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Palmitic acid induces central leptin resistance and impairs hepatic glucose and lipid metabolism in male mice

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Abstract

The consumption of diets rich in saturated fat largely contributes to the development of obesity in modern societies. A diet high in saturated fats can induce inflammation and impair leptin signaling in the hypothalamus. However, the role of saturated fatty acids on hypothalamic leptin signaling, and hepatic glucose and lipid metabolism remains largely undiscovered. In this study, we investigated the effects of intracerebroventricular (icv) administration of a saturated fatty acid, palmitic acid (PA, C16:0), on central leptin sensitivity, hypothalamic leptin signaling, inflammatory molecules and hepatic energy metabolism in C57BL/6J male mice. We found that the icv administration of PA led to central leptin resistance, evidenced by the inhibition of central leptin's suppression of food intake. Central leptin resistance was concomitant with impaired hypothalamic leptin signaling (JAK2-STAT3, PKB/Akt-FOXO1) and a pro-inflammatory response (TNF- α , IL1- β , IL-6 and pI κ Ba) in the mediobasal hypothalamus and paraventricular hypothalamic nuclei. Furthermore, the pre-administration of icv PA blunted the effect of leptin-induced decreases in mRNA expression related to gluconeogenesis (G6Pase and PEPCK), glucose transportation (GLUT2) and lipogenesis (FAS and SCD1) in the liver of mice. Therefore, elevated central PA concentrations can induce pro-inflammatory responses and leptin resistance, which are associated with disorders of energy homeostasis in the liver as a result of diet-induced obesity.

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Palmitic acid induces central leptin resistance and impairs hepatic glucose and lipid metabolism in male mice

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Abstract:

The consumption of diets rich in saturated fat largely contributes to the development of obesity in modern societies. A diet high in saturated fats can induce inflammation and impair leptin signaling in the hypothalamus. However, the role of saturated fatty acids on hypothalamic leptin signaling, and hepatic glucose and lipid metabolism remains largely undiscovered. In this study, we investigated the effects of intracerebroventricular (icv) administration of a saturated fatty acid, palmitic acid (PA, C16:0), on central leptin sensitivity, hypothalamic leptin signaling, inflammatory molecules, and hepatic energy metabolism in C57BL/6J male mice. We found that the icv administration of PA led to central leptin resistance, evidenced by the inhibition of central leptin's suppression of food intake. Central leptin resistance was concomitant with impaired hypothalamic leptin signaling (JAK2-STAT3, PKB/Akt-FOXO1), and a pro-inflammatory response (TNF- α , IL1- β , IL-6, and pI κ Ba) in the mediobasal hypothalamus and paraventricular hypothalamic nuclei. Furthermore, the pre-administration of icv PA blunted the effect of leptin-induced decreases in mRNA expression related to gluconeogenesis (G6Pase and PEPCK), glucose transportation (GLUT2), and lipogenesis (FAS and SCD1) in the liver of mice. Therefore, elevated central PA concentrations can induce pro-inflammatory responses and leptin resistance, which are associated with disorders of energy homeostasis in the liver as a result of diet-induced obesity.

Key words: Palmitic acid, leptin resistance, hypothalamus, inflammation, glucose metabolism, lipid metabolism

Introduction:

The hypothalamus is capable of sensing nutritional status [1, 2], and as a result nutrients can influence brain function [3]. Understanding the mechanisms by which specific nutrients, such as fatty acids influence signaling within the brain regulating energy balance will help to prevent and even treat obesity and other metabolic disorders.

Leptin is secreted by adipocytes, and its circulating levels reflect the amount of energy stored in fat. This hormone acts centrally, particularly in the hypothalamus, to reduce food intake and body weight [4]. Leptin can bind to the hypothalamic leptin receptor and activates the JAK2 (Janus kinase-2)-STAT3 (signal transducer and activator of transcription-3) pathway that promotes negative energy balance [5]. Besides the JAK2-STAT3 pathway, leptin also acts through the serine/threonine protein kinase B (PKB)/Akt signaling pathway to induce forkhead box protein O1 (FOXO1) phosphorylation and degradation, and decrease FOXO1 activity in the hypothalamus [6]. FOXO1 binds to STAT3 and prevents STAT3 from interacting with the POMC promoter complex, and consequently, inhibits STAT3-mediated leptin action [7]. Phosphorylation of FOXO1 results in FOXO1 release from the nucleus and allows pSTAT3 to bind to neuropeptide promoters, stimulating the transcription of anorexigenic pro-opiomelanocortin (POMC) and inhibiting orexigenic agouti-related protein (AgRP) expression [8].

Obesity results in resistance to the effect of leptin. For example, the administration of leptin to obese subjects failed to decrease body weight and food intake [9]. Furthermore, an intracerebroventricular (icv) injection of leptin failed to inhibit food intake and body weight in chronic high-saturated-fat diet-induced obese mice [10, 11]. This suggests that resistance to leptin in the central nervous system (CNS) compromises the ability of leptin to regulate food intake and body weight in the presence of a diet high in saturated fat. However, the cause of obesity and leptin resistance in most forms of human and rodent obesity is still poorly understood. It is known that

leptin binds to long-form LepRb on neurons in several regions in the hypothalamus, including the mediobasal hypothalamus (MBH) and paraventricular hypothalamic nuclei (PVN), to regulate food intake and energy homeostasis [12]. El-Haschimi et al. [13] showed that leptin failed to induce STAT3 activation in hypothalamic extracts from obese mice induced by a high-fat diet, suggesting hypothalamic leptin resistance.

Evidence suggests that the CNS is a critical target for leptin regulation of glucose and lipid metabolism in the peripheral tissue, such as liver, muscle, and adipose tissue. For example, the icv infusion of leptin increases glucose turnover and glucose uptake, but decreases hepatic glycogen content without changing plasma glucose in wild-type mice [14]. Central treatment with leptin decreased mRNA expression of the hepatic gluconeogenic enzymes, glucose 6-phosphatase (G6Pase), and phosphoenolpyruvate carboxykinase (PEPCK) in streptozotocin-induced diabetic rats [15]. In addition, chronic icv administration of leptin caused the down-regulation of genes encoding stearoyl-CoA desaturase-1 (SCD1), acetyl-coenzyme A-carboxylase (ACC), and fatty acid synthase (FAS) in the liver when compared with vehicle-infused pair-fed rats [16]. This suggests that leptin acts in the brain to suppress liver lipogenic gene expression independent of feeding. Furthermore, the attenuation of leptin-mediated Akt signaling in LepRb neurons causes decreased sympathetic tone in the liver and increases hepatic steatosis [17].

