Cell Metabolism
Article

Leptin Engages a Hypothalamic Neurocircuitry to Permit Survival in the Absence of Insulin

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http://dx.doi.org/10.1016/j.cmet.2013.08.004

SUMMARY

The dogma that life without insulin is incompatible has recently been challenged by results showing the viability of insulin-deficient rodents undergoing leptin monotherapy. Yet, the mechanisms underlying these actions of leptin are unknown. Here, the metabolic outcomes of intracerebroventricular (i.c.v.) administration of leptin in mice devoid of insulin and lacking or re-expressing leptin receptors (LEPRs) only in selected neuronal groups were assessed. Our results demonstrate that concomitant re-expression of LEPRs only in hypothalamic γ-aminobutyric acid (GABA) and pro-opiomelanocortin (POMC) neurons is sufficient to fully mediate the lifesaving and antidiabetic actions of leptin in insulin deficiency. Our analyses indicate that enhanced glucose uptake by brown adipose tissue and soleus muscle, as well as improved hepatic metabolism, underlies these effects of leptin. Collectively, our data elucidate a hypothalamic-dependent pathway enabling life without insulin and hence pave the way for developing better treatments for diseases of insulin deficiency.

INTRODUCTION

Insulin deficiency is caused by (i) autoimmune-mediated destruction of pancreatic β cells (as seen in type 1 diabetes mellitus [T1DM]) and (ii) metabolic-stress-induced pancreatic β cell dysfunction and death, dedifferentiation (as seen in aging and type 2 diabetes mellitus [T2DM]), or complete pancreatectomy (Butler et al., 2007; Coppari and Bjørbæk, 2012; Talchai et al., 2012). If untreated, this defect leads to hyperglycemia, polyuria, ketoacidosis, and death. To date, insulin therapy is the only lifesaving intervention available to several millions of people suffering from insulin deficiency. Thus, daily insulin administrations and frequent glucose monitoring are quotidian activities of these patients. Despite the undisputable fact that therapeutic insulin has converted a previously lethal defect into a life-compatible malady, this approach does not restore metabolic homeostasis and may even cause serious unwanted effects. For example, probably owing to the established lipogenic actions of the hormone (Horton et al., 2002), long-term insulin treatment is suspected to underlie the excessive ectopic lipid deposition (i.e., in nonadipose tissues) and the extremely high incidence of coronary artery disease observed in diabetic subjects (Larsen et al., 2002; Orchard et al., 2003). It is likely that these lipogenic actions of insulin promote a vicious cycle of lipid-induced insulin resistance in liver and skeletal muscle and hence lead to an increased insulin requirement in the long-term management of diabetes (Shulman, 2000). Furthermore, due to the potent and fast-acting glycemia-lowering effects of insulin, intensive insulin therapy significantly increases the risk of hypoglycemia (an event that is disabling and can even be fatal) (Cryer, 2009). Thus, better therapies for the treatment of diseases characterized by insulin deficiency are needed.

A major barrier to the development of superior treatments has been the dogma that life without insulin is not possible. However, while insulin appears to be an absolute requirement for normal organismal development, the idea that insulin is also indispensable for survival in adulthood needs to be revised. Indeed, we and others have shown that leptin monotherapy reverses several metabolic aberrancies and permits survival of adult rodents rendered insulin deficient. Notably, leptin therapy in this context

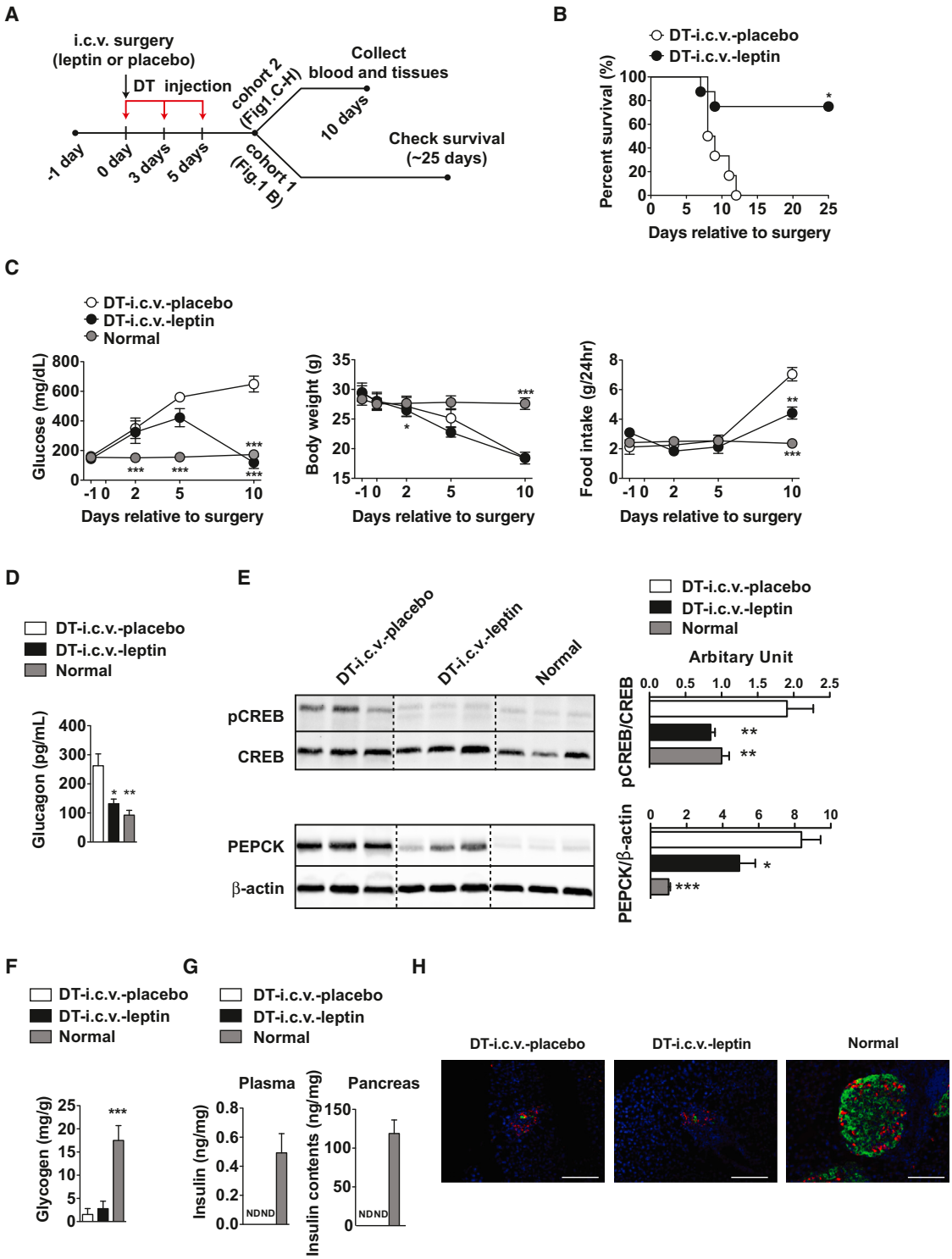

Figure 1. i.c.v. Leptin Administration Reverses Lethality and Improves Hyperglycemia Caused by Complete Insulin Deficiency

(A) Experimental design using *RIP-DTR* mice (Thorel et al., 2010). Leptin (25 ng/hr) or placebo (PBS) was intracerebroventricularly (i.c.v.) administered starting at day 0 in DT-i.c.v.-leptin or DT-i.c.v.-placebo mice, respectively. DT-i.c.v.-leptin and DT-i.c.v.-placebo mice were rendered insulin deficient by intraperitoneal (i.p.) diphtheria toxin (DT) administration at days 0, 3, and 5. Age-matched, nondiabetic controls were used to gather parameters in surgically and DT-untreated normal mice (normal group).

(B) Kaplan-Meier survival analyses were performed on DT-i.c.v.-leptin and DT-i.c.v.-placebo mice. Statistical analyses were done using the Gehan-Breslow-Wilcoxon test. ***p < 0.001 versus DT-i.c.v.-placebo mice. The number of mice at day 0 of DT-i.c.v.-leptin and DT-i.c.v.-placebo was 8 and 6, respectively.

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exerts lipolytic actions but does not cause hypoglycemia (Fujikawa et al., 2010; Wang et al., 2010; Yu et al., 2008). Thus, exploiting the slow-acting glycemia-lowering effect of leptin and/or harnessing the component(s) underlying its effect may represent attractive alternative(s) or adjuvant(s) to insulin therapy.

In mammals able to produce insulin, the glycemia-lowering action of leptin is mediated by its direct action on its cognate receptors expressed by hypothalamic neurons. For example, unilateral restoration of leptin receptors (LEPRs) signaling only in the hypothalamic arcuate nucleus (ARC) normalizes hyperglycemia in mice otherwise deficient in LEPR signaling (Coppari et al., 2005; Morton et al., 2005). Mechanistically, this action seems to require intact hypothalamic phosphatidylinositol 3-kinase (PI3K) signaling because delivery of PI3K inhibitors directly to the ARC impairs the ability of leptin to suppress hyperglycemia in these diabetic rodents (Morton et al., 2005). Recently, the biochemical identity of the ARC neurons underlying these actions of leptin has been unraveled. Indeed, re-expression of LEPRs only in pro-opiomelanocortin (POMC) neurons has been shown to be sufficient to restore normoglycemia in mice otherwise deficient in LEPR signaling (Berglund et al., 2012; Huo et al., 2009). LEPRs in the ventromedial hypothalamic nucleus (VMH) are also thought to be important for mediating the glucoregulatory actions of leptin. In fact, microinjection of leptin into the VMH of lean mice increases glucose uptake in skeletal muscle, heart, and interscapular brown adipose tissue (iBAT) (Haque et al., 1999; Minokoshi et al., 1999). Together, these results support the notion that in animals able to produce insulin, the glycemia-lowering action of leptin is mainly mediated by ARC and VMH neurons.

