Supplementary information

Leptin engages a hypothalamic neurocircuitry to permit survival in the absence of insulin

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Running title: Hypothalamic pathway enabling life without insulin

Supplementary Figure 1

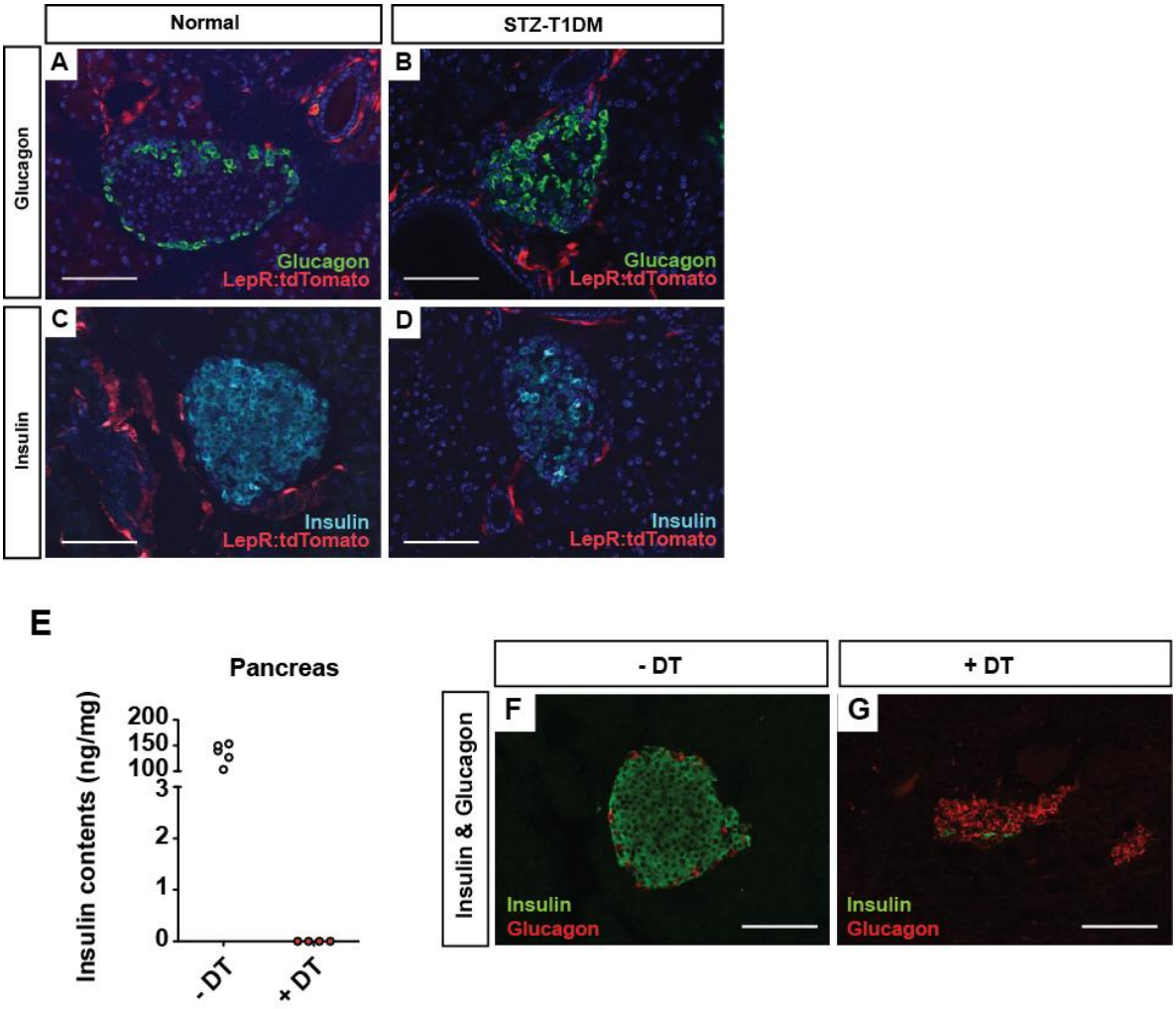

Figure S1. LEPR-B is not expressed by pancreatic insulin- or glucagon-producing cells and injections of diphtheria toxin (DT) abolishes pancreatic β-cells in *RIP-DTR* mice, Related to Figure 1. (**A** and **B**) Representative distribution of cells expressing LEPR-B (red) and glucagon (green) in the pancreas of non-diabetic (Normal) and STZ-induced insulin-deficient (STZ-T1DM) *LepR*-ires-Cre;*tdTomato*$^{flox/flox}$ mice (Scott et al., 2009). (**C** and **D**) Representative distribution of cells expressing LEPR-B (red) and insulin (sky-blue) in the pancreas of same mice as in A and B. Note that none of the LEPRs-expressing cells express