Recent evidence shows that hypothalamic inflammation induced by dietary saturated fats is implicated in the development of obesity and its associated leptin resistance [11]. Within one week of consuming a high-fat diet, there is low-grade hypothalamic inflammation and an increase in pro-inflammatory cytokines, evidenced by an increase in tumor necrosis factor alpha (TNF- α), interleukin-1-beta (IL-1 β), and interleukin 6 (IL-6) mRNA expression [18]. These cytokines activate the nuclear factor- κ B (NF- κ B) inflammatory signaling pathway by phosphorylating and degrading inhibitor kappa B alpha (I κ B α) [19]. Recent studies have also revealed that hypothalamic

inflammation plays an important role in mediating central leptin resistance and the interruption of leptin signaling in the hypothalamus of rodents. Constitutive activation of NF- κ B inflammatory signaling in the hypothalamus of mice induced central leptin resistance and impaired leptin signaling through pSTAT3 [20]. In contrast, a genetic or pharmacological blockade of hypothalamic inflammatory signaling has improved leptin sensitivity and elevated pSTAT3 [20, 21].

Palmitic acid (PA, C16:0) is the most common saturated fatty acid in human diets [22], accounting for approximately 65% of saturated fatty acids and 32% of total fatty acids in human serum [23]. Patients with metabolic syndrome have a significantly higher level of serum PA than controls [23], and also have high levels of PA, but not myristic acid or stearic acid within erythrocytes [24]. The central administration of PA significantly decreases the anorexigenic effect of leptin in mice [25]. In this study, we investigated hypothalamic leptin sensitivity, signaling, and inflammation in response to PA administration, and further examined the effect of icv PA and leptin on glucose levels and the expression of hepatic genes involved in glucose and lipid metabolism.

Materials and Methods:

Animals

Male C57BL/6J mice (10 weeks old, body weight: 22.74 ± 3.22 g) were obtained from the Animal Resource Centre (Perth, WA, Australia) and housed in environmentally controlled conditions (temperature 22 °C, 12 hour light/dark cycle). Mice were maintained on a normal lab chow diet (LC, Vella Stock feeds, Doonside, NSW, Australia) throughout this study. All experimental procedures were approved by the Animal Ethics Committee, University of Wollongong, Australia, and complied with the *Australian Code of Practice for the Care and Use of Animals for Scientific Purposes*.

Experiment Protocols

After 1 week of acclimatization, mice were anesthetized by isoflurane inhalation and placed in a stereotactic device. An icv cannula was implanted into the right lateral brain ventricle (0.25 mm posterior and 1.0 mm lateral relative to Bregma and 2.5 mm below the surface of the skull) as described in our previous study [10]. The accuracy of cannula implantation into the lateral ventricle was confirmed by examining the needle track in the brain sections of each animal (Fig S1).

Central leptin sensitivity test

Five days after the cannula implantation, a central leptin sensitivity test was performed as described previously [11]. The mice were randomly divided into 2 groups (n=24) and received icv injections of PA (25 pmol twice a day for 2.5 days, 5 injections in total) or vehicle [26]. At the end of day 2 of the test, the mice were fasted overnight, and each group of mice was divided into two subgroups (n=12) that received an icv injection of either leptin (0.5 µg in 2 µl) or vehicle (2 µl saline) one hour after the last PA injection. Food intake and animal weights were then measured 24 hours after the icv leptin or vehicle injection. As described previously [27], PA (P5585, Sigma-Aldrich, Australia) was dissolved in 96% ethanol, dried using nitrogen gas and then dissolved in 40% hydroxypropyl-β-cyclodextrin (HPB) (H107, Sigma-Aldrich) and stored at -20 °C. The working solution contained 25 pmol PA every injection.

Intraperitoneal glucose tolerance test (GTT)

After at least 3 day interval, the mice were repeated with PA and leptin as described for the leptin sensitivity test with individual mice assigned to the same previous treatment or vehicle group. Glucose tolerance tests were then performed 30 minutes after leptin injection. Blood glucose was measured at 0, 30, 60, and 120 min after glucose administration (0.5 g/kg glucose, ip) using a glucometer (Alameda, CA).

Tissue collection

Again, after at least 3 day interval, the mice were repeated with PA and leptin as described above. Thirty minutes after leptin injection, the mice were sacrificed by CO₂ asphyxiation. The brain and liver were immediately collected, snap frozen in liquid nitrogen, and stored at -80 °C for further processing and analysis. In a cryostat at a temperature of -18 °C, 500 µm frozen brain sections were cut from Bregma -0.58 mm to -2.72 mm according to a standard mouse brain atlas [28]. The mediobasal hypothalamus (MBH) and paraventricular nuclei (PVN) were dissected using a Stoelting Brain Punch (#57401, 0.5 mm diameter, Wood Dale, Stoelting Co, USA) from frozen coronal sections based on previously described coordinates [11, 28].

Western blot analysis

Western blotting was performed on protein extracts from frozen tissue as described in our previous study [10]. The expression of specific proteins was determined using the following antibodies: TNF- α (sc-8301), IL-1 β (sc-7884), IL-6 (sc-7920), pI κ B α (sc-8404), and pJAK2 (sc-21870) from Santa Cruz Biotechnology (City, State, Country), and pSTAT3 (Tyr705) (#9145), SOCS3 (#2932), pAkt (#9271), and pFOXO1 (#9461) from Cell Signaling Technology (Beverly, MA, USA). Bands corresponding to the proteins of interest were scanned and band density analysed using the automatic imaging analysis system, Quantity One (Bio-Rad). All quantitative analyses were normalized to β -actin, based on our previous studies [29]. Due to the small amount of tissue in the MBH and PVN of the hypothalamus, we used a previously-described modified multi-strip western blot, which allows the detection of multiple proteins with a smaller sample size than in a standard western blot [11].

RNA isolation and RT-PCR

Total RNA from the liver was extracted using the Aurum total RNA mini kit (Bio-Rad Laboratories, Hercules, CA) and reverse-transcribed to first-strand complementary DNA using the high-capacity cDNA reverse transcription kit (AB Applied Biosystems, CA, USA) according to the

manufacturer's instructions. Quantitative real-time PCR (qPCR) was performed in a 20 μ l final reaction volume using SYBR green I master on a Lightcycler 480 Real-time PCR System (F. Hoffmann-La Roche Ltd, Switzerland). Amplification was carried out with 45 cycles of 95 °C for 10 seconds, 60 °C for 30 seconds, and 72 °C for 30 seconds. The mRNA expression levels were normalized to GAPDH, which served as the internal control. Expression levels for each gene were calculated using the comparative threshold cycle value (Ct) method, using the formula $2^{-\Delta\Delta Ct}$ (where $\Delta\Delta Ct = \Delta Ct \text{ sample} - \Delta Ct \text{ reference}$) as described previously [30]. The primers used are listed in supplementary table 2.

