Leptin Signaling in the Arcuate Nucleus Reduces Insulin’s Capacity to Suppress Hepatic Glucose Production in Obese Mice

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SUMMARY

Insulin action in the hypothalamus results in the suppression of hepatic glucose production (HGP). Obesity is often associated with a diminished response to insulin, leading to impaired suppression of HGP in obese mice. Here, we demonstrate that blocking central leptin signaling in diet-induced obese (DIO) mice restores the liver’s ability to suppress glucose production. Leptin increases the expression of the insulin receptor phosphatase PTP1B, which is highly expressed in the hypothalamus of DIO mice. We demonstrate that the central pharmacological inhibition or ARH-targeted deletion of PTP1B restores the suppression of HGP in obese mice. Additionally, mice that lack PTP1B in AgRP neurons exhibit enhanced ARH insulin signaling and have improved glucose tolerance and insulin sensitivity. Overall, our findings indicate that obesity-induced increases in PTP1B diminish insulin action in the hypothalamus, resulting in unconstrained HGP and contributing to hyperglycemia in obesity.

INTRODUCTION

Leptin is a hormone released from adipose tissue in proportion to adiposity level (Maffei et al., 1995; Zhang et al., 1994). From the circulation, leptin enters the brain (Balland et al., 2014; Banks et al., 1996) and signals via its leptin receptor (LepRb), which is widely distributed throughout the brain with particularly high expression within the arcuate nucleus of the hypothalamus (ARH) (Scott et al., 2009; Tartaglia et al., 1995), to attenuate food intake and decrease body weight (Friedman and Halaas, 1998; Halaas et al., 1995). Importantly, obesity is characterized by hyperleptinemia and an inadequate response to this high level of endogenous leptin (Frederich et al., 1995). For a long time, DIO mice were considered to be resistant to leptin, but there is increasing evidence suggesting otherwise. At the cellular level, DIO mice display leptin signaling in ARH neurons under basal conditions, as revealed by STAT3 phosphorylation, consistent with the presence of a response to endogenous leptin in obese mice (Balland and Cowley, 2017; Balland et al., 2014). This observation is further supported by physiological evidence of leptin action in DIO mice because antagonizing leptin signaling results in increased food intake and body weight in DIO mice (Ottaway et al., 2015). Besides its weight-regulating effect, leptin is also implicated in glucose homeostasis (Elmquist et al., 2005; Gutiérrez-Juárez et al., 2004). Obesity is associated with hyperleptinemia, hyperglycemia, hyperinsulinemia, and type 2 diabetes. Although insulin exerts a profound effect on the regulation of blood glucose in the periphery, the central activation of insulin receptor (InsR) is also required to maintain glucose homeostasis (Brüning et al., 2000). Importantly, leptin and insulin share the same neuronal targets in the hypothalamus (Belgardt and Brüning, 2010; Garcia-Galiano et al., 2017), and the co-activation of both signaling pathways within the same ARH neurons have important implications in energy metabolism (Dodd et al., 2015).

In the present study, we provide in vivo evidence that blocking central leptin signaling facilitates hypothalamic insulin signaling and restores the suppression of hepatic glucose production (HGP) in DIO mice. Mechanistically, we show that protein tyrosine phosphatase 1B (PTP1B) represses the central insulin action on liver to control HGP. Pharmacological inhibition or genetic deletion of PTP1B in the ARH has similar metabolic improvements to LepRb antagonism in DIO mice. In addition, we show genetic evidence that these events are likely to occur in AgRP neurons. Altogether, our data indicate that leptin signaling in DIO mice is responsible for alterations in insulin-dependent central mechanisms involved in glucose homeostasis. These studies redefine our current understanding of central leptin action or resistance in obesity and highlight a previously unexplored mechanism by which leptin perturbs...
the central control of glucose metabolism in diet-induced obesity.

RESULTS

Central Inhibition of Leptin Signaling in DIO Mice Improves Glucose Tolerance and Hepatic Insulin Sensitivity