Because activation of either LEPRs or insulin receptor signaling partly impinges on the same molecules (e.g., PI3K) (Fukuda et al., 2008; Hill et al., 2008), and leptin enhances insulin sensitivity in insulin-resistant rodents and humans (German et al., 2009; Oral et al., 2002; Shimomura et al., 1999), the glycemia-lowering effects of leptin have been thought to be due to synergistic actions between administered leptin and endogenously secreted insulin. However, in insulin-deficient rodents, leptin administration is also very effective in ameliorating diabetes (Fujikawa et al., 2010; Wang et al., 2010; Yu et al., 2008). These latter findings may represent the groundwork for developing improved therapies for diseases of insulin deficiency. To reach this goal, a comprehensive understanding of the mechanisms (molecules and cell types) underlying the lifesaving and metabolic-improving actions of leptin in insulin deficiency is needed.

In this study, we used two different models of insulin deficiency: the streptozotocin (STZ)-induced (Fujikawa et al., 2010) and the diphtheria toxin (DT)-induced β cell depleted models (Thorel et al., 2010). By determining the metabolic outcomes of i.c.v. leptin administration in these insulin-deficient models lacking or re-expressing LEPRs only in select hypothalamic neurons, we established the identity of (i) the neuronal populations and (ii) peripheral components underlying the lifesaving and metabolic-improving actions of leptin in the context of insulin deficiency.

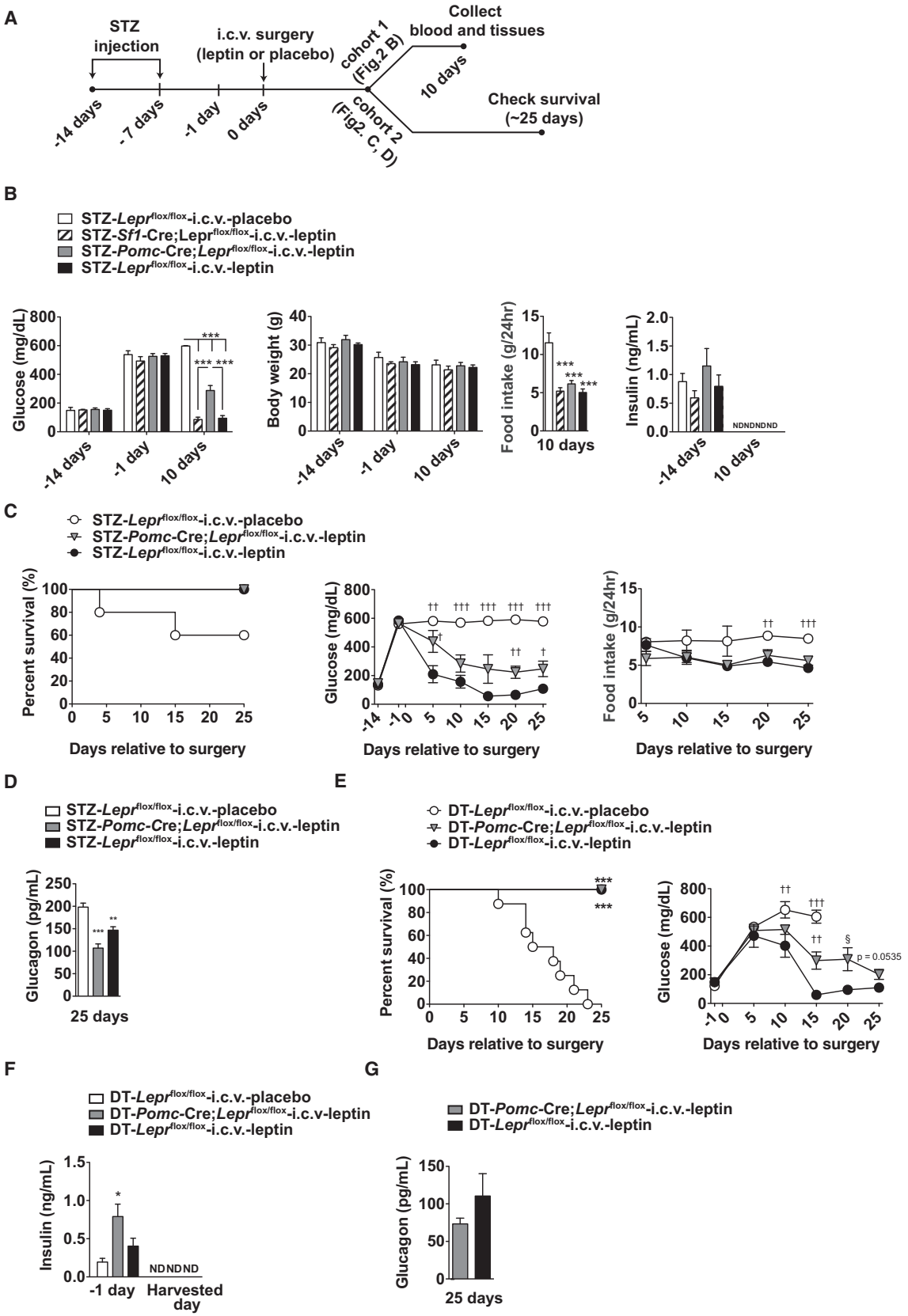
RESULTS

Brain LEPRs Mediate the Lifesaving and Metabolic Actions of Leptin in Insulin Deficiency

Experimental evidence suggests that LEPRs expressed by neurons within the central nervous system (CNS) underlie the beneficial effect of leptin administration in the context of insulin deficiency. First, the glucoregulatory actions of leptin are not mediated by hepatic LEPRs, as insulin-deficient mice lacking LEPRs only in liver respond normally to the hyperglycemia-lowering action of leptin administration (Denroche et al., 2011). Second, the idea that direct action of leptin on glucagon-secreting α cells underlies the anti-T1DM action of leptin seems to be at odds with our data indicating that pancreatic α cells do not express LEPR-B (the receptor isoform that mediates the majority of the biological actions of leptin) (Figures S1A–S1D available online). Third, we have previously reported that i.c.v. delivery of leptin permits survival and normalizes hyperglycemia of STZ-induced insulin-deficient mice (Fujikawa et al., 2010). However, STZ-treated mice retain minuscule amount of pancreatic insulin; therefore, it is unclear whether the residual insulin is required for the beneficial effect induced by leptin treatment (Fujikawa et al., 2010).

To address this issue, we used genetically engineered mice that can be rendered completely insulin deficient. *RIP-DTR* mice bear a rat insulin promoter (RIP)-DT receptor (DTR) allele cloned into the *Hprt* locus of the X chromosome. Three independent intraperitoneal (i.p.) DT administrations (at the dose of 0.5 μg/Kg of body weight each) in *RIP-DTR* mice have been shown to ablate virtually all insulin-producing pancreatic β cells (Thorel et al., 2010). Insulin deficiency in *RIP-DTR* mice was achieved by following the established protocol in Thorel et al. (2010) with a slight modification (Figure 1A). Accordingly, nearly all pancreatic β cells were ablated in DT-injected *RIP-DTR* mice (Figures S1E–S1G). Compared to normal mice, all DT-injected *RIP-DTR* mice that underwent i.c.v.-placebo treatment (DT-i.c.v.-placebo) displayed overtly high hyperglycemia, reduced body weight, hyperphagia, and hyperglucagonemia and inevitably succumbed within 15 days after the first DT administration (Figures 1B, 1C, and 1D). However, the vast majority of DT-injected *RIP-DTR* mice that underwent i.c.v. leptin treatment (DT-i.c.v.-leptin) were viable and had significantly improved hyperglycemia, hyperphagia, and hyperglucagonemia, albeit their body weight remained similar to DT-i.c.v.-placebo mice (Figures 1B, 1C, and 1D). In accordance with improved hyperglucagonemia, DT-i.c.v.-leptin mice had a hepatic level of phosphorylated cyclic AMP (cAMP) response element binding protein (pCREB; an established readout of glucagon receptor signaling) that was greatly reduced and indistinguishable compared to that of DT-i.c.v.-placebo and normal mice, respectively (Figure 1E).

(C–H) Glucose levels in the blood, body weight, and food intake (C); glucagon in the plasma (D); hepatic protein levels of pCREB and PEPCK (E); glycogen in the liver (F); insulin levels in the plasma and whole pancreas (G); and representative distribution of cells expressing insulin (green) and glucagon (red) in the pancreas of DT-i.c.v.-placebo, DT-i.c.v.-leptin, and normal mice (H). Statistical analyses were done using one-way ANOVA (Tukey's multiple comparison test). Values are mean ± SEM (n = 4–6). ***p < 0.001, **p < 0.01, *p < 0.05 versus DT-i.c.v.-placebo mice. ND, below the threshold of detection. Scale bar size = 100 μm. See also Figure S1.