Statistics

Data were analysed using the statistical package SPSS 19.0 (SPSS, Chicago, IL, USA). The two-tailed student's t-test was used to compare hypothalamic cytokine expression between the PA and vehicle groups. One-way analysis of variance (ANOVA) and the post hoc Tukey–Kramer honestly significant difference (HSD) test were used to analyse hypothalamic leptin signaling molecules, central leptin sensitivity, and mRNA expression of genes regulating hepatic glucose and lipid metabolism. $p < 0.05$ was regarded as statistically significant. Values are expressed as mean \pm SEM.

Results:

Palmitic acid can induce central leptin resistance

To directly address whether the saturated fatty acid PA impairs central leptin sensitivity *in vivo*, we examined the anorexigenic effect and body weight change in response to the icv injection of leptin (0.5 μ g) after icv PA injections (25 pmol twice a day for 2.5 days). As shown in Figure 1, central leptin administration in mice without PA treatment significantly suppressed food intake at 1 hour (-57.27%, $p < 0.001$), 4 hours (-57.55%, $p < 0.001$), 16 hours (-45.19%, $p < 0.001$) and 24 hours (-58.23%, $p < 0.001$), compared with the saline injection in the vehicle group (Fig. 1A). However, in the PA pre-treated group, leptin did not suppress food intake compared to the saline injection (Fig.

1A), with only the 21.47% decrease in food intake at 24 hours approaching significance ($p=0.03$), suggesting that PA induced central leptin resistance. Furthermore, 24 hours after administration leptin significantly decreased body weight by 2.66 g in the vehicle group ($p<0.001$), but only 1.54 g in the PA pre-treatment group, ($p<0.05$) (Fig. 1B).

Palmitic acid inhibits leptin signaling in the MBH and PVN

Hypothalamic leptin signaling regulates energy balance via the pJAK2-pSTAT3 and pAkt-pFOXO1 pathways [31, 32]. We examined the effects of the central administration of PA on the leptin mediation of these two pathways in the MBH and PVN. In both MBH and PVN, central leptin injection significantly increased the levels of pJAK2 (MBH: $p<0.01$, Fig. 2A; PVN: $p<0.01$, Fig. 2D), and pSTAT3 (MBH: $p<0.01$, Fig. 2B; PVN: $p<0.05$, Fig. 2E) in the vehicle group. However, with PA pre-treatment, there were no differences in pJAK2 and pSTAT3 between the leptin icv injection and the saline injection in the MBH and PVN, suggesting that the central administration of PA blunted leptin-pJAK2-pSTAT3 signaling. Interestingly, the PA central administration increased pSTAT3 in both the MBH and PVN (MBH: $p<0.01$, Fig. 2B; PVN: $p<0.05$, Fig. 2E). SOCS3 was increased after leptin and PA icv injection in the PVN ($p<0.05$, Fig. 2F,) but not in the MBH ($p>0.05$, Fig. 2C). Similarly, we found that leptin significantly increased pAkt and pFOXO1 in the MBH and PVN in vehicle mice but not in the mice pre-treated with PA (Fig. 3), suggesting that the leptin-Akt-FOXO1 signaling pathway was also impaired.

Hypothalamic inflammatory response in response to palmitic acid administration

Previously it has been shown that a high-saturated fat diet induces hypothalamic inflammation [33]. To determine the impact of central treatment with PA on hypothalamic inflammation, the expression of inflammatory molecules in the MBH and PVN of hypothalamus were examined. As depicted in Figure 4, the central administration of PA significantly increased TNF- α and pI κ B α in the MBH (both $p<0.05$, Fig. 4A). In the PVN, PA significantly induced an increase of TNF- α and

IL-1 β compared with the vehicle group (both $p < 0.05$, Fig. 4B). These results indicate that centrally administered PA can induce a pro-inflammatory response in the MBH and PVN.

Palmitic acid can attenuate the effectiveness of the central leptin regulation on hepatic glucose metabolism

A glucose tolerance test was performed after the central administration of PA and leptin. There were no significant changes in blood glucose levels during the glucose tolerance test after icv administration of leptin or PA. However, following PA treatment the icv injection of leptin increased blood glucose levels after overnight fasting and at the 30 minute time point of the glucose tolerance test (both $p < 0.05$, Table 1). These results imply that the icv injection of PA impaired the ability of central leptin to maintain normal blood glucose levels.

To investigate the action of central leptin in regulating glucose metabolism in the liver in response to the icv PA pre-treatment, we measured the level of mRNA expression for the genes involved in gluconeogenesis (G6Pase and PEPCK), glycolysis (glucokinase, GK), and glucose transportation (glucose transporter 2, GLUT2) in the liver. As shown in Figure 5, the mRNA expression of G6Pase ($p < 0.01$, Fig. 5A) and PEPCK ($p < 0.01$, Fig. 5B) decreased significantly after the central leptin injection in the vehicle group. However in PA group, the effect of leptin in decreasing G6Pase and PEPCK mRNA expression was blunted. The central administration of leptin significantly decreased GLUT2 mRNA expression ($p < 0.05$) in the liver, however this effect was not seen after pre-treatment with PA (Fig. 5C). Furthermore, the icv administration of PA decreased baseline GLUT2 mRNA expression in the liver compared with the vehicle group ($p < 0.05$, Fig. 5C). There was no significant difference in GK mRNA expression in the liver between the groups (Fig. 5D).

Palmitic acid attenuates the central leptin regulation of hepatic lipid and cholesterol metabolism

To examine the effect of icv PA on the regulation of hepatic lipid metabolism in response to leptin, we used quantitative RT-PCR to examine the mRNA expression of genes involved in hepatic lipogenesis, lipid beta-oxidation, and cholesterol metabolism. In the vehicle group, icv leptin significantly decreased the level of FAS and SCD1, indicating a reduced lipogenic effect in the liver (Fig. 6A & 6B, both $p < 0.05$). However, in the PA pre-treatment group, leptin did not decrease the level of FAS and SCD1. There was no significant difference in ACC mRNA expression between the groups.

Acyl-CoA oxidase (ACOX) and acetyl-CoA acetyltransferase 1 (ACAT1) are two markers of beta-oxidation of lipids in the liver. Leptin administered icv did not alter ACOX mRNA expression; however PA significantly increased basal ACOX mRNA expression compared with the vehicle group ($p < 0.01$, Fig. 6D). After pre-treatment with PA, leptin also significantly decreased ACOX mRNA expression, while no decrease was observed in the vehicle group ($p < 0.01$, Fig. 6D). Furthermore, with PA pre-treatment but not in the vehicle group, leptin significantly decreased ACAT1 mRNA expression ($p < 0.05$, Fig. 6E), suggesting that PA impairs central leptin modulation of beta-oxidation in the liver.

Moreover, we analysed the mRNA expression of key enzymes in cholesterol metabolism. The expression of 3-hydroxy-3-methylglutaryl-coenzyme reductase (HMG-CoA reductase, the key enzyme involved in the de novo synthesis of cholesterol) was down-regulated after central leptin injection in the vehicle group ($p < 0.05$, Fig. 6F), while this response was absent in the PA pre-treated group. There was no significant difference in the mRNA expression of Apo lipoprotein A1 (APoA1, the main component of HDL and a key player in cholesterol efflux) between the groups.