glucagon or insulin. (**E**) Pancreatic insulin contents and (**F**) Representative distribution of cells expressing insulin (green) and glucagon (red) in the

pancreas of *RIP-DTR* mice that received (+DT) or did not receive (-DT) intraperitoneal (ip) DT administration. *RIP-DTR* mice were administered 3 times with DT at day 0, 3, and 5 as previously indicated (legend in Figure 1). Data shown in **E** and **F** are from mice sacrificed 10 days after the first DT (or placebo) administration. Scale bar size = 100 μm.

Supplementary Figure 2

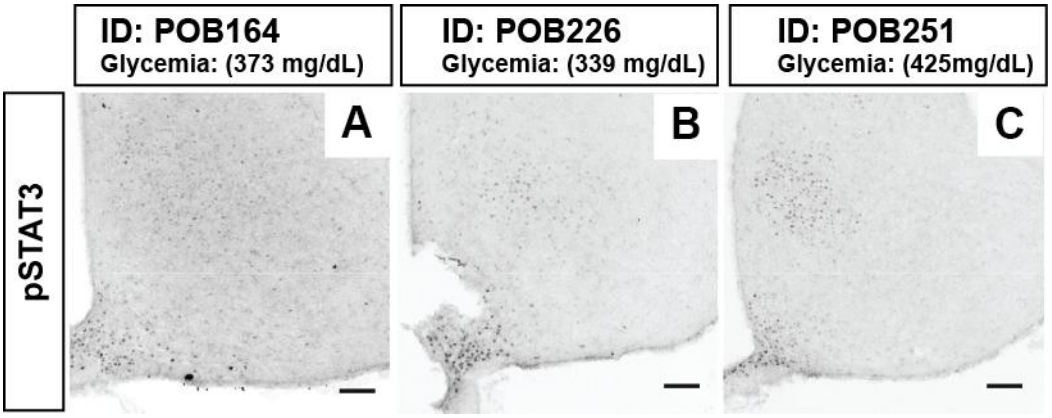

Figure S2. icv leptin administration evokes phosphorylation of STAT3 in the hypothalamus of insulin-deficient STZ-*Pomc*-Cre;*Lepr*$^{flox/flox}$ mice, Related to Figure 2. (**A**, **B** and **C**) Top-boxed text indicates the individual mouse ID number and its glucose levels in the blood 10 days after icv leptin administration. Scale bar size = 100 μ m.