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Hepatic phosphoenolpyruvate carboxykinase (PEPCK) protein expression levels were improved, but not completely normalized, by i.c.v. leptin administration (Figure 1E), and in line with our previous report (Fujikawa et al., 2010), hepatic glycogen levels were not improved by i.c.v. leptin administration (Figure 1F). Importantly, circulating and pancreatic insulin levels were virtually abolished in both DT-i.c.v.-placebo and DT-i.c.v.-leptin mice compared to normal mice (Figure 1G). Also, immunohistological assays revealed a minimal presence of pancreatic β cells in both DT-i.c.v.-placebo and DT-i.c.v.-leptin mice compared to the normal group (Figure 1H). These data suggest that the aforementioned effects of i.c.v. leptin administration are not secondary to pancreatic β cell regeneration. Collectively, our results establish that leptin administration permits survival and improves hyperglycemia of mice totally lacking insulin via CNS-dependent mechanisms.

Marginal Contribution of POMC^{LEPRs} to the Actions of Leptin in Insulin Deficiency

To determine the identity of the neuronal population(s) mediating the effect of leptin in insulin deficiency, we used genetically engineered mice that enabled the deletion of LEPRs in a neuron-type-specific fashion. Because hypothalamic POMC and steroidogenic factor 1 (SF1) neurons have been shown to regulate glucose metabolism in insulin-intact mammals (Berglund et al., 2012; Huo et al., 2009; Ramadori et al., 2011), we directly tested whether LEPRs in POMC or SF1 neurons (POMC^{LEPRs} or SF1^{LEPRs}, respectively) are required for the effects of leptin in the context of insulin deficiency. Notably, SF1 is encoded by the *Nr5a1* gene, which is only expressed in the VMH within the CNS (Choi et al., 2013). Thus, SF1 neurons are a representative neuronal population of the VMH (Dhillon et al., 2006). By breeding *Pomc*-Cre or *Sf1*-Cre allele to *Lepr*^{flox} allele, we obtained LEPR-intact control (*Lepr*$^{flox/flox}$ mice) and *Pomc*-Cre;*Lepr*$^{flox/flox}$ or *Sf1*-Cre;*Lepr*$^{flox/flox}$ mice that lack LEPRs only in POMC or SF1 neurons, respectively (Balthasar et al., 2004; Dhillon et al., 2006). Insulin deficiency in *Pomc*-Cre;*Lepr*$^{flox/flox}$, *Sf1*-Cre;*Lepr*$^{flox/flox}$, or *Lepr*$^{flox/flox}$ mice was achieved by i.p. STZ administrations (Fujikawa et al., 2010) (Figure 2A). Compared to STZ-injected *Lepr*$^{flox/flox}$ mice that underwent i.c.v.-placebo treatment (STZ-*Lepr*$^{flox/flox}$-i.c.v.-placebo), hyperglycemia was similarly reduced in STZ-injected *Sf1*-Cre;*Lepr*$^{flox/flox}$ mice that underwent i.c.v. leptin treatment (STZ-*Sf1*-Cre;*Lepr*$^{flox/flox}$-i.c.v.-leptin) and STZ-injected *Lepr*$^{flox/flox}$ mice that underwent i.c.v. leptin treatment (STZ-*Lepr*$^{flox/flox}$-i.c.v.-leptin) (Figure 2B). However, STZ-injected *Pomc*-Cre;*Lepr*$^{flox/flox}$ mice that underwent i.c.v. leptin treatment (STZ-*Pomc*-Cre;*Lepr*$^{flox/flox}$-i.c.v.-leptin) displayed a blunted response to the hyperglycemia-lowering action of the treatment (Figure 2B). Because food intake and body weight were comparable between STZ-*Pomc*-Cre;*Lepr*$^{flox/flox}$-i.c.v.-leptin, STZ-*Sf1*-Cre;*Lepr*$^{flox/flox}$-i.c.v.-leptin, and STZ-*Lepr*$^{flox/flox}$-i.c.v.-leptin mice, the diminished action of leptin on hyperglycemia in the STZ-*Pomc*-Cre;*Lepr*$^{flox/flox}$-i.c.v.-leptin group was not secondary to hyperphagia (Figure 2B). Similarly, the dampened action of the hormone was not due to impaired leptin delivery because phosphorylated signal transducer and activator of transcription 3 (pSTAT3; an established readout of leptin-induced LEPR signaling) was readily detectable in the brain of STZ-*Pomc*-Cre;*Lepr*$^{flox/flox}$-i.c.v.-leptin mice that displayed hyperglycemia (Figure S2). Notably, at 10 days after i.c.v. surgery, circulating insulin was below the threshold of detection in all groups (Figure 2B), suggesting that all of these mice were devoid of circulating insulin.

As the hormonal effects on glycemia were slightly dampened, we used a second cohort to assess whether the action of leptin on survival was also altered in STZ-*Pomc*-Cre;*Lepr*$^{flox/flox}$-i.c.v.-leptin mice. Similar to results obtained with the first cohort, food intake and body weight were comparable between STZ-*Pomc*-Cre;*Lepr*$^{flox/flox}$-i.c.v.-leptin and STZ-*Lepr*$^{flox/flox}$-i.c.v.-leptin mice (Figure 2C and data not shown). Also, glycemia was again moderately higher in the former group compared to the latter (Figure 2C). Interestingly, no difference in the percentage of survival between STZ-*Pomc*-Cre;*Lepr*$^{flox/flox}$-i.c.v.-leptin and STZ-*Lepr*$^{flox/flox}$-i.c.v.-leptin mice was noted (Figure 2C). Notably, at 25 days into the treatment, circulating glucagon levels in

Figure 2. LEPRs in POMC Neurons Are Required to Mediate a Marginal Component of Antidiabetic Action of Leptin in the Context of Insulin Deficiency

(A) Experimental design using streptozotocin (STZ)-treated mice (Fujikawa et al., 2010). To induce insulin deficiency, STZ was i.p. administered to mice lacking LEPRs selectively in POMC neurons (*Pomc*-Cre;*Lepr*$^{flox/flox}$) or SF1 neurons (*Sf1*-Cre;*Lepr*$^{flox/flox}$) and littermate control mice (*Lepr*$^{flox/flox}$). Leptin (25 ng/hr) was delivered i.c.v. to *Pomc*-Cre;*Lepr*$^{flox/flox}$, *Sf1*-Cre;*Lepr*$^{flox/flox}$, and *Lepr*$^{flox/flox}$ mice (STZ-*Pomc*-Cre;*Lepr*$^{flox/flox}$-i.c.v.-leptin, STZ-*Sf1*-Cre;*Lepr*$^{flox/flox}$-i.c.v.-leptin, and STZ-*Lepr*$^{flox/flox}$-i.c.v.-leptin group, respectively). Placebo (PBS) was delivered i.c.v. to *Lepr*$^{flox/flox}$ mice (STZ-*Lepr*$^{flox/flox}$-i.c.v.-placebo).

(B) Glucose levels in the blood, body weight, food intake, and insulin levels in the plasma of STZ-*Lepr*$^{flox/flox}$-i.c.v.-placebo, STZ-*Sf1*-Cre;*Lepr*$^{flox/flox}$-i.c.v.-leptin, STZ-*Pomc*-Cre;*Lepr*$^{flox/flox}$-i.c.v.-leptin, and STZ-*Lepr*$^{flox/flox}$-i.c.v.-leptin mice.

(C) Kaplan-Meier survival analyses were performed on STZ-*Lepr*$^{flox/flox}$-i.c.v.-placebo, STZ-*Pomc*-Cre;*Lepr*$^{flox/flox}$-i.c.v.-leptin, and STZ-*Lepr*$^{flox/flox}$-i.c.v.-leptin mice. Statistical analyses were done using the log rank test (number of mice = 5), glucose levels in the blood, and food intake of STZ-*Lepr*$^{flox/flox}$-i.c.v.-placebo, STZ-*Pomc*-Cre;*Lepr*$^{flox/flox}$-i.c.v.-leptin, and STZ-*Lepr*$^{flox/flox}$-i.c.v.-leptin mice.

(D) glucagon levels in the plasma of STZ-*Lepr*$^{flox/flox}$-i.c.v.-placebo, STZ-*Pomc*-Cre;*Lepr*$^{flox/flox}$-i.c.v.-leptin and STZ-*Lepr*$^{flox/flox}$-i.c.v.-leptin mice.

(E) Kaplan-Meier survival analyses were performed on DT-*Lepr*$^{flox/flox}$-i.c.v.-placebo, DT-*Pomc*-Cre;*Lepr*$^{flox/flox}$-i.c.v.-leptin, and DT-*Lepr*$^{flox/flox}$-i.c.v.-leptin mice. Statistical analyses were done using the log rank test (among all groups) followed by Gehan-Breslow-Wilcoxon test (each group versus DT-*Lepr*$^{flox/flox}$-i.c.v.-placebo). ***$p < 0.001$. The number of mice at day 0 of DT-*Lepr*$^{flox/flox}$-i.c.v.-placebo, DT-*Pomc*-Cre;*Lepr*$^{flox/flox}$-i.c.v.-leptin, and DT-*Lepr*$^{flox/flox}$-i.c.v.-leptin was 8, 9, and 6, respectively. Glucose levels in the blood and food intake of the DT-*Lepr*$^{flox/flox}$-i.c.v.-placebo, DT-*Pomc*-Cre;*Lepr*$^{flox/flox}$-i.c.v.-leptin, and DT-*Lepr*$^{flox/flox}$-i.c.v.-leptin mice.