Palmitic acid can attenuate elevated hypothalamic tyrosine hydroxylase (TH) expression stimulated by leptin

Hypothalamic TH mediates leptin-induced sympathetic activity and energy expenditure [34]. We found that the icv injection of leptin significantly increased TH protein levels in the MBH and PVN (MBH: $p < 0.05$, Fig. 7A; PVN: $p < 0.05$, Fig. 7B), while PA pre-treatment blunted these effects in both the MBH and PVN.

Discussion:

A high-saturated-fat diet induces obesity-associated leptin resistance in humans and experimental animals [11, 35-37], and PA is a major source of saturated fatty acids in our diet [22]. We have shown that the elevation of central PA can reduce leptin sensitivity by suppressing food intake and inhibiting body weight gain in mice. In this situation, central leptin is unable to activate its downstream signaling molecules in the hypothalamus (e.g. JAK2-STAT3 and Akt/FOXO1 pathways). Liver glucose and lipid metabolism (which are regulated by central leptin) are also impaired due to pre-treatment with PA.

Defective leptin JAK2-STAT3 signaling in the hypothalamus is involved in the development of central leptin resistance in high-saturated-fat diet-induced obesity in rodents [38]. In the current study, we found that leptin JAK2-STAT3 signaling was impaired in the MBH and PVN after the elevation of central PA. Münzberg and colleagues showed that pSTAT3 immunoreactivity was reduced in the MBH in chronic high-fat DIO mice following an intraperitoneal injection of leptin [39]. Leptin receptor mRNA and immunoreactivity was observed in the PVN neurons [40, 41]. An icv injection of leptin elevates c-Fos immunoreactivity in the PVN [42]. However, the reported effects of leptin in the regulation of JAK2-STAT3 signaling in the PVN are not consistent. Previously, it has been reported that icv leptin administration increases pSTAT3 immunoreactivity in the PVN neurons of rats [43], and increases pSTAT3 protein in the PVN of mice [44]. However,

in the study of Münzberg and colleagues, pSTAT3 immunoreactivity was not altered in the neurons of PVN in mice following the leptin challenge [39]. In the current study, icv leptin increased pSTAT3 and its upstream activator pJAK2 in the PVN in the control group, suggesting that leptin regulates energy balance via the PVN. We also showed that this increase induced by leptin was absent following pre-treatment with PA, indicating that PA can impair the leptin signaling pathway in the PVN. A previous study has also reported that mice resistant to leptin during pregnancy have defective leptin JAK2-STAT3 signaling in the PVN [45]. SOCS3 is a leptin-inducible inhibitor of leptin JAK2-STAT3 signaling, and it has been suggested to mediate central leptin resistance in obesity [46]. We have shown that SOCS3 was significantly increased in the PVN following icv injection of PA, which may contribute to impaired leptin STAT3 signaling in the hypothalamus and the development of central leptin resistance.

The hypothalamic Akt-FOXO1 signaling pathway plays a significant role in leptin activation in the brain [31, 32]. For example, an icv injection of leptin activates hypothalamic Akt and improves glucose tolerance in skeletal muscle [47]. Akt can phosphorylate and inactivate FOXO1, a transcriptional factor in the hypothalamus [32]. The over expression of FOXO1 in the arcuate nucleus (Arc) of MBH decreases leptin sensitivity and increases food intake and body weight in mice; while conversely, an icv infusion of FOXO1-antisense oligonucleotide promotes negative energy balance and increases insulin sensitivity in DIO rats [48]. Our study found that an icv injection of leptin stimulates Akt-FOXO1 phosphorylation not only in the MBH, but also in the PVN of the hypothalamus. Hypothalamic Akt-FOXO1 signaling is also downstream of central insulin action. Previous studies show that an icv injection of PA inhibits the activation of pAkt induced by insulin in the hypothalamus [49]. However, the effect of PA on the pAkt-pFOXO1 signaling pathway in response to leptin has not been studied. We found that leptin activation of Akt-FOXO1 signaling was impaired in the MBH and PVN following an icv injection of PA in mice. Previously, leptin-stimulated activation of the hypothalamic PI3K-Akt signaling pathway was

impaired in high-saturated-fat DIO mice [50]. Thus our findings suggest that centrally administered saturated fatty acids such as PA can directly attenuate leptin Akt-FOXO1 signaling in the hypothalamus, which may contribute to central leptin resistance in obesity induced by a high-saturated-fat diet.

Studies have shown that a high-saturated-fat diet increases TNF- α and IL-1 β mRNA expression in the hypothalamus, which can lead to insulin and leptin resistance [18, 33]. The current study found that central PA administration directly promotes inflammation, with increased levels of TNF- α , IL-1 β , and pI κ B α observed in the MBH and the PVN. Smith and Nagura have shown that fatty acids can readily enter the brain in a linear fashion over time, with 40% of C¹⁴-labeled palmitate incorporated within 45 seconds [51]. These studies and ours included, suggest that the over-consumption of saturated fatty acids increases the level of saturated fatty acids in the brain, and induces a hypothalamic inflammatory response. Hypothalamic inflammation leads to central leptin resistance in high-fat diet-induced obesity in rodents [20]. Furthermore, the hypothalamic infusion of palmitate induces hypothalamic inflammation and central insulin resistance [33]. Constitutive activation of IKK β , upstream of I κ B α , in the hypothalamus induces central leptin resistance and impairs leptin signaling through pSTAT3 by increasing SOCS3 mRNA expression in mice [20]. Therefore, the activation of hypothalamic TNF- α , IL-1 β , and pI κ B α may play a key role in high-saturated-fat diet-induced obesity and central leptin resistance by impairing leptin-STAT3 and Akt signaling in the MBH and PVN.

Hypothalamic leptin signaling regulates liver glucose and lipid metabolism [52, 53]. Our present study showed that central leptin decreased liver PEPCK and G6Pase mRNA expression in normal mice, indicating suppression of hepatic gluconeogenesis. A similar study previously demonstrated that icv leptin injection can decrease hepatic G6Pase and PEPCK mRNA expression in rats with STZ diabetes [15]. Hepatic GLUT2 transports glucose from the liver to the bloodstream [54], and

we further demonstrated that hepatic GLUT2 mRNA expression decreased after an icv leptin injection. Therefore, both decreased hepatic gluconeogenesis and glucose transportation after the icv leptin injection should result in reduced blood glucose levels. However, in the present study, blood glucose did not change after the icv leptin injection. This may be due to the icv leptin injection decreasing hepatic glycogen content, which was reduced by 30% in mice in a previous study [14]. In the current study, PA pre-treatment also impaired the central action of leptin in maintaining normal blood glucose during a glucose tolerance test, with increased blood glucose levels observed at fasting and at 30 minutes of the glucose tolerance test following icv leptin. Obese rats induced by a high-saturated-fat diet showed increased levels of G6Pase and PEPCK mRNA expression in the liver [21]. The current study showed that PA pre-treatment abolished the effects of leptin in suppressing hepatic G6Pase, PEPCK, and GLUT2 mRNA expression. This suggests that PA alters the action of central leptin in the maintenance of blood glucose homeostasis, by preventing leptin from suppressing gluconeogenesis and glucose transportation in the liver.