To test whether the central leptin signaling interferes with insulin action in DIO mice, we injected control and DIO mice intracerebroventricularly (i.c.v.) with leptin antagonist (LAN) (Enrion et al., 2011), to block endogenous leptin from activating LepRb-associated signaling pathways in the brain, prior to subjecting mice to hyperinsulinemic-euglycemic clamps (Figure 1). Hypothalamic insulin has previously been shown to inhibit HGP in rats (Obici et al., 2002; Pocai et al., 2005a) and mice (Könner et al., 2007). Therefore, we investigated whether the blockade of endogenous leptin signaling could promote central insulin action and modify glucose homeostasis in DIO mice. We performed hyperinsulinemic-euglycemic clamp studies following vehicle (artificial cerebro-spinal fluid [aCSF]) or LAN i.c.v. infusions (see Figures 1A and 1B for experimental design). We also used the insulin receptor antagonist S961 (Vikram and Jena, 2010), delivered i.c.v., to determine whether the physiological effect exerted by LAN requires insulin signaling. LAN pre-treatment did not change body weight of either control (Figure S1H) or DIO mice (Figure S1A). Interestingly, DIO mice treated with LAN required a higher glucose infusion rate (GIR) to maintain euglycemia when compared to aCSF-treated group (Figures 1C–1E). In parallel, HGP during the clamp was significantly reduced in the LAN group (Figures 1F and S1B). The glucose disposition rate (GDR) was not affected in the basal state, albeit a marked decrease in GDR was observed in the clamped mice treated with S961 and LAN + S961 (Figure 1G). Interestingly, the LAN-dependent improvements in GIR and HGP were completely blunted by the co-administration of insulin receptor antagonist S961 i.c.v. (Figures 1C, 1E, and 1F). Of note, blocking central LepRb signaling prior to hyperinsulinemic-euglycemic clamps perturbed normal glucose homeostasis in lean control mice (Figures S1D–S1I). Specifically, GIR and GDR were significantly reduced in control mice during the clamp (Figures S1F and S1G). This was accompanied by a marked increase in HGP (Figure S1).

Figure 1. Central Inhibition of Leptin Signaling Decreases HGP in DIO Mice during Hyperinsulinemic-Euglycemic Clamp

(A) Schematic representation of the experimental procedure.

(B) Schematic representation of the hyperinsulinemic-euglycemic clamp procedure.

(C) Glucose infusion rates during a 2-hr hyperinsulinemic-euglycemic clamp in DIO mice.

(D) Blood glucose levels were maintained at $\pm 0.5$ mmol/L during the clamp procedure ($p < 0.05$ between aCSF versus LAN+S961; $***p < 0.001$ between aCSF versus S961).

(E) Average glucose infusion rate during steady state.

(F) HGP measured under basal and clamped conditions.

(G) Rate of glucose disposal under basal and clamped conditions.

All data are represented as mean ± SEM (n = 6–8 mice/group). ns, $p > 0.05$; *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; ****$p < 0.0001$ as determined by one-way ANOVA followed by Tukey’s post hoc test (E) and two-way ANOVA followed by Bonferroni’s post hoc test (D, F, and G).

See also Figure S1.
Central Pharmacological Inhibition or ARH-Targeted Deletion of PTP1B Recapitulates the LAN-Dependent Restoration of Hypothalamic Insulin Signaling and Improves the Hepatic Response to Insulin in DIO Mice

PTP1B is a negative regulator of leptin and insulin signaling (Bence et al., 2006; Eichebly et al., 1999; Galic et al., 2005; Goldstein et al., 1998; Zabolotny et al., 2002; Zhang et al., 2015). Previous studies have shown that hypothalamic PTP1B is elevated in obesity (Dodd et al., 2017; Wu et al., 2005; Zabolotny et al., 2008; Zhang et al., 2015) and that leptin can promote PTP1B expression as part of a negative feedback loop (White et al., 2009). We tested whether the sustained activation of LepRb signaling in obesity was coupled to an increased in PTP1B-mediated inhibition of insulin signaling, resulting in decreased HGP. To achieve this, we used the PTP1B inhibitor claramine (Han et al., 2008) to repeat the protocol of i.c.v. injection followed by clamps as we previously used with LAN injections. The central inhibition of PTP1B was associated with a decrease in HGP (Figures 2D and S2B) and an increase in GIR during the clamp (Figures 2A and 2C). Notably, the improvement in the suppression of HGP was similar to the LAN group and there was no additive effect when both LAN and claramine were co-injected (Figures 2A–2E). Body weights did not vary among the treatment groups (Figure S2A). Importantly, LAN treatment normalized the elevated expression of PTP1B observed in the mediobasal hypothalamus (MBH) of DIO mice (Figure 2F). In addition, the increase in hepatic gluconeogenic genes mRNA levels in DIO mice, namely Pck1 and G6pc, was completely abolished following LAN treatment (Figures 2G and 2H). These results suggest that the attenuation of insulin signaling caused by central LepRb signaling is mediated through the induction of PTP1B in DIO mice. To further evaluate the role of PTP1B in ARH insulin resistance, we fed Ptpn1fl/fl mice a high fat diet (HFD) for 20 weeks and then performed intra-ARH rAAV-cytomegalovirus (CMV)-Cre-GFP injections to specifically ablate PTP1B in ARH neurons of DIO mice (Figures 4A and 4B). Additionally, AgRP-1B mice had lower body weight and respiratory exchange ratio (RER) (Figures S4C, S4D, and S4G). Strikingly, AgRP-1B mice displayed a greater sensitivity to insulin when compared to control mice as indicated by elevated insulin-induced Akt phosphorylation (pAkt) in the ARH (Figures 4A and 4B). Importantly, these differences were independent of changes in energy expenditure, oxygen consumption and RER (Figures S4E–S4G). Upon high-fat feeding, AgRP-1B mice developed diet-induced obesity but gained significantly lower body weight compared to their Ptpn1fl/fl littermates after 16 weeks of HFD (Figure 4H). The differences in body weight and insulin sensitivity between Ptpn1fl/fl and AgRP-1B mice would confound the interpretation of hyperinsulinemic-euglycemic clamps as we