(F) Glucagon levels in the DT-*Lepr*$^{flox/flox}$-i.c.v.-placebo, DT-*Pomc*-Cre;*Lepr*$^{flox/flox}$-i.c.v.-leptin, and DT-*Lepr*$^{flox/flox}$-i.c.v.-leptin mice. Harvested day means the date of death of succumbed DT-*Lepr*$^{flox/flox}$-i.c.v.-placebo mice and 25 days after i.c.v. leptin administration in DT-*Pomc*-Cre;*Lepr*$^{flox/flox}$-i.c.v.-leptin and DT-*Lepr*$^{flox/flox}$-i.c.v.-leptin mice. Statistical analyses were done using one-way ANOVA (Tukey's or Dunnett's multiple comparison test). Values are mean ± SEM (n = 3–9). ***$p < 0.001$, **$p < 0.01$ versus STZ- or DT-*Lepr*$^{flox/flox}$-i.c.v.-placebo or STZ- or DT-*Pomc*-Cre;*Lepr*$^{flox/flox}$-i.c.v.-leptin mice. $^{\dagger\dagger\dagger}p < 0.001$, $^{\dagger\dagger}p < 0.01$, $^{\dagger}p < 0.05$ versus STZ- or DT-*Lepr*$^{flox/flox}$-i.c.v.-leptin. ND, below the threshold of detection. See also Figure S2.

STZ-*Pomc*-Cre;*Lepr*$^{flox/flox}$-i.c.v.-leptin and STZ-*Lepr*$^{flox/flox}$-i.c.v.-leptin mice were similarly reduced compared to STZ-*Lepr*$^{flox/flox}$-i.c.v.-placebo mice (Figure 2D).

To further assess the role of POMC^{LEPRs} on the lifesaving and hyperglycemia-lowering actions of leptin in the absence of insulin, we used our DT model of insulin deficiency. By following the breeding strategy described in the Supplemental Experimental Procedures, the *RIP-DTR* allele was introduced into mice lacking LEPRs only in POMC neurons and their controls. Insulin deficiency in these mice was achieved by i.p. DT injections as described above and shown in Figure 1A. Data presented in Figure 2E indicate that i.c.v. leptin administration exerted similar survival effects in DT-treated *RIP-DTR* mice lacking LEPRs only in POMC neurons compared to their LEPR-intact controls. However, also in this model of insulin deficiency, i.c.v.-leptin-treated mice lacking LEPRs in POMC neurons were slightly hyperglycemic compared to i.c.v.-leptin-treated LEPR-intact controls (Figure 2E). Notably, circulating insulin was undetectable in all groups (Figure 2F). In line with data in Figure 2D, POMC^{LEPRs} were dispensable for the effects of i.c.v. leptin administration on glucagon also in the DT-induced model of insulin deficiency. Indeed, at 25 days into the treatment, circulating glucagon levels in DT-*Pomc*-Cre;*Lepr*$^{flox/flox}$-i.c.v.-leptin and DT-*Lepr*$^{flox/flox}$-i.c.v.-leptin mice were indistinguishable (Figure 2G). Collectively, our results from experiments conducted on two different mouse models of insulin deficiency establish that POMC^{LEPRs} are (i) required for mediating only a marginal component of the hyperglycemia-lowering effect and (ii) dispensable for the lifesaving, hyperphagia-suppressing, and hyperglucagonemia-lowering actions of leptin in the context of insulin deficiency.

POMC^{LEPRs} Are Not Sufficient for Mediating the Actions of Leptin in Insulin Deficiency

Although our data shown in Figure 2 indicate that POMC^{LEPRs} are marginally important, these data do not establish whether POMC^{LEPRs} are sufficient to mediate the anti-T1DM effects of leptin. To directly test this hypothesis, mice expressing LEPRs only in POMC neurons were generated. The *Pomc*-Cre allele was bred to a Cre-conditional *Lepr* null reactivatable (*Lepr*^{TB}) allele that allows for the re-expression of endogenous LEPRs upon Cre-mediated excision of a *loxP*-flanked transcriptional blocking cassette (Berglund et al., 2012). Importantly, *Pomc*-Cre;*Lepr*$^{TB/TB}$ mice express LEPRs only in POMC neurons (Berglund et al., 2012). By following the breeding strategy described in the Supplemental Experimental Procedures, the *RIP-DTR* allele was introduced into *Pomc*-Cre;*Lepr*$^{TB/TB}$ mice and their controls. Insulin deficiency in these mice was achieved by i.p. DT injections as described above and in Figure 1A. In agreement with data presented in Figures 1B and 1C, i.c.v. leptin administration reversed mortality and improved hyperglycemia in the vast majority of DT-injected *RIP-DTR* mice (DT-i.c.v.-leptin) (Figures 3A and 3B). However, it failed to do so in DT-injected *RIP-DTR* mice expressing LEPRs only in POMC neurons (DT-*Pomc*-Cre;*Lepr*$^{TB/TB}$-i.c.v.-leptin) and DT-injected *RIP-DTR* mice deficient in LEPRs (DT-*Lepr*$^{TB/TB}$-i.c.v.-leptin), both of which had similar body weights (Figures 3A and 3B). Notably, the recalcitrant response to treatment displayed by DT-*Lepr*$^{TB/TB}$-i.c.v.-leptin and DT-*Pomc*-Cre;*Lepr*$^{TB/TB}$-i.c.v.-leptin mice occurred in the presence of a minuscule amount of circulating insulin (Figure 3B). Also, while i.c.v. leptin administration induced pSTAT3 in several hypothalamic sites (including ARC and VMH) of DT-i.c.v.-leptin mice, it failed to exert this action in DT-*Lepr*$^{TB/TB}$-i.c.v.-leptin mice (Figure 3C), hence proving that the latter are indeed LEPR-deficient mice. Furthermore, pSTAT3 was detected only in ARC (the site at which POMC neurons are located) of DT-*Pomc*-Cre;*Lepr*$^{TB/TB}$-i.c.v.-leptin mice (Figure 3C). These results further bolster the idea that these mutants express functional LEPRs only in POMC neurons (Berglund et al., 2012).

The refractory response to leptin administration displayed by DT-*Pomc*-Cre;*Lepr*$^{TB/TB}$-i.c.v.-leptin mice could be due to (i) failure to deliver the hormone into the brain of these mice, (ii) DT injections causing ablation of POMC neurons, and/or (iii) non-POMC neurons, which are crucial components of this neurocircuitry. The first possibility is ruled out by data showing pSTAT3 staining in ARC of DT-*Pomc*-Cre;*Lepr*$^{TB/TB}$-i.c.v.-leptin mice (Figure 3C). The likelihood that DT injections caused ablation of POMC neurons is also ruled out because hypothalamic *Pomc* messenger RNA (mRNA) levels in *RIP-DTR* mice did not change 24 hr after DT injection, whereas the pancreatic *preproinsulin* mRNA level drastically decreased in these mice at the same time point (Figures S3A and S3B). As expected, glucose levels in the blood increased 48 hr after DT injection (Figure S3C). Also, the anatomical distribution of β-endorphin (a product of POMC) in the hypothalamus was comparable between *RIP-DTR* mice treated with or without DT (Figures S3D–S3I). On the other hand, the anatomical distribution of pancreatic β cells was dramatically different between these two groups (Figures S3D–S3I). The possibility that DT injections caused ablation of non-POMC neurons that are crucial components of this neurocircuitry is also unlikely. In fact, our previously published results (Fujikawa et al., 2010) and our data in Figure 1 strongly indicate that i.c.v. leptin administration exerts very similar survival and antidiabetic effects in the DT-induced and STZ-induced models of insulin deficiency. Nevertheless, *Dtr* was detected in pancreas and hypothalamus of *RIP-DTR* mice (Figure S3J). Thus, we cannot rule out the possibility that DT injections ablated other types of cells within this brain structure. Taken together, our results indicate that even if DT administration killed some hypothalamic cells in mice bearing the *RIP-DTR* allele, these cells are likely dispensable for mediating the beneficial effects of leptin administration. Collectively, our results in Figures 2, 3, S2, and S3 establish that POMC^{LEPRs} exert only a minor role in mediating the beneficial effects of leptin administration in the context of insulin deficiency.

Importance of Concomitant Expression of LEPRs in GABAergic and POMC Neurons

To determine the identity of the important neurons in the neurocircuitry engaged by leptin, we took advantage of several lines of evidence. First, our data in Figure 2 excluded a role for LEPRs expressed by SF1 neurons that represent a large population of leptin-responsive glutamatergic neurons (Vong et al., 2011). Second, our data in Figures 2, 3, S2, and S3 establish that LEPRs on POMC neurons (also in part glutamatergic) (Vong et al., 2011) play only a marginal role in mediating the effects of leptin in insulin deficiency. Thus, LEPRs on glutamatergic neurons are unlikely to exert a major function in this pathway. Therefore, we investigated the role of LEPRs expressed by GABAergic neurons. To directly test the possibility that LEPRs on GABAergic neurons are the