FAS and SCD1 are critical lipogenic enzymes in the liver [55, 56]. FAS catalyzes fatty acid biosynthesis from malonyl-CoA to palmitic acid [57]. SCD1 catalyzes the cellular synthesis of monounsaturated long-chain fatty acids, particularly oleate (C18:1n-9) and palmitoleate (C16:1n-7), which are the major components of membrane phospholipids, triglycerides, and cholesterol esters [58]. In our current study, we found that icv leptin can significantly decrease the mRNA expression of FAS and SCD1 in the liver of control mice, indicating that central leptin suppresses lipogenesis in the liver. SCD1 has recently become a target of interest for the reversal of hepatic steatosis and insulin resistance [59]. Liver-specific knockout of SCD1 in mice can protect against diet-induced obesity and hepatic steatosis [60]. Interestingly, our study found that PA can impair the inhibitory effect of central leptin in the liver FAS and SCD1 mRNA expression, which may contribute hepatic steatosis in obesity.

Previously, Prieur et al. reported that an icv injection of leptin (1 µg twice a day for 2.5 days, for 5 times) not only decreased lipogenesis, but also activated beta-oxidation by increasing the mRNA expression of genes, such as ACOX and ACAT1, in the liver of *ob/ob* mice [61]. Our present study showed that icv leptin (0.5 µg) suppressed lipogenesis but did not increase ACOX and ACAT1 mRNA expression in the liver. These contrasting results for ACOX and ACAT1 may be due to the differences between the doses or the animal models used in the studies. In addition, we found that icv PA administration increased ACOX mRNA expression in the liver. This is in agreement with a previous study showing that a low level of a saturated fatty acid (stearic acid) in the brain was accompanied by decreased ACOX mRNA expression in the liver of aged mice [62]. However, the exact mechanism in the brain which regulates hepatic beta-oxidation requires further study.

Tyrosine hydroxylase (TH) is a rate-limiting enzyme for the synthesis of catecholamines. In our study, the icv leptin injection significantly increased TH protein levels in the MBH and PVN. A previous study showed that microinjection of leptin into the ventromedial hypothalamus of rats activates sympathetic tone by increasing catecholamine secretion [63]. Our study suggests that the icv leptin injection not only activates TH in the MBH (including ventromedial hypothalamus), but also in the PVN. It has also been shown that TH-positive neurons in the VMH and PVN directly project to brainstem autonomic regions such as the nucleus tractus solitarius of the brain stem and A1/C1 cell groups [64]. From there, the catecholamine-synthesizing neurons in the brainstem send efferent signals to the spinal cord and exert autonomic control in many organs, including the liver, to regulate sympathetic nervous system-mediated glucose and lipid metabolism. TH expression in the MBH and PVN has been identified as a major candidate on account for the down-regulation of sympathetic outflow in obese mice [34, 65]. James et al. showed that the attenuation of leptin-mediated phosphatidylinositol 3-kinase signaling (PI3K, upstream of Akt) in the hypothalamus decreases sympathetic tone in the liver of obese mice [17]. The present study found that PA

decreased leptin-induced activation of TH in the MBH and PVN, suggesting that PA can impair the function of leptin-activated sympathetic outflow, which may occur via altered leptin STAT3 and PI3K-Akt signaling in the hypothalamus.

In summary, we have demonstrated that PA (as a major source of saturated fatty acids) plays a causal role in central leptin resistance, increased food intake, and increased body weight gain. An increased level of central PA can lead to hypothalamic inflammation and impaired hypothalamic leptin JAK2-STAT3 and leptin Akt-FOXO1 signaling pathways in the MBH and PVN. More importantly, this can alter the central regulation of hepatic glucose and lipid metabolism. Therefore, PA may play a key role in the alteration of hypothalamic regulation of peripheral energy metabolism.

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Figure legends:

Fig 1

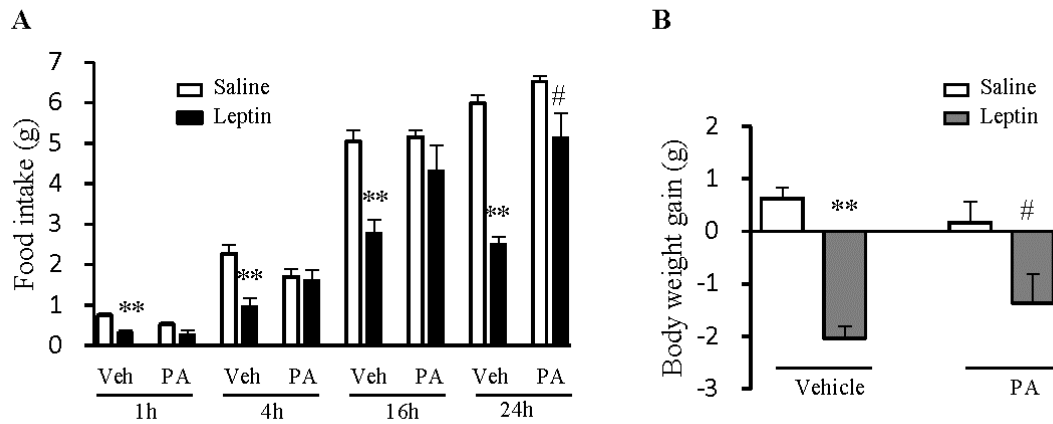


Fig. 1 Icv PA induces central leptin resistance.

Food intake at 1, 4, 16, and 24 hours (A), and body weight gain (B) at 24 hours was significantly decreased after icv injection of leptin compared with saline injection in the vehicle mice, but not in the PA icv treated mice. ** $p < 0.01$ vs vehicle + saline; # $p < 0.05$ vs PA + saline.