**Figure 2. Central Inhibition of PTP1B Restores Insulin-Induced Akt Activation in the ARH of DIO Mice and Decreases HGP during Hyperinsulenic-Euglycemic Clamp**

(A) Glucose infusion rates during a 2-hr hyperinsulenic-euglycemic clamp in DIO mice.

(B) Blood glucose levels were maintained at ~7 ± 0.5 mmol/L during the clamp procedure (n = 5 mice/group).

(C) Average glucose infusion rate during steady state.

(D) HGP measured under basal and clamped conditions.

(E) Rate of glucose disposal under basal and clamped conditions.

(F) Representative western blots and quantitative comparison of PTP1B and vinculin in MBH explants from control and DIO mice after 3 days of aCSF, leptin, or LAN i.c.v. injections.

(G) Graph representing Pck1 mRNA level in the liver following 3 days of aCSF, leptin (1 μg), or LAN (5 μg) i.c.v. injections in control and DIO mice.

(H) Graph representing the G6pc mRNA level in the liver following 3 days of aCSF, leptin (1 μg), or LAN (5 μg) i.c.v. injections in control and DIO mice. All data are represented as mean ± SEM. ns, p > 0.05; *p < 0.05; **p < 0.01; ***p < 0.001; ****p < 0.0001 as determined by one-way ANOVA followed by Tukey’s post hoc test (C: n = 5–10 mice/group; F: n = 6–7 mice/group; G and H: n = 3–4 mice/group) and two-way ANOVA followed by Bonferroni’s post hoc test (D and E: n = 5–10 mice/group).

See also Figure S2.
Figure 3. Acute Deletion of PTP1B in the ARH after the Onset of Obesity Decreases HGP during Hyperinsulinemic-Euglycemic Clamp

(A) Representative photomicrographs (10×) showing GFP expression (green) 5 weeks following the injection of rAAV-Cre-GFP into the ARH of Ptpn1 fl/fl mice (scale bar 75 μm). The picture illustrates a missed injection (left) and a hit (right) injection, respectively.

(B) Graph representing the mean body weight of Ptpn1 fl/fl mice over 4 weeks, following rAAV-Cre-GFP injection into the ARH (n = 7–8 mice/group).

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had previously performed in this series of studies. Therefore, to investigate the contribution of HGP regulation in the improved glucose homeostasis of AgRP-1B/HFD mice despite body weight (BW) difference between groups, we performed a pyruvate tolerance test in AgRP-1B/HFD and Ptpn1<sup>fl/fl</sup>/HFD mice. High-fat-fed AgRP-1B mice had a markedly improved tolerance to pyruvate relative to their Ptpn1<sup>fl/fl</sup>/HFD littermates. In line with our clamp studies, 3 days of LAN i.c.v. injection improved pyruvate tolerance in Ptpn1<sup>fl/fl</sup>/HFD mice, matching AgRP-1B/HFD mice response to pyruvate. Noteworthy, there was no further improvement of pyruvate tolerance in response to LAN treatment in AgRP-1B/HFD mice (Figures 4f and 4j). These findings are consistent with the leptin-induced repression of insulin signaling in AgRP neurons and the promotion of HGP in DIO mice due to the induction of PTP1B in AgRP neurons.

**DISCUSSION**

The failure of exogenous leptin to reduce appetite and to cause weight loss in obese subjects has led to the concept of leptin resistance. In the current study, we sought to re-assess leptin resistance in the hypothalamus and its influence on glucose homeostasis in obesity. Our results demonstrate that the activation of leptin signaling in ARH is responsible, at least in part, for the uncontrolled hepatic glucose production (HPG) causing impaired blood glucose regulation in obesity.