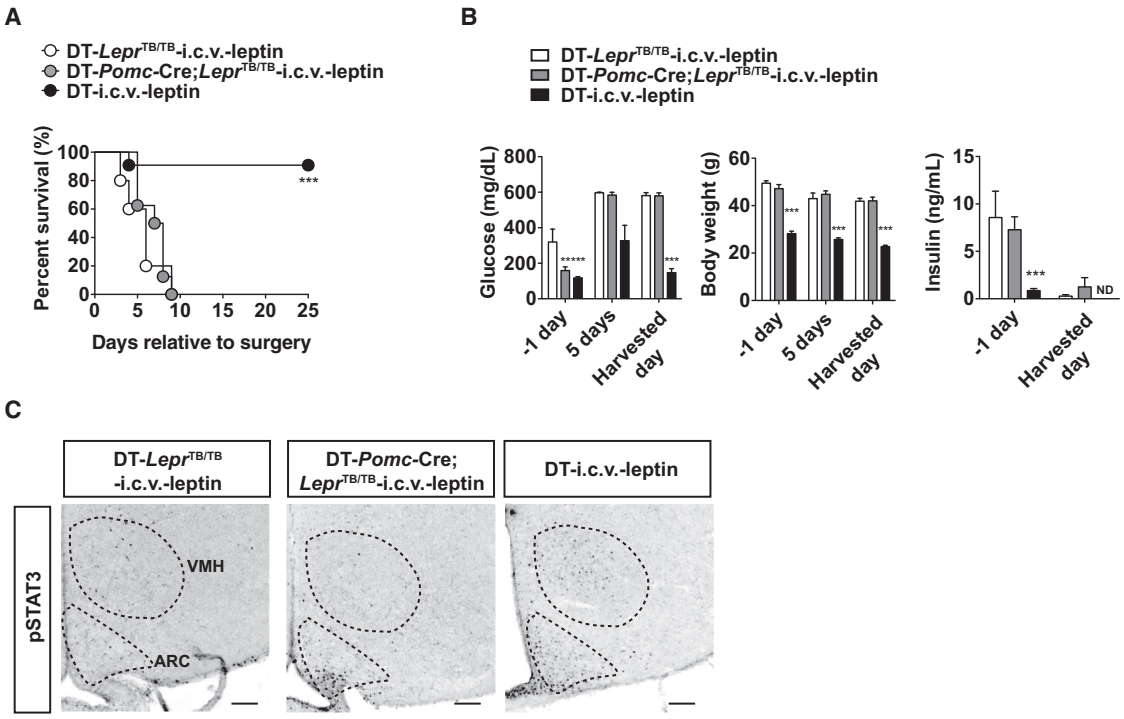


Figure 3. LEPRs in POMC Neurons Are Not Sufficient to Mediate the Antidiabetic Action of Leptin in the Context of Insulin Deficiency

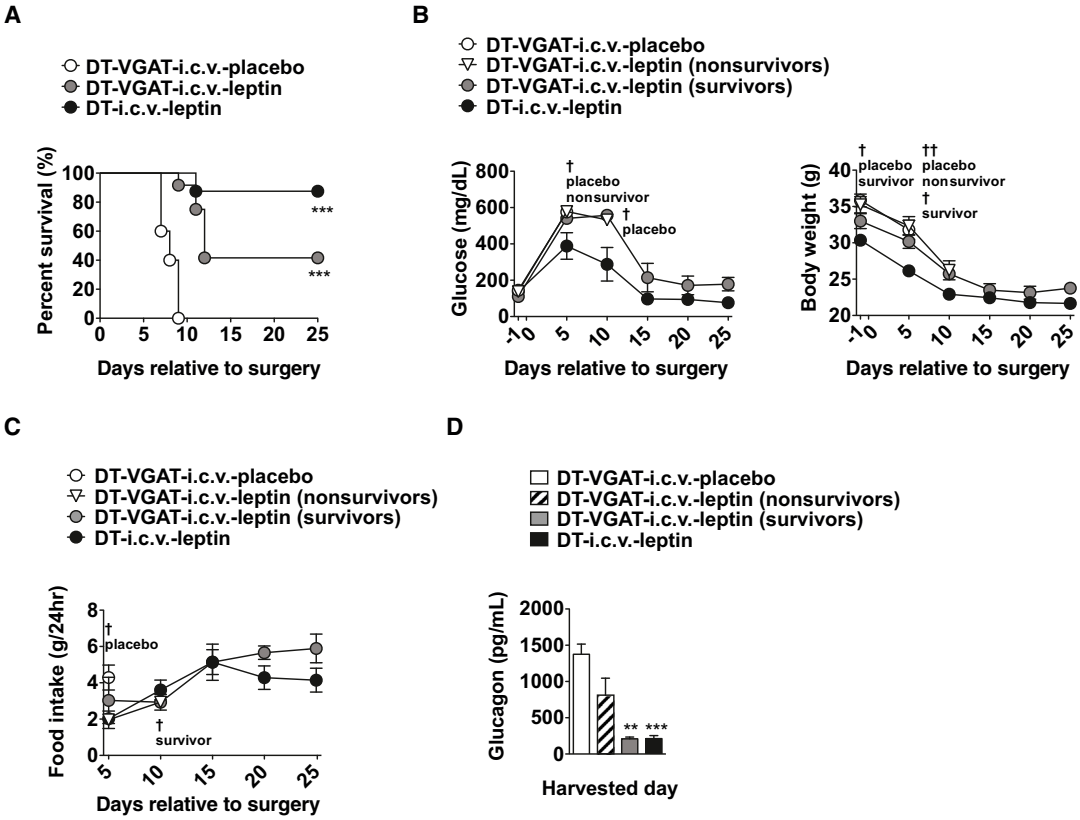
(A) Kaplan-Meier survival analyses were performed on insulin-deficient mice expressing LEPRs selectively in POMC neurons, LEPR-intact control (*Lepr*$^{+/+}$ and *Pomc*-Cre;*Lepr*$^{+/+}$), and LEPR null littermates that received i.c.v. leptin (25 ng/hr) administration (DT-*Pomc*-Cre;*Lepr*$^{TB/TB}$-i.c.v.-leptin, DT-i.c.v.-leptin, DT-*Lepr*$^{TB/TB}$-i.c.v.-leptin mice, respectively). Statistical analyses were done using the log rank test (among all groups) followed by the Gehan-Breslow-Wilcoxon test (each group versus DT-*Lepr*$^{TB/TB}$-i.c.v.-leptin). ***$p < 0.001$. The number of mice at day 0 of DT-*Lepr*$^{TB/TB}$-i.c.v.-leptin, DT-*Pomc*-Cre;*Lepr*$^{TB/TB}$-i.c.v.-leptin, and DT-i.c.v.-leptin was 11, 8, and 5, respectively.

(B and C) Glucose levels in the blood, body weight, and insulin levels in the plasma (B) and representative distribution of cells expressing phosphorylated STAT3 (pSTAT3) in the mediobasal hypothalamus of DT-*Lepr*$^{TB/TB}$-i.c.v.-leptin, DT-*Pomc*-Cre;*Lepr*$^{TB/TB}$-i.c.v.-leptin, and DT-i.c.v.-leptin mice (C). Harvested day means the date of death for succumbed DT-*Pomc*-Cre;*Lepr*$^{TB/TB}$-i.c.v.-leptin and DT-i.c.v.-leptin mice and 25 days after i.c.v. leptin administration for DT-i.c.v.-leptin mice. Statistical analyses were done using one-way ANOVA (Tukey's multiple comparison test). Values are mean ± SEM (n = 5–11). ***$p < 0.001$, **$p < 0.01$ versus DT-*Lepr*$^{TB/TB}$-i.c.v.-leptin. ND, below the threshold of detection. Scale bar size = 100 μm. ARC, arcuate nucleus; VMH, ventromedial hypothalamic nucleus. See also Figure S3.

crucial mediators of the effects of leptin in insulin deficiency, we assessed the consequence of i.c.v. leptin administration in insulin-deficient mice expressing LEPRs only in GABAergic neurons. The vesicular GABA transporter (VGAT) is encoded by the gene *Slc32a1* and biochemically characterizes GABAergic neurons. By breeding the *Vgat*-ires-Cre allele (known to drive Cre-mediated recombination of *loxP*-flanked alleles in all GABAergic neurons) (Vong et al., 2011) to the *Lepr*^{TB} allele mice expressing LEPRs only in GABAergic neurons (*Vgat*-ires-Cre;*Lepr*$^{TB/TB}$ mice) were obtained. By following the breeding strategy described in the Supplemental Experimental Procedures, the *RIP-DTR* allele was introduced into *Vgat*-ires-Cre;*Lepr*$^{TB/TB}$ mice and their controls. Insulin deficiency in these mice was achieved by i.p. DT injections as described above and shown in Figure 1A.

As expected, no DT-treated *Vgat*-ires-Cre;*Lepr*$^{TB/TB}$;*RIP-DTR* mice undergoing i.c.v. placebo administration (DT-VGAT-i.c.v.-placebo) survived for longer than 10 days into treatment (Figure 4A). In line with our results in Figure 1, 7 out of 8 DT-i.c.v.-leptin controls survived 25 days into treatment (Figure 4A). Notably, 5 out of 12 DT-treated *Vgat*-ires-Cre;*Lepr*$^{TB/TB}$;*RIP-DTR* mice undergoing i.c.v. leptin administration (DT-VGAT-i.c.v.-leptin) also survived 25 days into treatment (Figure 4A). Remarkably, all of these survivors displayed significant hyperglycemic improvements, albeit these were not as complete as the ones shown by DT-i.c.v.-leptin controls that have intact LEPRs (Figure 4B). Insulin in the plasma was undetectable in both DT-VGAT-i.c.v.-leptin survivors and DT-i.c.v.-leptin controls, hence supporting the notion that DT-VGAT-i.c.v.-leptin survivors were truly insulin deficient (data not shown). Food intake was also similar between DT-VGAT-i.c.v.-leptin survivors and DT-i.c.v.-leptin controls, while body weight tended to be slightly higher in the former compared to the latter group (Figures 4B and 4C). Interestingly, the ability of i.c.v. leptin administration to suppress hyperglucagonemia was fully intact and only partial in DT-VGAT-i.c.v.-leptin survivors and nonsurvivors, respectively, compared to DT-i.c.v.-leptin controls (Figure 4D).