Fig 2

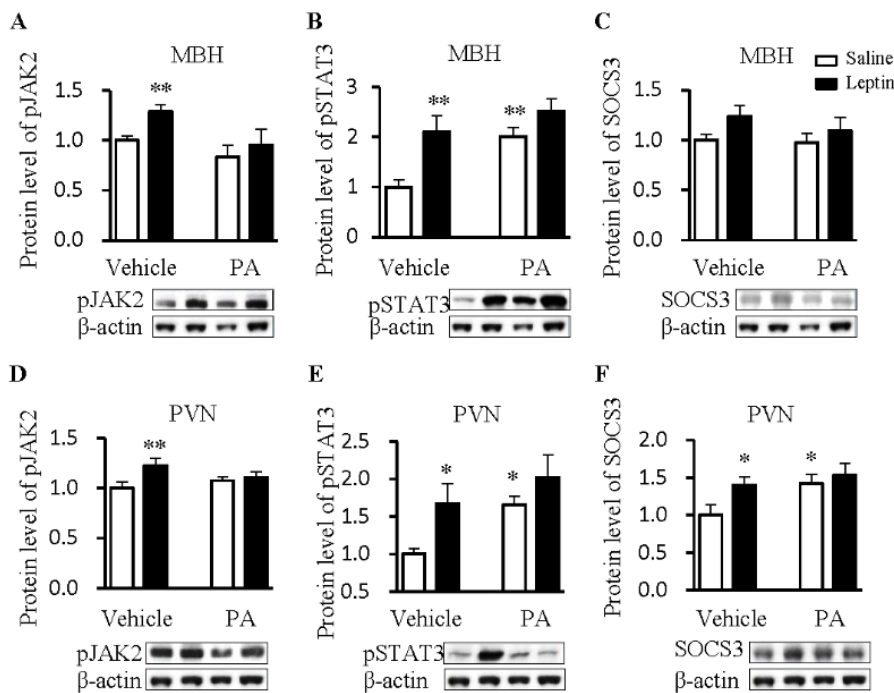


Fig. 2 Icv PA attenuates leptin JAK2-STAT3 signaling in the hypothalamus.

Phospho-JAK2 (A, D), phospho-STAT3 (pSTAT3) (B, E), and SOCS3 (C, F) protein expression in the MBH and PVN of the hypothalamus was detected by western blot in mice treated with an icv injection of leptin or saline, after the icv injection of PA and vehicle. * $p < 0.05$, ** $p < 0.01$ vs vehicle + saline. MBH: mediobasal hypothalamus, PVN: paraventricular nucleus.

Fig 3

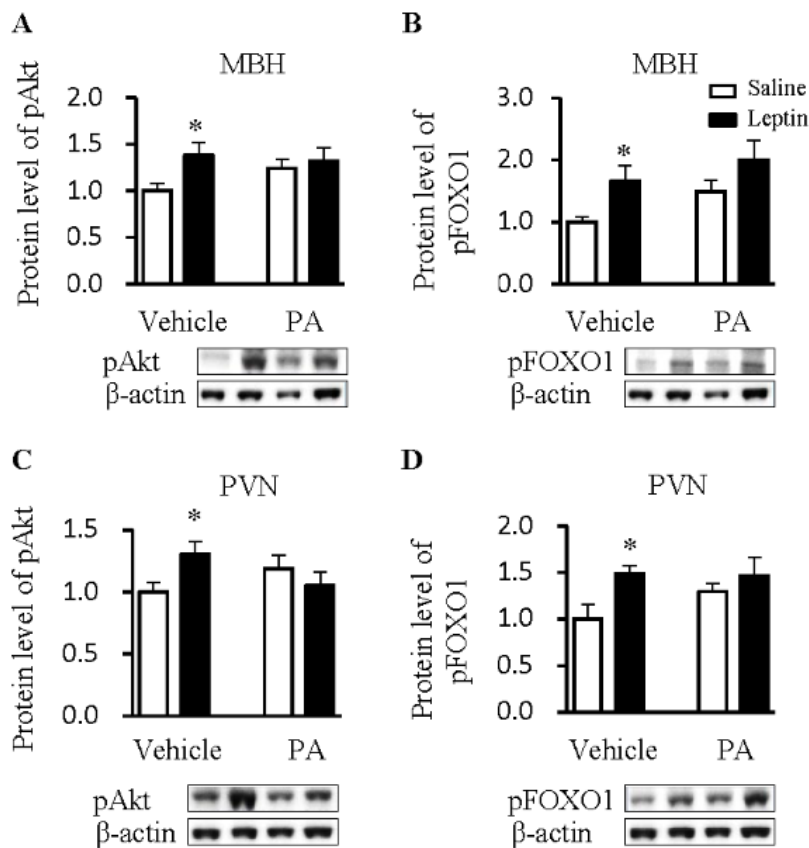


Fig. 3 Icv PA attenuates leptin Akt-FOXO1 signaling in the hypothalamus.

Phospho-Akt (pAkt) (A, C) and phospho-FOXO1 (pFOXO1) (B, D) protein expression in the MBH and PVN of hypothalamus were detected by western blot in mice treated with an icv injection of leptin or saline, after the icv injection of PA and vehicle. * $p < 0.05$, vs vehicle + saline. MBH: mediobasal hypothalamus, PVN: paraventricular nucleus.

Fig 4

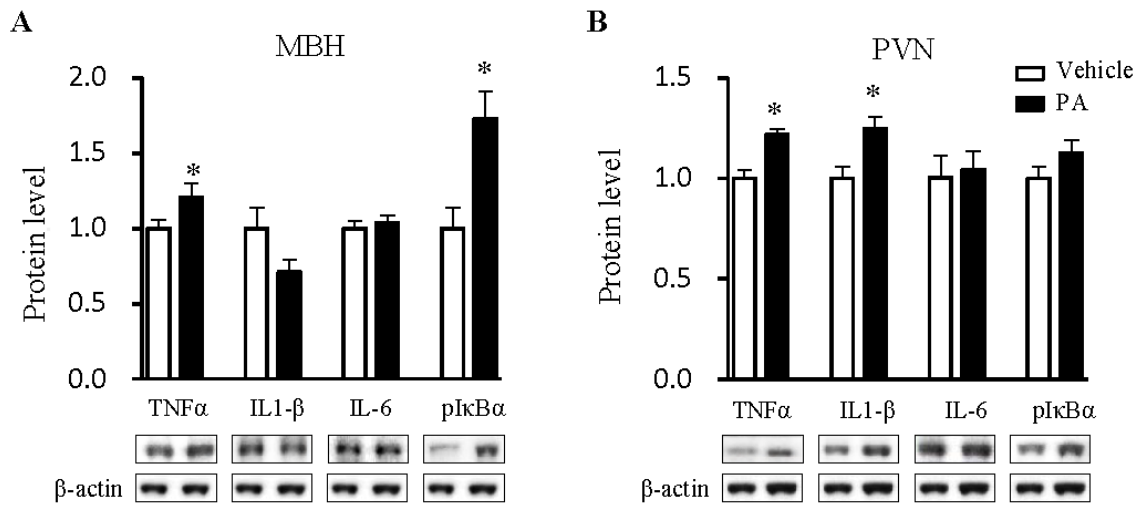


Fig. 4 Effects of icv PA on the inflammatory response in the hypothalamus.