Recently, Ottaway and colleagues (Ottaway et al., 2015) demonstrated that endogenous leptin still regulates energy homeostasis in DIO mice. They showed that pegylated-LAN treatment increased food intake and body weight similarly in control and DIO mice, demonstrating that endogenous leptin activity is still present in DIO mice (Ottaway et al., 2015). In our study, we showed that the use of non-pegylated LAN (shorter bioactivity) did not affect feeding and body weight of control and DIO mice, thereby eliminating confounding factors, which could modify the response to hyperinsulinemic-euglycemic clamps.

Leptin and insulin share neuronal targets in the brain (Belgardt and Bruning, 2010), and a substantial body of evidence shows they both have crucial roles in regulating energy balance (Bruening et al., 2000; Dodd et al., 2015; Halaas et al., 1995; Woods et al., 1979) and glucose homeostasis (Berglund et al., 2012; Fujikawa et al., 2013; Hill et al., 2010; Obici et al., 2002; Pocai et al., 2005b). A recent study has reported that at least 50% of arcuate LepR-expressing neurons were also responsive to central administration of insulin (Garcia-Galiano et al., 2017), and previous work from our group demonstrated the physiological importance of leptin and insulin co-action on ARH neurons to regulate energy balance (Dodd et al., 2015). The existence of a cross-talk between leptin and insulin signaling in ARH neurons led us to hypothesize that hyperleptinemia in DIO mice could lead to an imbalance between leptin and insulin signaling in ARH neurons and consequently impair the regulation of glucose homeostasis in obesity.

Hypothalamic insulin resistance has been reported in obese humans (Henri et al., 2014, 2015). Intranasal insulin administration is known to suppress endogenous glucose production in lean men. However, in overweight or obese insulin-resistant men, the same dose of intranasal insulin was ineffective during a pancreatic clamp (Xiao et al., 2018). In rodents, the inhibition of central InsR signaling induces hyperglycemia and glucose intolerance (Vikram and Jena, 2010). Central insulin signaling is known to control HGP (Obici et al., 2002) via the vagus nerve (Kimura et al., 2016; Vikram and Jena, 2010) in rodents. Our studies reaffirm the importance of central InsR signaling. The restored suppression of HGP induced by i.c.v. LAN in DIO mice was lost when central insulin receptor was blocked with the specific InsR antagonist, S961, demonstrating that the control of HGP relies largely on functional insulin signaling in the CNS of DIO mice. Given the modest reduction in GDR after i.c.v. S961 in DIO mice, this highlights the involvement of central InsR signaling in regulating insulin sensitivity in other peripheral tissues. In fact, mice with specific InsR ablation in NPY neurons display a significant decrease in glucose uptake into skeletal muscle during clamp (Loh et al., 2017). These studies all point to the notion that alterations in ARH insulin signaling are involved in the perturbation of systemic glucose homeostasis associated with obesity. Although the central effect of insulin on HGP does not appear to extend to dogs (Rammnan et al., 2013), a recent study has utilized fMRI and hyperinsulinemic-euglycemic clamps to show that intranasal insulin acts on the hypothalamus to inhibit endogenous glucose production in humans (Henri et al., 2017). Thus, we propose that, in obese individuals, hyperleptinemia might constantly drive LepRb signaling to concurrently impede insulin action on neurons involved in glucose homeostasis. We used an in vivo approach to corroborate findings from previous studies, which reported that intact insulin signaling in the ARH is required to regulate HGP (Kimura et al., 2016; Obici et al., 2002). Indeed, LAN treatment restored hepatic insulin sensitivity and pyruvate tolerance in DIO mice. Further, we also showed that central blockade of InsR exacerbated the insulin-resistant state in DIO mice.

The central action of leptin on glucose homeostasis is highlighted in our clamp studies performed in lean mice with LepRb...
Figure 4. Acute Deletion of PTP1B in the AgRP Neurons Increases Insulin Sensitivity and Ameliorates Glucose Homeostasis

(A) Representative photomicrographs (10x) showing pAkt-labeled cells (black) 15 min following intraperitoneal (i.p.) insulin injection in control mice (Ptpn1fl/fl) and mice lacking PTP1B expression in AgRP neurons (AgRP-1B) (scale bar 75 μm).

(B) Graphs representing the mean number of ARH neurons per hemi-section displaying pAkt labeling in Ptpn1fl/fl and AgRP-1B mice.

(C) Graphs representing the mean blood glucose level in Ptpn1fl/fl and AgRP-1B mice in fed and fasted (6 hr) conditions.