Notably, leptin-responsive GABAergic neurons are localized only in ARC, lateral hypothalamic area (LHA), and dorsomedial hypothalamus (DMH) within the CNS (Vong et al., 2011). Accordingly, robust pSTAT3 staining was detected in the ARC, LHA, and DMH of DT-VGAT-i.c.v.-leptin, but not in DT-VGAT-i.c.v.-placebo, mice (Figure 4E). The different intragroup survival outcomes and metabolic profiles displayed by DT-VGAT-i.c.v.-leptin mice could be due to (i) failure to deliver the hormone



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into the brain, (ii) altered leptin sensitivity of DT-VGAT-i.c.v.-leptin nonsurvivors, and/or (iii) DT injections ablating a significant proportion of leptin-responsive VGAT-expressing neurons in DT-VGAT-i.c.v.-leptin nonsurvivors. The aforementioned possibilities are unlikely because the anatomical distribution of brain pSTAT3 was comparable between DT-VGAT-i.c.v.-leptin survivors and nonsurvivors (Figures S4A–S4F).

Thus, LEPRs on other non-GABAergic neuronal group(s) must play a required role. Because our results in Figure 2 indicated that POMC^{LEPRs} are required, we investigated whether re-expression of LEPRs in GABAergic and POMC neurons restores a full responsiveness to leptin administration. By following the breeding strategy described in the Supplemental Experimental Procedures, mice expressing LEPRs selectively in GABAergic and POMC neurons (*Vgat*-ires-Cre;*Pomc*-Cre;*Lepr*$^{TB/TB}$;*RIP-DTR* mice) were obtained. Insulin deficiency in these mice was achieved by i.p. DT injections as described above and shown in Figure 1A. Re-expression of LEPRs in GABAergic and POMC neurons had greater effects in mediating the lifesaving and hyperglycemia-improving action of leptin administration compared to re-expression of LEPRs only in GABAergic neurons (Figures 5A and 5B). In fact, DT-VGAT-i.c.v.-leptin mice again displayed a partial improvement in lethality and hyperglycemia, while these parameters were similarly rescued in i.c.v.-leptin-treated insulin-deficient mice expressing LEPRs concomitantly only in GABAergic and POMC neurons (DT-VGAT-POMC-i.c.v.-leptin group) compared to DT-i.c.v.-leptin controls (Figures 5A and 5B). In line with data shown in Figure 4D, DT-VGAT-i.c.v.-leptin survivors were again found to display similar levels of circulating glucagon compared to DT-i.c.v.-leptin controls (Figure 5C). Also, DT-VGAT-POMC-i.c.v.-leptin mice had circulating glucagon levels indistinguishable from DT-VGAT-i.c.v.-leptin survivors and DT-i.c.v.-leptin controls (Figure 5C). At 25 days after induction of insulin deficiency, insulin in the plasma was undetectable in all groups (data not shown).

Altogether, our results demonstrate that concomitant expression of LEPRs only in GABAergic and POMC neurons is sufficient to fully mediate the lifesaving and antidiabetic effects of leptin administration in the context of insulin deficiency and that the vast majority of these actions are via LEPRs in GABAergic neurons.

Improved Liver, Soleus, and iBAT Metabolism Underlie the Actions of Leptin in Insulin Deficiency

To gather further insights into the mechanisms underpinning the leptin-dependent pathway enabling life without insulin, we interrogated the role of β-adrenergic receptors. These receptors have been previously indicated as important components of the hypothalamic-dependent pathway governing glucose metabolism (Haque et al., 1999; Minokoshi et al., 1999). Thus, to directly test whether β-adrenergic receptors are downstream effectors of the neurocircuitry engaged by leptin, we assessed the metabolic outcomes of i.c.v. leptin administration in mice devoid of the three known β-adrenergic receptors (β-less mice) (Bachman et al., 2002). As shown in Figure 6A, i.c.v. leptin administration rescued hyperglycemia to similar extents in STZ-treated wild-type (STZ-wild-type-i.c.v.-leptin) and β-less (STZ-β-less-i.c.v.-leptin) mice. These genetic results were further corroborated by pharmacological data. Indeed, administration of the β-adrenergic receptor blocker timolol did not alter the antidiabetic actions of i.c.v. leptin administration in STZ-treated wild-type mice (Figures 6B and 6C). Collectively, these data indicate that β-adrenergic receptors are dispensable for the antidiabetic action of leptin in the context of insulin deficiency.

We and others have reported that the improved hyperglycemia engendered by leptin administration to insulin-deficient rodents is not the result of increased glucose excretion (Fujikawa et al., 2010; Wang et al., 2010; Yu et al., 2008). To determine the fate of glucose in insulin-deficient mice undergoing leptin administration, we used glucose-tracing analyses (Figure 6D). Our results indicate that i.c.v. leptin administration does not affect glucose uptake in the brain or gastrocnemius and vastus skeletal muscle, while it significantly enhances glucose uptake in soleus muscle and iBAT of DT-treated *RIP-DTR* mice (Figure 6E). To further elucidate the peripheral effects of i.c.v. leptin administration in insulin-deficient mice, hepatic gene expression and metabolite levels in STZ-*Lepr*$^{flox/flox}$-i.c.v.-leptin and STZ-*Lepr*$^{flox/flox}$-i.c.v.-placebo mice (same animals as shown in Figure 2B) were assessed by transcriptomics and metabolomics assays, respectively. The transcriptomics analysis revealed that more than 800 genes were significantly ($p < 0.001$) down- or upregulated by i.c.v. leptin administration (Figure S5A). The metabolomics analysis indicated that 23 metabolites were significantly ($p < 0.01$) altered by i.c.v. leptin administration (Figure S5B and Table S1). Notably, pathway analysis indicated that several metabolic pathways, including those relevant to glucose metabolism, were significantly altered ($p < 0.001$) by i.c.v. leptin administration (Figure S5C and Table S2). We particularly focused on glucose and glycogen metabolism because lower circulating glucagon levels brought on by i.c.v. leptin administration may affect those pathways. In line with our results that hepatic

Figure 4. LEPRs in GABAergic Neurons Are Sufficient to Largely Mediate the Survival and Antidiabetic Action of Leptin in the Context of Insulin Deficiency

(A) Kaplan-Meier survival analyses were performed on DT-treated mice expressing LEPRs selectively in GABAergic neurons that received i.c.v. leptin (25 ng/hr) administration (DT-VGAT-i.c.v.-leptin) or placebo (PBS) administration (DT-VGAT-i.c.v.-placebo) and LEPR-intact control (composed of *Vgat*-ires-Cre;*Lepr*$^{+/+}$;*RIP-DTR* and *Lepr*$^{+/+}$;*RIP-DTR* mice) littermate mice that received i.c.v. leptin administration (DT-i.c.v.-leptin). Statistical analyses were done using the log rank test (among all groups) followed by the Gehan-Breslow-Wilcoxon test (compared each group versus DT-VGAT-i.c.v.-placebo). ***$p < 0.001$. The number of mice at day 0 of DT-VGAT-i.c.v.-placebo, DT-VGAT-i.c.v.-leptin, and DT-i.c.v.-leptin was 5, 12, and 8, respectively.

(B–D) Glucose levels in the blood and body weight (B), food intake (C), and glucagon levels in the plasma (D) in DT-VGAT-i.c.v.-placebo, DT-VGAT-i.c.v.-leptin (nonsurvivors), DT-VGAT-i.c.v.-leptin (survivors), and DT-i.c.v.-leptin mice.

(E) Representative distribution of cells expressing phosphorylated STAT3 (a readout of leptin-responsive neurons) in the lateral hypothalamic area (LHA), hypothalamic arcuate nucleus (ARC), and dorsomedial nucleus (DMH) of DT-VGAT-i.c.v.-placebo, DT-VGAT-i.c.v.-leptin, and DT-i.c.v.-leptin mice. Anatomical locations of LHA, ARC, and DMH are shown in red, dashed-line boxes (Franklin and Paxinos, 2008). Statistical analyses were done using one-way ANOVA (Dunnett's multiple comparison test). Values are mean ± SEM (n = 3–12). Scale bar size = 100 μm. $^{\dagger\dagger\dagger}p < 0.001$, $^{\dagger\dagger}p < 0.01$, $^{\dagger}p < 0.05$ versus DT-i.c.v.-leptin. See also Figure S4.

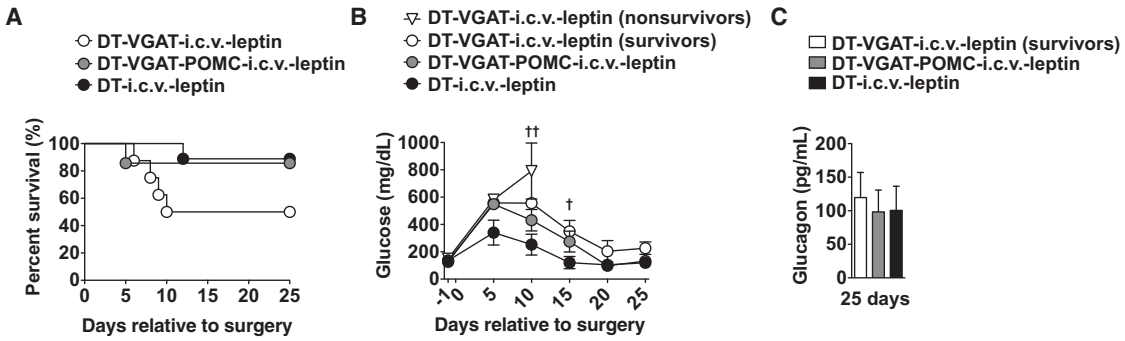

Figure 5. LEPRs in GABAergic and POMC Neurons Are Sufficient to Fully Mediate the Antidiabetic Action of Leptin in the Context of Insulin Deficiency

(A) Kaplan-Meier survival analyses were performed on DT-treated mice expressing LEPRs selectively in both GABAergic and POMC neurons (*Vgat*-ires-Cre;*Pomc*-Cre;*Lepr*$^{TB/TB}$;*RIP-DTR* mice), GABAergic neurons (*Vgat*-ires-Cre;*Lepr*$^{TB/TB}$;*RIP-DTR* mice), and LEPR-intact control mice (composed of *Vgat*-ires-Cre;*Pomc*-Cre;*Lepr*$^{+/+}$;*RIP-DTR*, *Vgat*-ires-Cre;*Lepr*$^{+/+}$;*RIP-DTR*, *Pomc*-Cre;*Lepr*$^{+/+}$;*RIP-DTR*, and *Lepr*$^{+/+}$;*RIP-DTR* mice) that received i.c.v. leptin (25 ng/hr) administration (DT-VGAT-POMC-i.c.v.-leptin, DT-VGAT-i.c.v.-leptin, and DT-i.c.v.-leptin). Statistical analyses were done using the log rank test. The number of mice at day 0 of DT-VGAT-i.c.v.-leptin, DT-VGAT-POMC-i.c.v.-leptin, and DT-i.c.v.-leptin was 8, 7, and 9, respectively.