Supplementary Figure 3

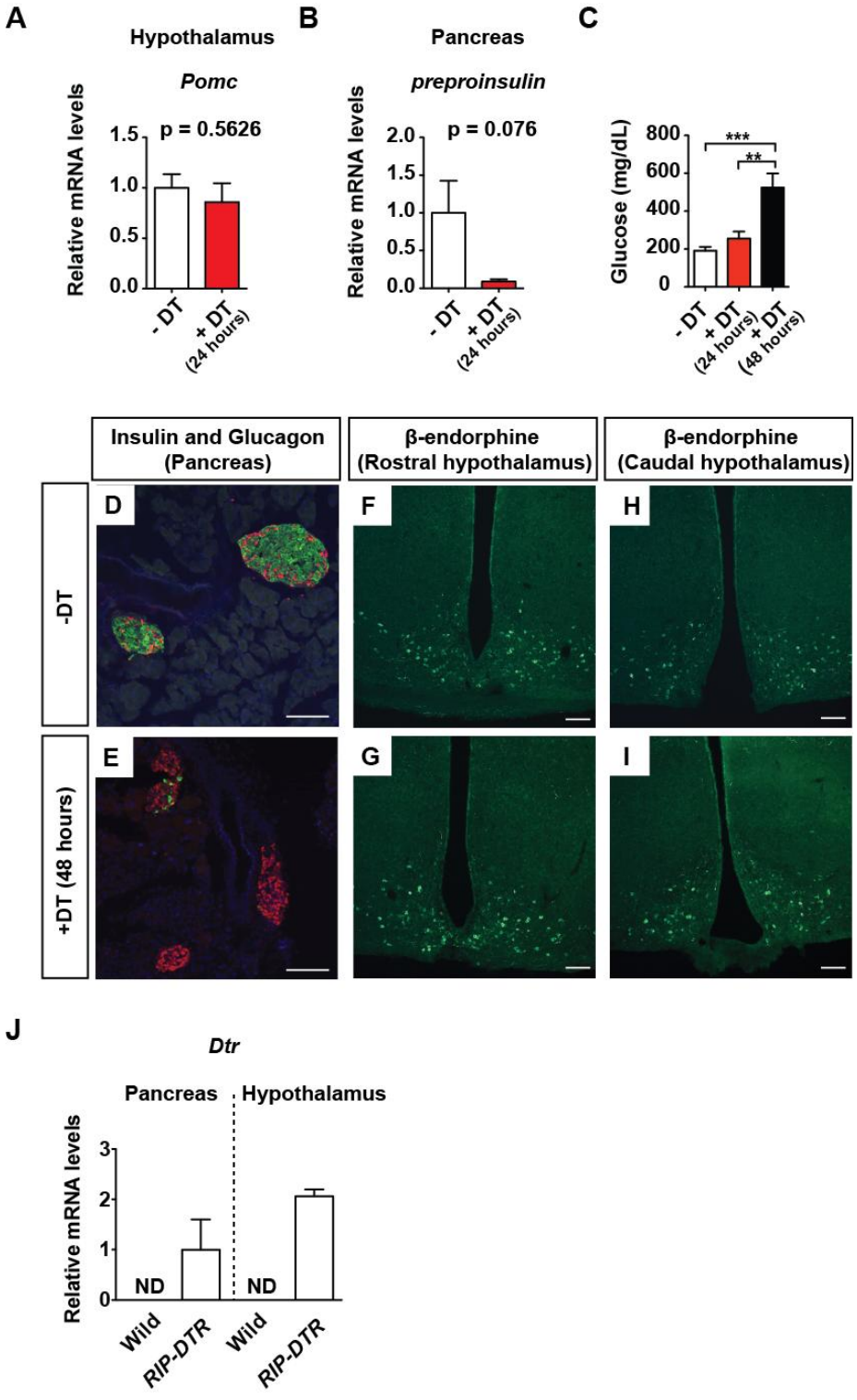

Figure S3. DT administration does not ablate POMC neurons, Related to Figure 3 (**A**) hypothalamic *Pomc* and (**B**) pancreatic *preproinsulin* mRNA expression levels and (**C**) glucose levels in the blood of *RIP-DTR* mice 24 or 48 hours after ip DT administration (time is indicated between parenthesis). (**D** and **E**) representative distribution of cells expressing pancreatic insulin (green) and glucagon (red) in *RIP-DTR* mice 48 hours after ip DT administration (+DT) or DT-untreated control mice (-DT). (**F**-**I**) Representative distribution of cells expressing β-endorphin (green) in rostral and caudal hypothalamus of +DT or –DT mice. (**J**) Pancreatic and hypothalamic *Dtr* mRNA expression levels in *RIP-DTR* and control mice. Statistical analyses were done using unpaired t-test and one-way ANOVA (Tukey's Multiple Comparison Test). Values are mean ± S.E.M. (n = 4-5). ***P<0.001, **P<0.01.