(D) Glucose tolerance test in Ptpn1fl/fl and AgRP-1B mice.

(E) Graph showing the mean area under the curve of glucose tolerance test in Ptpn1fl/fl and AgRP-1B mice.

(F) Insulin tolerance test in Ptpn1fl/fl and AgRP-1B mice.

(G) Graph showing the mean area under the curve of insulin tolerance test in Ptpn1fl/fl and AgRP-1B mice.

(H) Graph representing the mean body weight of Ptpn1fl/fl and AgRP-1B mice from 6 weeks of age during 16 weeks of diet (n = 9–12 mice per group).

(I) Pyruvate tolerance test following 3 days of aCSF or LAN i.c.v. injections in Ptpn1fl/fl and AgRP-1B mice fed with HFD for 20 weeks.

(J) Graph showing the mean area under the curve of pyruvate tolerance test following 3 days of aCSF or LAN i.c.v. injections in Ptpn1fl/fl and AgRP-1B mice fed with HFD for 20 weeks.

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inhibition. These mice displayed a severe impairment in whole-body glucose homeostasis, as evidenced by reduced GIR and GDR and increased HGP. These findings show that, under normal conditions, intact central leptin signaling is critical in regulating glucose homeostasis. Indeed, previous work from Rossetti’s group has described the role of leptin in mediating hepatic glucose fluxes through melanocortin-dependent and melanocortin-independent pathways (Gutiérrez-Juárez et al., 2004). Hence, it may be expected that the presence of excessive leptin levels in obese mice might similarly alter the regulation of HGP. In the current study, obese mice display increased body weight and elevated HGP associated with impaired leptin and insulin action. Specifically, when central insulin fails to regulate blood glucose, the blockade of leptin signaling in the brain is capable of restoring insulin signaling in the ARH to suppress HGP in DIO mice.

In obese mice, hyperleptinemia in the ARH increases the expression of SOCS3 (Björbaek et al., 1998; Münzberg et al., 2004), PTP1B (Elchebly et al., 1999; Klaman et al., 2000; Zabolotny et al., 2002), and TCPTP (Loh et al., 2011). Normally, these regulators exert a negative feedback upon activation of the LepRb-signaling cascade. This notion is supported by a significant reduction in SOCS3 mRNA levels in DIO mice with chronic LAN treatment (Ottaway et al., 2015) and the enhanced leptin signaling and sensitivity accompanying PTP1B and TCPTP deletion in the CNS (Bence et al., 2006; Loh et al., 2011).

Our data suggest a cross-talk between leptin and insulin signaling whereby leptin signaling inhibits insulin signaling, leading to impaired glucose homeostasis in DIO. This notion is supported by a recent study showing that SOCS3 inactivation in LepRb-expressing neurons protected mice from diet-induced insulin resistance independently of body weight (Pedroso et al., 2014). PTP1B is another leptin-induced negative feedback molecule (Zabolotny et al., 2002), and it is a potent negative regulator of insulin signaling dephosphorylating the insulin receptor and insulin receptor substrate-1 (Elchebly et al., 1999; Galic et al., 2005; Goldstein et al., 1998; Zhang et al., 2015). PTP1B also represses leptin signaling (Bence et al., 2006; Zabolotny et al., 2002; Zhang et al., 2015), and mice lacking PTP1B in LepRb-expressing neurons are hypersensitive to leptin and resistant to high-fat feeding (Tsou et al., 2012). Moreover, high-fat-fed mice with neuronal deletion of PTP1B display increased insulin sensitivity and improved glucose tolerance (Bence et al., 2006).

Because leptin and insulin-responsive neurons in the ARH can overlap (Dodd et al., 2015; García-Galiano et al., 2017), hyperleptinemia-induced leptin signaling could result in sustained PTP1B activity to blunt insulin signaling in the ARH neurons of DIO mice. We investigated the role of PTP1B in ARH neurons to determine how LepRb signaling may contribute to hypothalamic insulin resistance in DIO mice. We found that the central inhibition of PTP1B in DIO mice restored hypothalamic insulin signaling and the suppression of HGP during hyperinsulinemic-euglycemic clamps. Importantly, the degree of restoration of hypothalamic insulin signaling was similar between the central inhibition of PTP1B and the blockade of leptin signaling with LAN. It remains tempting to postulate that the increase in PTP1B protein as a consequence of hyperleptinemia perturbs insulin-mediated control of HGP in DIO mice through the melanocortin-independent pathway described by Rossetti and colleagues (Gutiérrez-Juárez et al., 2004). Furthermore, we employed temporal ARH-targeted PTP1B deletion after the onset of obesity to discriminate between effects in the ARH versus the whole-brain inhibition of phosphatase. Specifically, we found that our control (AAV-Cre missed) mice remained insulin resistant, and mice with successful Cre-mediated recombination (AAV-Cre hit) in the ARH displayed a similar improvement in hepatic insulin sensitivity to that achieved with pharmacological LAN inhibition. Thus, we propose that obesity-induced hyperleptinemia causes persistent leptin signaling and thereby increases PTP1B protein in ARH neurons to repress insulin signaling, so that insulin fails to appropriately suppress HGP, thereby contributing to hyperglycemia in obesity.