(B and C) Glucose levels in the blood of DT-VGAT-i.c.v.-leptin (nonsurvivors), DT-VGAT-i.c.v.-leptin (survivors), VGAT-POMC-i.c.v.-leptin, and DT-i.c.v.-leptin (C) and glucagon levels in the plasma in DT-VGAT-i.c.v.-leptin (survivors), DT-VGAT-POMC-i.c.v.-leptin, and DT-i.c.v.-leptin at 25 days (C). Statistical analyses were done using one-way ANOVA (Dunnett's multiple comparison test). Values are mean ± SEM (n = 3–9). $^{\dagger\dagger}p < 0.05$, $^{\dagger}p < 0.05$ versus DT-i.c.v.-leptin.

glycogen levels were not altered by i.c.v. leptin administration (Figure 1F) (Fujikawa et al., 2010), we found many genes and metabolites relevant to this pathway that were not significantly altered by i.c.v. leptin administration (Figure S5D and Table S2). On the other hand, hepatic glucose content and the expression of glyceraldehyde-3-phosphate dehydrogenase and phosphoglycerate kinase 1 (both of which encode for enzymes of the glycolytic pathway) were reduced by i.c.v. leptin administration (Figure S5D). These results indicate that although not normalized, the liver of i.c.v.-leptin-treated insulin-deficient mice displayed improved metabolomics and transcriptomics profiles compared to i.c.v.-placebo-treated insulin-deficient mice. Collectively, our results suggest that increased glucose uptake and utilization by soleus and iBAT and improved hepatic metabolism are components of the mechanism underlying the antidiabetic action of leptin in the context of insulin deficiency.

DISCUSSION

In this study, we unraveled a neurocircuitry whereby leptin engages hypothalamic GABA^{LEPRs} and POMC^{LEPRs} to permit survival and maintain euglycemia in the context of complete insulin deficiency. This conclusion was drawn mainly because concomitant re-expression of LEPRs in GABAergic and POMC neurons is sufficient to mediate virtually all of the lifesaving and antidiabetic actions of leptin administration in insulin-deficient mice (Figure 5). Our results also clarified the contribution that each one of the two aforementioned neuronal populations exerts on this pathway. As shown in Figure 2, the effects of i.c.v. leptin administration on survival, food intake, and circulating glucagon level do not require POMC^{LEPRs}. Although our results indicate that POMC^{LEPRs} are required for mediating the effect on hyperglycemia, their contribution to this action is marginal. In fact, data shown in Figure 2 indicate that the ability of i.c.v. leptin administration to suppress hyperglycemia in the STZ- and DT-induced models of insulin deficiency is only slightly diminished in mutants lacking POMC^{LEPRs}. Also, these receptors alone are not sufficient for mediating any of the effects of i.c.v. leptin administration in the context of insulin deficiency (Figure 3). On the other hand, our data strongly suggest that LEPRs in GABAergic neurons mediate a large component of the hormonal effects on survival and glucose metabolism in the context of complete insulin deficiency (Figure 4). Thus, the beneficial effects of leptin administration in insulin-depleted mice are largely mediated by direct action of the hormone leptin on GABA^{LEPRs}, while only a minimal component of these effects is mediated by POMC^{LEPRs}.

Unger and colleagues have provided experimental support for the idea that leptin improves diabetes of insulin-deficient rodents in part via diminishing circulating glucagon content and action (Lee et al., 2012; Wang et al., 2010; Yu et al., 2008). Our results are in line with this notion. Indeed, in our leptin-treated insulin-deficient mouse models displaying ameliorated hyperglycemia, circulating glucagon level and action were also improved (Figures 1F, 2D, 2G, 4D, and 5C). Combined with data shown in Figures S1A–S1D indicating that LEPR-B is not expressed by pancreatic α cells, our results suggest that the hyperglucagonemia-lowering effect of leptin is not via direct action of the hormone on pancreatic α cells, but rather via indirect action through hypothalamic GABAergic neurons. Further studies will be needed to unravel all components of this GABAergic neuron-pancreatic α cell axis.

Although GABAergic neurons are widely distributed throughout the CNS, the subgroup of GABAergic neurons also able to express LEPRs is restricted to in the ARC, DMH, and LHA (Figure 4E); notably, these results are in line with previously reported data (Vong et al., 2011). Therefore, the direct action of leptin on one or several of the aforementioned three sites must be important for the lifesaving and antidiabetic action of the hormone in the context of insulin deficiency (Figure 4E). Among

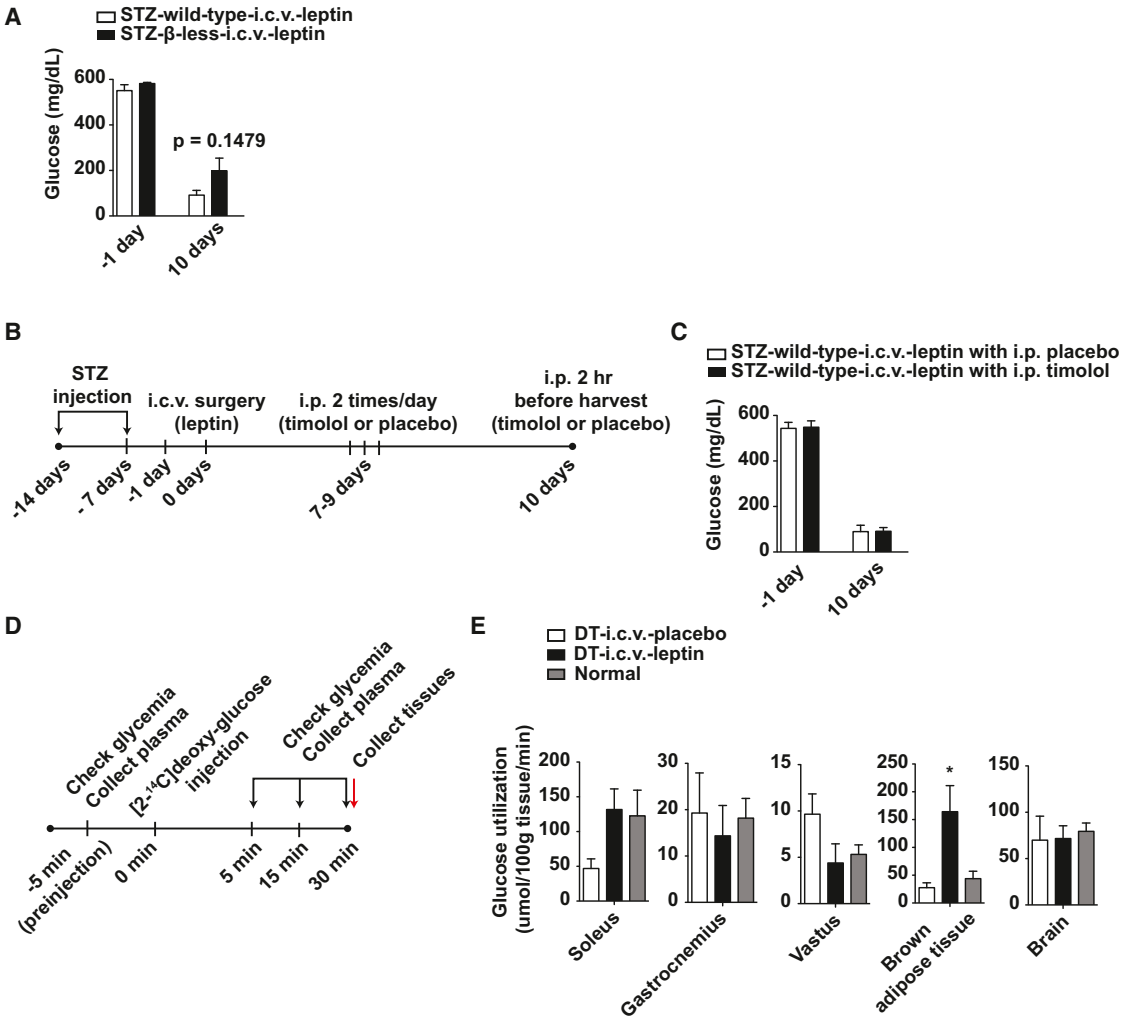

Figure 6. Leptin Administration Induces Glucose Uptake in Soleus and iBAT of Insulin-Deficient Mice

(A) Glucose level in the blood of insulin-deficient β-less mice (that do not express any types of β-adrenergic receptors) and their wild-type controls that received i.c.v. leptin (25 ng/hr) administration (STZ-β-less-i.c.v.-leptin and STZ-wild-type-i.c.v.-leptin groups, respectively). Insulin deficiency was induced by administration of STZ, as shown in Figure 2.