TNF- α , IL-1 β , IL-6, and pI κ B α protein expression in the MBH (A) and PVN (B) of the hypothalamus were detected by western blot in mice treated with an icv injection of PA and vehicle. * $p < 0.05$, vs vehicle + saline. MBH: mediobasal hypothalamus, PVN: paraventricular nucleus.

Fig 5

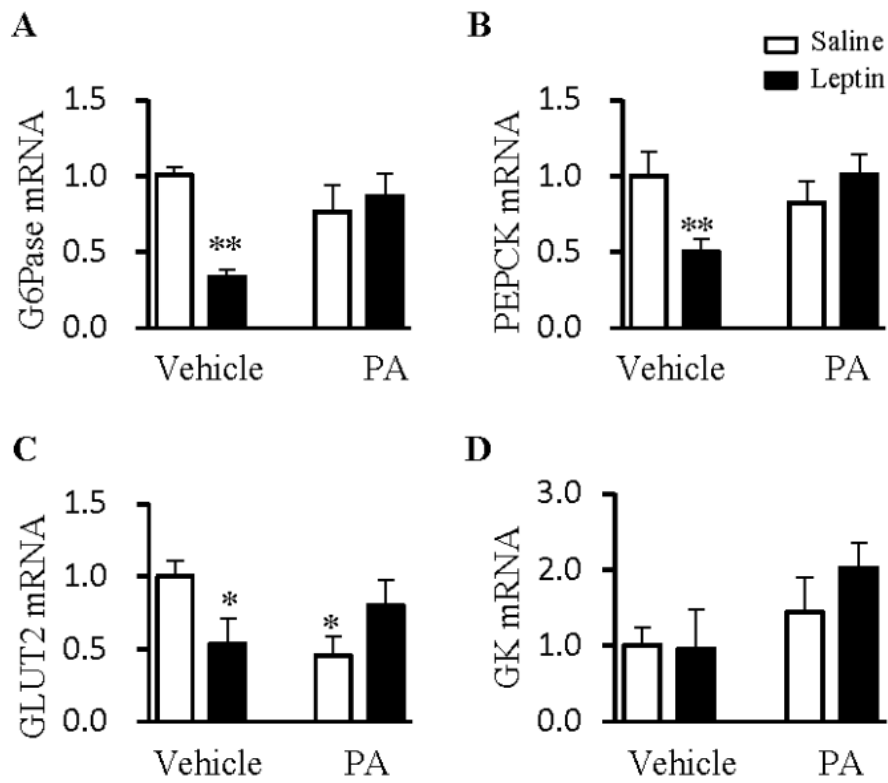


Fig. 5 Effects of icv PA on mRNA expression of genes involved in gluconeogenesis, glucose transportation, and glycolysis in the liver

The mRNA levels of G6Pase (A), PEPCK (B), GLUT2(C), and GK (D) in the liver were measured by quantitative real-time PCR in mice treated with an icv injection of leptin or saline, after the icv injection of PA and vehicle. * $p < 0.05$, ** $p < 0.01$ vs. vehicle + saline.

Fig 6

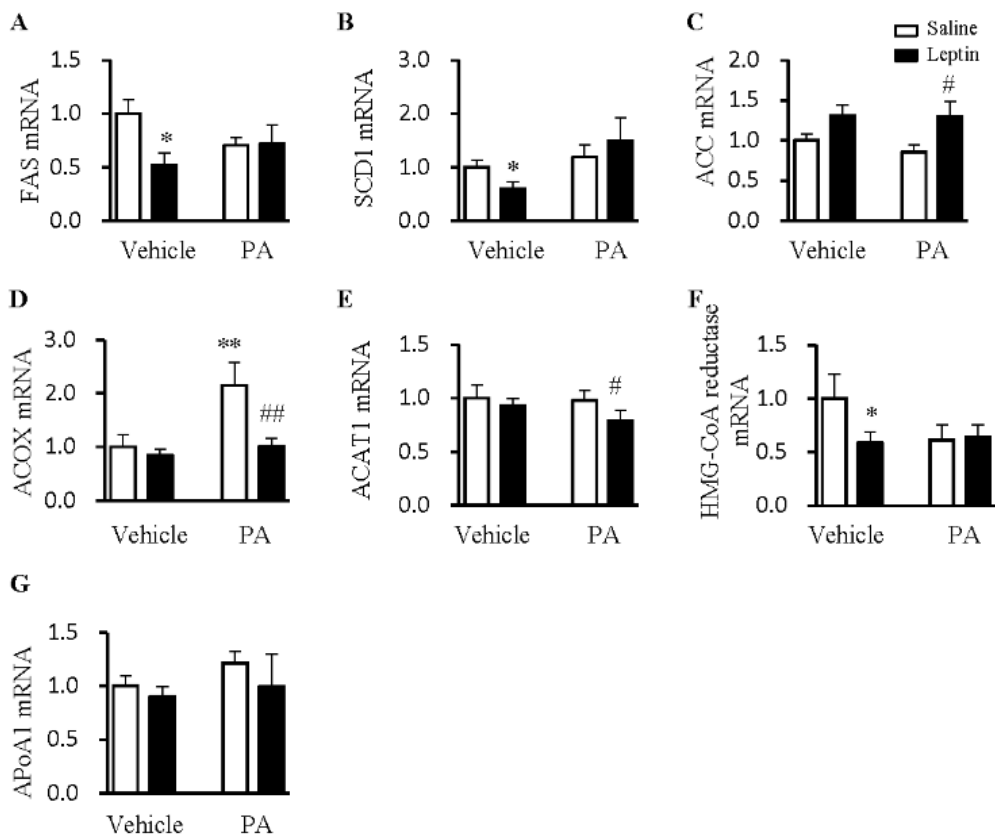


Fig. 6 Effects of icv PA on the mRNA expression of genes involved in lipogenesis, lipid beta-oxidation, and cholesterol metabolism

The mRNA levels of FAS (A), SCD1(B), ACC (C), ACOX (D), ACAT1 (E), HMG-CoA reductase (F), and APoA1 (G) in the liver were measured by quantitative real-time PCR in mice treated with an icv injection of leptin or saline, after the icv injection of PA and vehicle. * $p < 0.05$, ** $p < 0.01$ vs vehicle + saline; # $p < 0.05$, ## $p < 0.01$ vs PA + saline.