To date, AgRP and proopiomelanocortin (POMC) neurons have been largely implicated in the regulation of glucose homeostasis, given that they express high levels of LepRb and InsR (Belgardt and Brüning, 2010). Preliminary studies of insulin signaling in POMC and AgRP neurons by immunohistochemistry suggested that insulin response specifically in AgRP neurons could be altered by high leptin levels (data not shown).

Building on this notion, we generated mice with PTP1B deletion only in AgRP neurons (AgRP-1B). Interestingly, AgRP-1B mice displayed improved glucose tolerance and insulin sensitivity, in accordance with our in vivo pharmacological inhibition and ARH genetic deletion studies. Noteworthy, AgRP-1B mice retain the capacity to control HGP, even in the obese state. This illustrates the involvement of AgRP neurons in the mechanisms of hypothalamic insulin resistance linked to obesity and the associated hyperglycemia. In line with this notion, overexpressing PTP1B specifically in AgRP neurons may cause uncontrolled HGP and ultimately lead to hyperglycemia.

In conclusion, our study indicates that leptin signaling persists in ARH neurons in obesity, which impairs insulin action in AgRP neurons through the induction of PTP1B. Our findings demonstrate that pathological leptin signaling in the ARH underpins the alteration of hypothalamic insulin signaling, and it accounts, at least in part, for the failure of insulin action in the hypothalamus-liver axis, which suppresses HGP, leading to the perturbation of glucose homeostasis in obesity.

STAR METHODS

Detailed methods are provided in the online version of this paper and include the following:

- KEY RESOURCES TABLE
- CONTACT FOR REAGENT AND RESOURCE SHARING

All data are represented as mean ± SEM. ns, p > 0.05; *p < 0.05; **p < 0.01; as determined by one-way ANOVA followed by Tukey’s post hoc test (J: n = 6–8 mice/group), two-way ANOVA followed by Tukey’s post hoc test (B: n = 4 mice/group; C: n = 10–11 mice/group), two-tailed unpaired t test (E and G; n = 10–11 mice/group), and two-way ANOVA with repeated measures (H: n = 9–12 mice/group; interaction: ***p < 0.0001; time: ****p < 0.0001; genotype: *p < 0.05).

See also Figure S4.
AUTHOR CONTRIBUTIONS

E.B. designed the experiments. E.B., W.C., G.T.D., and G.C. conducted experiments. E.B. and W.C. wrote the manuscript. G.T.D. and G.C. conducted experiments and supervised the work and edited the manuscript.

DECLARATION OF INTERESTS

The authors declare no competing interests.

REFERENCES


Impaired insulin action in the human brain: causes and metabolic consequences. 


STAR METHODS

KEY RESOURCES TABLE

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CONTACT FOR REAGENT AND RESOURCE SHARING

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Michael Cowley (michael.cowley@monash.edu).

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Animals

All animal procedures were approved by Monash University Animal Ethics Committee. Two- to three-month-old male C57BL6 mice (Monash Animal Services) were housed in a controlled environment with constant temperature and 12hr light/12hr dark cycle in a high-barrier facility (Monash ARL). Mice were either fed ad libitum with standard chow diet (8.5% fat, Barastoc, Ridley AgriProducts, Australia) or HFD (43% energy from fat, SF04-001, Specialty Feeds, Western Australia, Australia) for 12 to 20 weeks. To generate Agrp-Ires-Cre; Ptpn1<sup>fl/fl</sup>, mice (Bence et al., 2006) were bred with Agrp-Ires-Cre mice (Tong et al., 2008).