(B and C) Experimental design (B) and glucose level in the blood of STZ-treated Friend virus B (FBV)/NIH Jackson (NJ) mice that received i.c.v. administration of leptin (25 ng/hr) followed by administrations of β-adrenergic receptor blocker timolol (1 mg/kg BW) or placebo (0.9% saline solution) (C). Mice were injected i.p. twice a day from day 7 up to day 9 and once more at 2 hr before sacrifice.

(D) Experimental design of assessment of basal glucose utilization. The experiment was carried out 10 days after i.c.v. administration, as described in Figure 1A.

(E) Glucose utilization levels in soleus muscle, gastrocnemius muscle, vastus muscle, interscapular brown adipose tissue and brain of DT-i.c.v.-leptin, DT-i.c.v.-placebo, and normal mice. Values are mean ± SEM (n = 4–6). Statistical analyses were done using unpaired t test or one-way ANOVA (Tukey's multiple comparison test). *$p < 0.05$ versus DT-i.c.v.-placebo mice. See also Figure S5 and Tables S1 and S2.

the plausible candidates are agouti-related protein (AgRP)-expressing neurons, as they are (i) located in the ARC, (ii) GABAergic, and (iii) known to express LEPRs (Vianna and Coppari, 2011). Thus, to directly test whether AgRP neurons are indeed the crucial neurons in this neurocircuitry, assessing the outcomes of leptin administration in insulin-deficient mice expressing LEPRs only in AgRP neurons appears to be the ideal experimental approach. Such mutants can theoretically be generated by breeding the $Lepr^{TB}$ allele to the *Agrp*-ires-Cre transgene (Tong et al., 2008). Nevertheless, our attempts to achieve this goal failed, due to the nearly 100% frequency of detectable Cre-recombined $Lepr^{TB}$ allele in sites in which AgRP neurons are not present in *Agrp*-ires-Cre;$Lepr^{TB/TB}$ mice. Tackling the relevance of LEPRs in DMH and/or LHA is currently a technically difficult task mainly because of the lack of DMH- and/or LHA-specific-Cre mouse lines. Thus, future studies aimed at sorting out the contribution that LEPRs expressed by GABAergic neurons within the ARC, DMH, and LHA on the lifesaving and antidiabetic action of leptin in the context of insulin deficiency are warranted.

It is of interest that while $POMC^{LEPRs}$ are sufficient to mediate the effects of leptin on glucose metabolism in rodents able to

secrete insulin (Berglund et al., 2012), they are not sufficient to mediate these glucoregulatory effects of the hormone in rodents devoid of insulin (Figure 3). These results demonstrate that insulin is necessary for POMC neurons to trigger the cascade of events that bring about reduced hyperglycemia induced by leptin. Along these lines, in rodents able to produce insulin, hypothalamic-mediated control of glucose metabolism (as, for example, in skeletal muscle and iBAT) relies on the activation of sympathetic nervous system (SNS) neurons (these cells secrete catecholamines whose targets are adrenergic receptors) (Minokoshi et al., 1999; Ramadori et al., 2011). However, our genetic and pharmacological data in Figures 6A and 6C indicate that β-adrenergic receptors are dispensable for hypothalamic-dependent glucoregulatory actions of leptin in rodents unable to produce insulin. It is important to note that our results cannot rule out the possibility that other types of adrenergic receptors (e.g., α-adrenergic receptors) and/or receptors whose ligands secreted by SNS neurons are not catecholamines are important components of the aforementioned mechanism. Nevertheless, we suggest that alternative non-SNS pathways are engaged by leptin to increase glucose uptake in skeletal muscle and iBAT in the context of insulin deficiency (Figure 6E). These pathways may include parasympathetic nervous system neurons and/or humoral factor(s), and future studies will be needed to directly test this hypothesis. Collectively, our data and previously published results mentioned above indicate that different pathways can be engaged by hypothalamic neurons in order to keep glucose metabolism in check in the presence versus absence of insulin.

From a therapeutic viewpoint, our results could pave the way for developing the urgently needed improved therapies for the millions of people affected by insulin deficiency (e.g., T1DM and some T2DM patients) (Butler et al., 2007; Coppari and Bjørbæk, 2012; Talchai et al., 2012). In fact, the sole therapeutic available to these patients is insulin administration that, as discussed in the Introduction, bears several limitations and can even underlie some of the serious morbidities seen in these patients (Larsen et al., 2002; Orchard et al., 2003). Although these preclinical results indicate that leptin therapy may represent an ideal alternative, its applicability in insulin-deficient humans seems improbable. Indeed, several clinical trials failed to show effectiveness of leptin administration in patients who are not severely hypoleptinemic or totally lacking leptin; yet, the vast majority of T2DM and insulin-treated T1DM subjects are not known to be severely hypoleptinemic or leptin deficient (Coppari and Bjørbæk, 2012). Therefore, targeting the cellular and molecular components underlying the effects of leptin in insulin deficiency may prove efficacious in the clinical arena. Here, we identified crucial components of this pathway; hence, we believe that our results bear important clinical significance. In fact, harnessing hypothalamic GABAergic and POMC neurocircuitry and/or its downstream effector components (e.g., brown adipocytes) may permit survival and greatly improve hyperglycemia in people suffering from diseases characterized by insulin deficiency.

EXPERIMENTAL PROCEDURES

Animals

All mice were housed with standard chow diet and water available ad libitum in a light- and temperature-controlled environment. We used male mice. Tail DNA was collected from each mouse to determine its genotype. Genotyping primers are described in Table S3. Care of mice was within the Institutional Animal Care and Use Committee (IACUC) guidelines, and all the procedures were approved by the University of Texas Southwestern Medical Center IACUC.

Induction of Insulin Deficiency by Diphtheria Toxin or Streptozotocin Injection

We used 3- to 4-month-old male *RIP-DTR* mice whose body weights (BWs) were approximately 30 g. Diphtheria toxin (Sigma) was dissolved in sterile 0.9% NaCl and i.p. administered into *RIP-DTR* mice to ablate pancreatic β cells (0.5 μg/kg BW; on days 0, 3, and 5). Streptozotocin (Sigma) was dissolved in cold-sterile 0.9% NaCl and immediately i.p. administered (150 mg/kg BW; two times at one week intervals) into mice (Fujikawa et al., 2010).

i.c.v. Leptin Administration

Leptin (PeproTech; 25 ng/hr/0.11 μL) was chronically i.c.v. administered by the osmotic pump (ALZET) as previously described (Fujikawa et al., 2010). A sterile PBS (pH = 7.4; Invitrogen) solution was administered into the control group as placebo treatment.

Assessment of Energy Substrates and Hormone Levels

Daily glycemia, plasma, and pancreatic insulin were measured as previously described (Fujikawa et al., 2010). Plasma glucagon was measured as previously described (Berglund et al., 2012).

Assessment of mRNA and Protein Contents

mRNA levels and protein contents in the hypothalamus, pancreas, and liver were determined by the previously described methods with slight modifications (Fujikawa et al., 2010).

Immunohistochemistry

Phosphorylation of STAT3 in the brain was determined as previously described (Scott et al., 2009). Pancreatic glucagon and insulin distribution was determined as previously described (Fujikawa et al., 2010)

Assessment of Basal, Tissue-Specific Glucose Uptake

Mice were fasted for 1.5 hr and then i.p. injected with 150 uCi of [2-^{14}C]deoxyglucose. Samples to measure blood glucose and plasma ^{14}C-specific activity were taken at t = −5 (preinjection), 5, 15, and 30 min. Mice were then euthanized immediately after the final sample, and tissues were quickly dissected and frozen in liquid nitrogen. Methods and calculations to determine tissue-specific glucose uptake (Rg) have been previously described (Fueger et al., 2003; Kraegen et al., 1985).

Statistical Analysis

With the exception of microarray and metabolomics results, all data sets were analyzed for statistical significance using Prism (GraphPad) as indicated in each of the figure legends. Error bars in all figures represent SEM.

ACCESSION NUMBERS

The microarray data were deposited to The National Center for Biotechnology Information under the accession number GSE48598.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, five figures, and three tables and can be found with this article online at http://dx.doi.org/10.1016/j.cmet.2013.08.004.

ACKNOWLEDGMENTS

We thank Drs. Mirco Gallie and Kim Ki Woo for providing technical advice; Massimo Attanasio, Claes Wollheim, Christian Bjørbæk, and Paolo Sassone-Corsi for critical reading of the manuscript; and Joyce Repa for providing some of the quantitative real-time PCR primers. This work was supported by

the Juvenile Diabetes Research Foundation (postdoctoral fellowship 3-2011-405 to T.F.), the American Heart Association (postdoctoral fellowship to G.R. and Scientist Development Grant to R.C.), the National Science Foundation (NSFIIS-0513376 to P.B.), and National Institutes of Health Grants (FDK092083A to E.D.B.; F32 DK078478 to L.V; R01 DK089044, R01 DK075632, P30 DK046200, and P30DK057521 to B.B.L.; RL1 DK081185 and R37 DK053301 to J.K.E.; LM010235 and NLM T15LM07443 to P.B.; and R01 DK080836 and R01 DK091680 to R.C.). This work has also received the unrestricted support of the Louis-Jeantet Foundation (to R.C.).

Received: March 15, 2013
Revised: May 31, 2013
Accepted: July 26, 2013
Published: September 3, 2013

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