Supplementary Figure 4

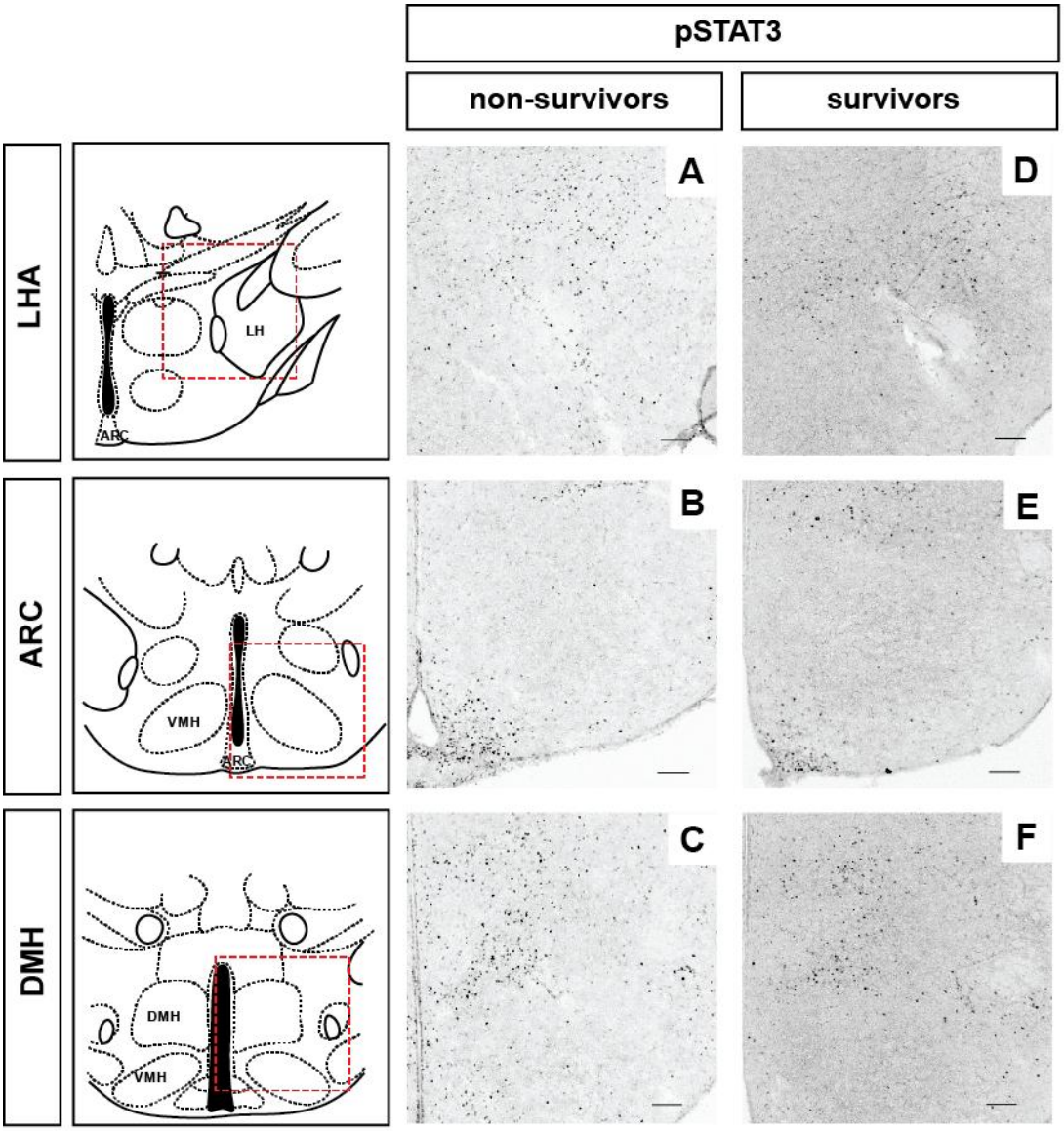

Figure S4. icv leptin administration evokes phosphorylation of STAT3 in the hypothalamus of insulin-deficient DT-*Vgat*-ires-Cre;*Lepr*$^{TB/TB}$;*RIP-DTR* mice, Related to Figure 4. Representative distribution of cells expressing phosphorylated STAT3 (p-STAT3) in the lateral hypothalamic area (LHA), hypothalamic arcuate nucleus (ARC) and dorsomedial nucleus (DMH) in (**A**-**C**) DT-VGAT-icv-leptin (non-survivors) and (**D**-**F**) DT-VGAT-icv-leptin (survivors) mice shown in Figure 4. Scale bar size = 100 µ m. ARC, arcuate nucleus; LHA, lateral hypothalamic area; VMH, ventromedial hypothalamic