METHOD DETAILS

Genotyping

Genotyping was performed by PCR on DNA extracted from tail biopsies using primers previously described for the Agrp-Ires-Cre (Tong et al., 2008) and Ptpn1<sup>fl/fl</sup> mice (Bence et al., 2006).
Intra-ARH rAAV injection
Following 20 weeks of HFD, Ptpn1fl/fl mice were stereotaxically injected with rAVV expressing Cre recombinase and GFP (rAAV-CMV-Cre-GFP; UNC Vector Core) bilaterally into the ARH (coordinates, bregma: anterior-posterior, –1.40 mm; dorsal-ventral, –5.80 mm; lateral, +/-0.20 mm, 100 nl/side). Mice were allowed to recover for 4 weeks post-surgery before jugular catheter implantation for unrestrained clamps. Brains were frozen and cut after the clamp procedure and ARH targeting was confirmed by post-mortem GFP detection using a fluorescent microscope (See Figure S7 for injections hit map).

Lateral ventricle cannulation and icv infusions
Cannulas were stereotaxically inserted (1.0 mm lateral and 0.2 mm rostral to bregma). Each surgery was followed by one week of recovery before performing additional procedures on cannulated mice. For the clamp studies, aCSF (1 μl), LAN (5 μg; Protein Laboratories Rehovot, Israel) or claramine (5 μg; Sigma-Aldrich) were infused once daily for two consecutive days and a third infusion one hour before starting the clamp procedure. For studies with double central inhibition of leptin receptor and insulin receptor, LAN was injected as previously described and S961 was injected 30min before starting the clamp procedure.

Western blot
Proteins were extracted from microdissected MBH in 100 μL of lysis buffer (25mM Tris pH 7.4, 50 mM β-glycerophosphate, 1.5 mM EGTA, 0.5 mM EDTA, 1 mM sodium pyrophosphate, 1 mM sodium orthovanadate, 10 mg/ml leupeptin and pepstatin, 10 mg/ml aprotinin, 100 mg/ml phenylmethylsulfonyl fluoride, and 1% Triton X-100). Tissues were homogenized by successive passing through 23- and 26-gauge needles. The lysates were centrifugated at 13,300 rpm for 15min at 4°C. Protein content was determined using the Bradford method (Bio-Rad, Hercules, CA). Aliquots of protein extracts containing the same amount of protein were prepared in 1X sample buffer containing 12.5% β-mercaptoethanol and stored at –80°C until use. Samples were boiled for 5 min and loaded in 3%–7% Tris-acetate polyacrylamide gels (Invitrogen). After electrophoresis, the proteins were transferred onto nitrocellulose membranes (Invitrogen) for 1.5 hr on ice at room temperature. Blots were blocked in TBS containing 0.05% Tween 20 and 5% skimmed milk for 1 hr at room temperature and then incubated overnight at 4°C with PTP1B antibody (Abcam #ab201974; 1:1000) in TBST-milk buffer. To visualize the immunoreaction, the blots were incubated with horseradish-peroxidase-conjugated (HRP-conjugated) secondary antibodies (Antibody Australia; 1:1000) for 1 hr at room temperature and developed using enhanced chemiluminescence (SuperSignal West Pico substrate, Invitrogen). Vinculin was used as a marker of total protein content. Membranes were incubated with vinculin primary antibody (Sigma Aldrich #V9131; 1:10000) at room temperature for 30min, followed by incubation in HRP-conjugated secondary antibody (Antibody Australia; 1:1000) for 1 hr at room temperature and developed similarly to PTP1B.

Hyperinsulinemic-Euglycemic clamp
Previously ICV cannulated mice were subjected to jugular vein catheterization 5 days prior to hyperinsulinemic euglycemic clamp studies. Mice were anesthetized with isoflurane (2%–3% in oxygen) while an indwelling silastic catheter was inserted into the right internal jugular vein and exteriorized through the back of the neck. The catheters were kept patent with heparin sodium (10 IU/ml; Pfizer). Mice were allowed 5 days postsurgical recovery. Food pellets were placed at the bottom of the cage to facilitate recovery. Body weight was recorded daily, and mice that had less than 5% weight loss were subsequently studied. In brief, all mice received a single icv injection of either aCSF (1 μl), LAN (5 μg in 1 μl), or claramine (5 μg in 1 μl) at D17 and D18 and 1h before the commencement of clamp (Figures 1A and 1B). For the insulin receptor inhibition clamp, mice received icv aCSF or LAN (5 μg in 1 μl) at D17 and D18 and 1h before the commencement of clamp. 30min prior to the commencement of the clamp, mice received icv injection of S961 (0.5 μg in 1 μl; Novo Nordisk).

