mHypo cells showed a marked diminution of FGFR1 and KLB expressions associated with a significant inhibition of FGFR1 phosphorylation, suggesting a direct effect of resistin on the modulation of FGFR expression and activity at the neuronal level. Recently, it has been shown that FGF21 acts directly in the hypothalamus to modulate energy balance and insulin sensitivity (41,42). Additionally, mice lacking the KLB gene are refractory to the insulin-sensitizing effects of FGF21 (42). In line with these reports, we showed that intracerebroventricular resistin infusion reduced the expression of FGF21 and its receptor components FGFR1 and KLB in the hypothalamus of rats and mice that could contribute to the defect of hypothalamic FGF21 signaling. In peripheral tissues, intracerebroventricular resistin increases FGF21 expression in the liver and WAT of rats, accompanied by increased circulating levels of FGF21. This was associated with a marked

cells (*K*). Representative immunoblot analysis of FGF21-dependent phosphorylation of FGFR1 and ERK1/2 in SH-SH5Y (*L*) and mHypo (*M*) cells treated with or without resistin for 16 h. *N*: FGFR1 phosphorylation on liver crude membranes from vehicle- and resistin-treated rats. All data are expressed as means ± SEM (n = 3–6 /group). *$P < 0.05$, **$P < 0.01$, ***$P < 0.005$ compared with vehicle-treated animals or control SH-SY5Y cells. Resistin preT, with resistin treatment; W/O preT, without resistin treatment.

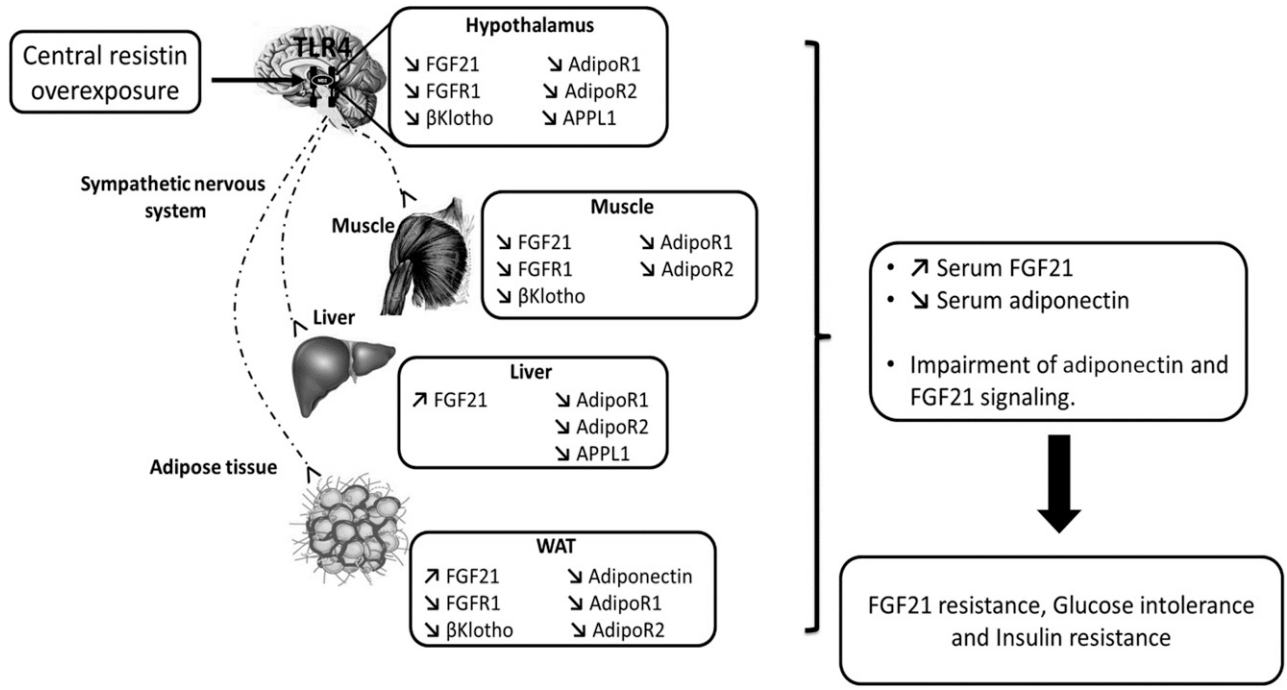

Figure 8—Schematic illustration of resistin/TLR4-dependent impairment of adiponectin and FGF21 signaling in the hypothalamus and peripheral insulin-sensitive tissues.

downregulation of FGFR1 expression in both muscle and WAT and decreased KLB expression in WAT.

Next, we examined the role of TLR4 in resistin-dependent regulation of FGF21 and FGFRs gene expressions. Interestingly, we found that the repression of TLR4 in SH-SY5Y cells almost completely abrogates resistin effects. Importantly, the resistin-dependent downregulation of FGF21, FGFR1, and KLB in the hypothalamus was abolished in TLR4-KO mice, suggesting a critical role of TLR4 in mediating resistin effects on FGF21 signaling cascade in vivo. Recently, it has been reported that FGF21 upregulates WAT adiponectin expression and secretion and that FGF21 critically depends on adiponectin to exert its insulin-sensitizing effect (40). Here, we showed that central resistin markedly decreased WAT adiponectin expression, thereby reducing serum adiponectin levels. This could contribute to the impairment of the insulin-sensitizing effects of adiponectin in resistin-treated rats.

Thus, we have demonstrated that chronic intracerebroventricular resistin treatment increased blood glucose and FGF21 levels and decreased those of adiponectin. Insulin and resistin plasma levels remained unchanged. Furthermore, intracerebroventricular resistin treatment impaired adiponectin action and increased FGF21 plasma levels, thus promoting hyperglycemia.

In summary, our study brings new insights concerning the mechanisms involved in resistin-dependent insulin resistance and provides a novel mechanism by which a central resistin-TLR4 pathway promotes whole-body insulin resistance through the impairment of adiponectin signaling and by promoting FGF21 resistance (Fig. 8).

Duality of Interest. No potential conflicts of interest relevant to this article were reported.

Author Contributions. Y.B. and M.T. designed and supervised experiments. Y.B. performed most experiments. H.A., D.C., S.A.R., L.R., and M.T. contributed to research data. Y.B. and M.T. performed data analysis. A.G. contributed to the manuscript revision and discussion and produced recombinant resistin. Y.B. and M.T. wrote the manuscript. M.T. is the guarantor of this work and, as such, had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

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