Fig 7

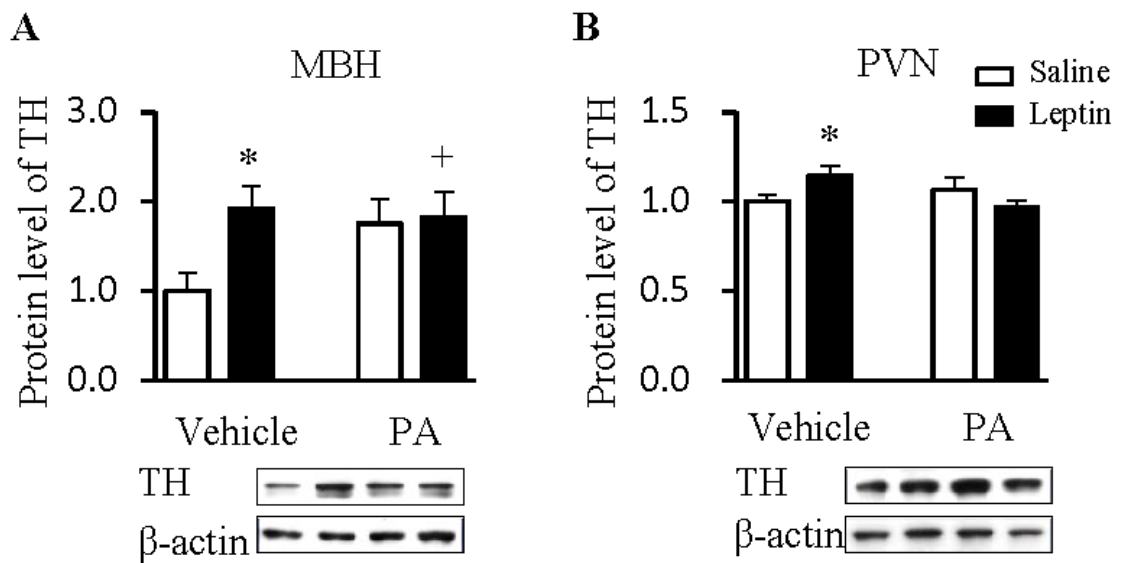


Fig. 7 Effects of icv PA on the TH level in the hypothalamus in response to icv leptin

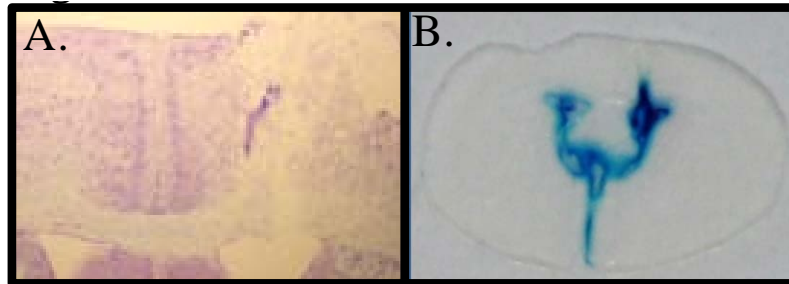
The level of TH protein expression in the MBH (A) and PVN (B) of the hypothalamus were detected by western blot in mice treated with an icv injection of leptin or saline, after the icv injection of PA and vehicle. * $p < 0.05$, vehicle + saline, ⁺ $p < 0.10$, > 0.05 vs vehicle + saline. MBH: mediobasal hypothalamus, PVN: paraventricular nucleus.

Table 1. Influence of icv PA on central leptin's effect on glucose tolerance test

Blood glucose level (mM)				
Group	0 min	30 min	60 min	120 min
Veh+Saline	7.15±1.06	9.08±1.23	8.95±0.93	7.73±1.45
Veh+Leptin	7.22±1.50	10.72±2.41	9.00±2.41	7.44±1.71
PA+Saline	6.94±0.87	9.06±1.11	9.10±1.5	7.80±1.62
PA+Leptin	8.33±0.95*	10.23±1.09*	9.28±0.59	7.88±0.41

Blood glucose was measured at 0, 30, 60, and 120 min after glucose administration (0.5 g/kg glucose, ip) using a glumeter. * $p < 0.05$ vs PA + saline.

Fig S1



The accuracy of cannula implantation into the lateral ventricle was confirmed by examining the needle track on the brain sections of each animal (A). A pre-experimental confirmation of the correct location of the injection was also carried out using a Methylene Blue injection (B).

Supplementary Table 1. List of antibodies.

Peptide	Name of Antibody	Manufacturer, catalog #	Species raised in; monoclonal or polyclonal	Dilution
TNF- α	TNF α Antibody (H-156)	Santa Cruz Biotechnology, sc-8301	rabbit, polyclonal	1:200
IL-1 β	IL-1 β Antibody (H-153)	Santa Cruz Biotechnology, sc-7884	rabbit polyclonal	1:200
IL-6	IL-6 Antibody (H-183)	Santa Cruz Biotechnology, sc-7920	rabbit polyclonal	1:200
p-I κ B α	Phospho-I κ B α (Ser32) (14D4)	Santa Cruz Biotechnology, sc-8404	rabbit, Monoclonal	1:1000
pJAK2	pJAK2(Tyr1007/1008)	Santa Cruz Biotechnology, sc-21870	Goat, Monoclonal	1:1000
p-STAT3	pSTAT3(Y705)(D3A7)	Cell Signaling Technology, #9145S	rabbit, Monoclonal	1:2000
SOCS3	SOCS3 (L210)	Cell Signaling Technology, #2932S	rabbit polyclonal	1:1000
pAkt	pAkt (S473)	Cell Signaling Technology, #9271S	rabbit polyclonal	1:1000
pFOXO1	pFoxO1(S256)	Cell Signaling Technology, #9461S	rabbit polyclonal	1:1000

Supplementary table 2. The primers used in qPCR for neuropeptide mRNA measurement

GENE	Forward primer	Reverse primer	NCBI reference
G6Pase	CTGTGAGACCGGACCAGGA	GACCATAACATAGTATACACCTGCTGC	NM_008061.3
PEPCK	CAGGATCGAAAGCAAGACAGT	AAGTCCTCTTCCGACATCCAG	NM_011044.2
GLUT2	ACCCTGTTCCCTAACCGGG	TGAACCAAGGGATTGGACC	NM_031197.2
GK	GTGGTGCTTTTGAGACCCGTT	TTCAATGAAGGTGATTTTCGCA	NM_010292.4
FAS	AGGGGTCGACCTGGTCCTCA	GCCATGCCAGAGGGTGGTT	NM_007988.3
SCD1	CTTCTTGCGATACTACTCTGG	TGAATGTTCTTGTCGTAGGG	NM_009127.4
HMG-CoA reductase	CACCTCTCCGTGGGTAAAA	GAAGAAGTAGGCCCCCAATC	NM_008255.2
APoAI	GTGGCTCTGGTCTTCCTGAC	ACGGTTGAACCCAGAGTGTC	NM_009692.3
ACAT1	CCGAGACA ACTACCCAAGGA	CACACACAGGACCAGGACAC	NM_009230.3
ACOX	ATGAATCCCGATCTGCGCAAGGAGC	AAAGGCATGTAACCCGTAGCACTCC	NM_015729.2
ACCa	GAAGTCAGAGCCACGGCACA	GGCAATCTCAGTTCAAGCCAGTC	NM_133360.2
GAPDH	TGAAGCAGGCATCTGAGGG	CGAAGGTGGAAGAGTGGGAG	NM_008084.2