nucleus; DMH, dorsomedial hypothalamic nucleus. Anatomical location of LHA, ARC and DMH is shown in red-colored, dashed-line boxed area in top panels (Franklin and Paxinos, 2008). Values are mean ± S.E.M.

Supplementary Figure 5

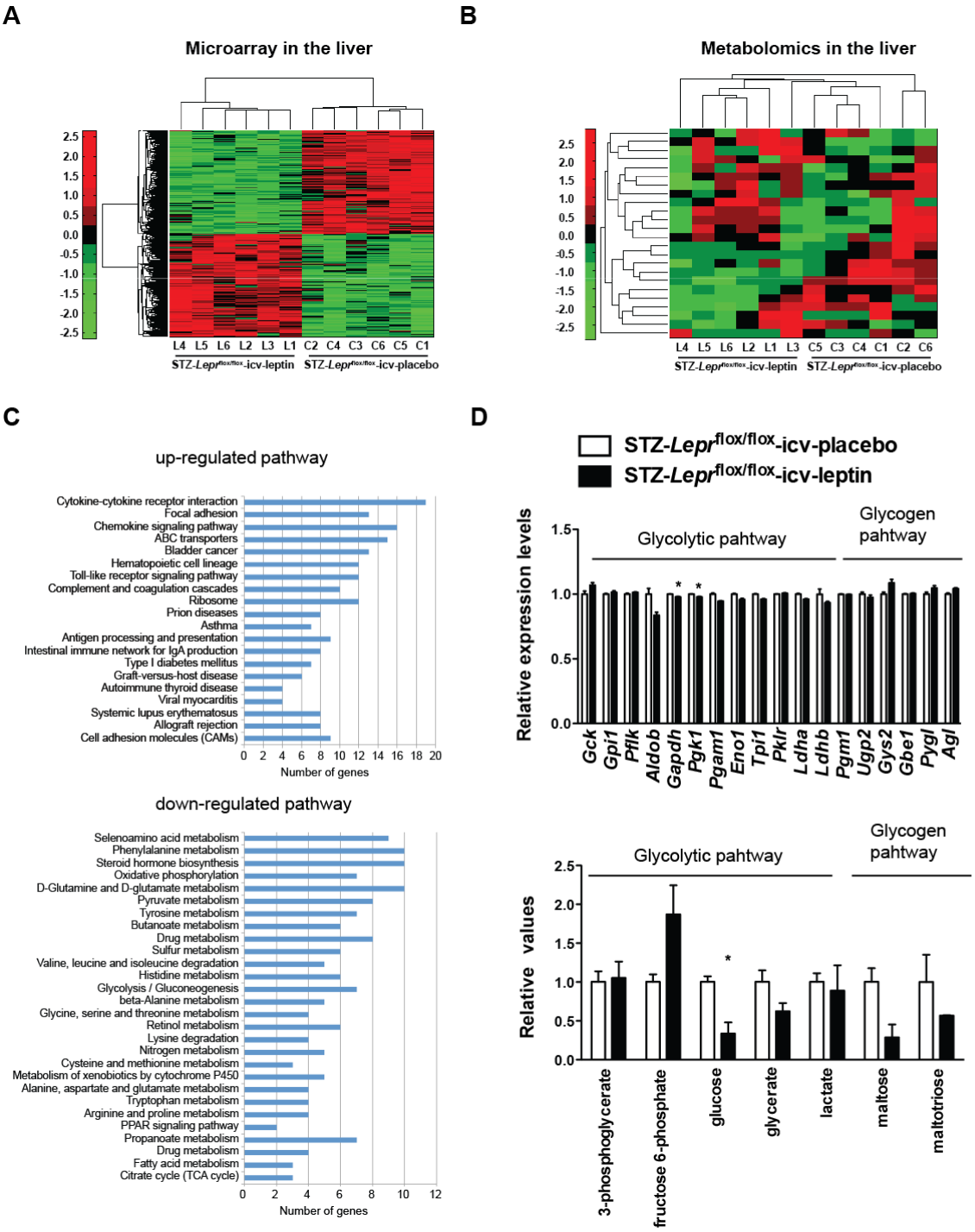

Figure S5. Microarray and Metabolomics analysis in the liver of STZ-*Lepr*$^{flox/flox}$-icv-leptin and –placebo mice, Related to Figure 6, and Table S1 and S2. (A)

Hierarchical clustering of genes that were differentially expressed (p<0.001) and (**B**) metabolites that were significantly different (p<0.01) in the liver of STZ-*Lepr*$^{flox/flox}$-icv-leptin and STZ-*Lepr*$^{flox/flox}$-icv-placebo mice. (**C**) Pathway enrichment analysis of differentially expressed genes. In the up and down regulated categories, pathways are ordered based on p-values from DAVID starting with the most significant pathway. (**D**) Glycolytic and glycogen relevant gene expression levels (upper: * p<0.001) and metabolites levels (lower: * p<0.01) in the liver of STZ-*Lepr*$^{flox/flox}$-icv-leptin and STZ-*Lepr*$^{flox/flox}$-icv-placebo mice. Values are mean ± S.E.M. *Gck*, glucokinase; *Gpi1*, glucose phosphate isomerase 1; *Pfkl*, phosphofructokinase, liver, B-type; *Aldob*, aldolase B; *Gapdh*, glyceraldehyde-3-phosphate dehydrogenase; *Pgk1*, phosphoglycerate kinase 1; *Pgam1*, phosphoglycerate mutase 1; *Eno1*, enolase 1; *Tpi1*, triosephosphate isomerase 1; *Pklr*, pyruvate