Hyperinsulinemic euglycemic clamp performances were performed on 4h fasted, conscious and unrestrained mice as described previously (Enriori et al., 2016). The infusion protocol consisted of a 60min tracer equilibration period (t = −60 to 0min), followed by a 120-min experimental period (t = 0 to 120min). A bolus of [3-3H]glucose (2.5 μCi; PerkinElmer) was administered at t = −60min, followed by a constant infusion of 0.05 μCi/min for 60min, starting at 11h30. At t = −15 and −5min, blood samples (−50 μL) were obtained from a small nick in the tail vein for the assessment of basal plasma glucose concentration, plasma insulin, and glucose-specific activity. At 12h30, hyperinsulinemic, euglycemic clamp was initiated at t = 0min with an infusion of human insulin (4 mU/kg/min in DIO; 2 mU/kg/min in control; ActRapid; Novo Nordisk). To minimize changes in glucose specific activity, the continuous infusion of high-pressure liquid chromatography-purified [3-3H]glucose was increased to 0.1 μCi/min and maintained throughout the procedure. Blood glucose was measured every 5min, and 15% glucose was infused at variable rates to maintain euglycemia (~6.5 ± 0.5mmol/L). Blood samples (10 μL) were taken every 10min from t = 90 to 120min to determine glucose-specific activity. At the end of the clamp procedure, mice were anesthetized with sodium pentobarbital (60 mg/kg), tissues were extracted within minutes and stored in −80°C for later analysis. Plasma insulin was determined from samples obtained from cardiac puncture.

Determination of plasma and tissue radioactivity
Blood samples (10 μL) were deproteinized with equal molar volume (0.3N) of barium hydroxide and zinc sulfate, [3-3H]glucose radioactivity in the supernatant was assessed by liquid scintillation counting. Whole body glucose kinetics such as the rate of glucose appearance (HGP: endogenous R30) and disappearance (glucose disposal: R30) were calculated using isotopic steady-state equations.
of Steel. Endogenous glucose production during the clamp was calculated by subtracting the glucose infusion rate (GIR) from whole body $R_d$ (GDR). The insulin-stimulated component of the $R_d$ (IS-$R_d$) is equal to clamp $R_d$ minus the basal glucose turnover rate.

**Metabolic measurements**

Glucose, insulin and pyruvate tolerance tests (GTT, ITT and PTT) were performed on 6h (Figures 4D and 4E) and 4h (Figures 4F, 4G, 4I, and 4J) fasted conscious mice by injecting D-glucose (2mg/g body weight), insulin (0.65mU/g body weight) or sodium pyruvate (1.5mg/g body weight) into the peritoneal cavity and measuring glucose in tail blood immediately before and at 15, 30, 60, 90 and 120min after ip injection using a Accu-Check glucometer (Roche, Germany). For the PTT realized in Ptpn1fl/fl and AgRP-1B mice fed with HFD (Figures 4I and 4J), aCSF and LAN icv injection were performed as described in “icv infusions” section above.

Body composition (lean and fat mass) was measured using EchoMRI (Echo Medical Systems, Houston, TX). Metabolic phenotyping of mice was carried out using a Promethion Metabolic Screening System (Sable Systems International, NV) fitted with indirect open circuit calorimetry and food consumption and activity monitors to measure activity, food intake and energy expenditure. Energy expenditure and the respiratory exchange ratio (RER = VCO$_2$/VO$_2$) were calculated from the gas exchange data. Data was smoothed to plus/minus one data point.

**Quantitative PCR**

RNA was extracted from snap frozen liver biopsies. Quantitative real-time PCR was performed as previously described (Dodd et al., 2015). TaqMan gene expression assays were used for the following genes: Pck1 (Mm01247058_m1) and G6pc (Mm00839363_m1).

**Immunoperoxidase labeling**

Mice were injected intraperitoneally with insulin (0.85mU/g; Novo Nordisk) and perfused 15 later with 4% paraformaldehyde. Brain were frozen, cut and sections were treated and incubated as described above. pAkt-labeled cells were processed for immunoperoxidase staining using rabbit IgG VECTASTAIN ABC Elite and DAB (3,30-diaminobenzidine) Peroxidase Substrate Kits (Vector Laboratories, UK); rostral-caudal hypothalamic pAkt-labeled cells counted using bright field.

**QUANTIFICATION AND STATISTICAL ANALYSIS**

**Statistics**

Data were analyzed on GraphPad PRISM and are represented as mean with error bars indicating SEM. All the statistical details of experiments can be found in the figure legends. P values were calculated using one-way ANOVA followed by Tukey’s multiple-comparison test, two-way ANOVA followed by Bonferroni’s post hoc test, or t test. Significances were defined as ns: $p > 0.05$; *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; ****$p < 0.0001$.

**DATA AND SOFTWARE AVAILABILITY**

All data produced for this manuscript are available from the lead contact (michael.cowley@monash.edu) upon reasonable request.