kinase; *Pgm1*, phosphoglucomutase 1; *Ugp2*, UDP-glucose pyrophosphorylase 2; *Gys2*, glycogen synthase 2; *Gbe1*. glucan (1,4-alpha-), branching enzyme 1; *Pygl*, liver glycogen phosphorylase; *Agl*, amylo-1,6-glucosidase, 4-alpha-glucanotransferase.

Supplementary Table 1.

Table S1. Metabolomic analysis of liver from insulin-deficient mice treated with icv leptin, Related to Figure 6 and S5. STZ was used to induce insulin deficiency in mice as described in Experimental Procedures. Mice were sacrificed 10 days into treatment as indicated in Figure 2A and liver sampleswere collected. The number of mice was 6 in each group.

Supplementary Table 2.

Table S2. Pathway analysis of hepatic microarray data frominsulin-deficient mice treated with icv leptin, Related to Figure 6 and S5. Microarray analysis was performed from same liver samples as indicated in Table S2. Pathway analysis was performed as described in Supplementary Experimental Procedures. The number of mice was 6 in each group.

Supplementary Table 3.

Genotype	Primer ID	Sequence (5'→3')	Alleles	approximately bp
RIP-DTR	RIPDTR1	ACGGCTGCTTCATCTACAAGG	Transgenic allele	300
	RIPDTR2	TTGGTGTCCACGTAGTAGTAG	All sample	450
	PY6R	CCCTCCTCACCTTTCCCTTA		
	PY7F	CTGTCCTCAGAAAGCCTTGG		
Wild for	HPRT3	CTCCGGAAAGCAGTGAGGTAAG	Wild type allele	300
	HPRT5	GAGGGAGAAAAATGCGGA	All sample	450
	PY6R	CCCTCCTCACCTTTCCCTTA		
	PY7F	CTGTCCTCAGAAAGCCTTGG		
LeprTB	LepreacF	CAGTCTGGACCGAAGGTGTT	Transgenic allele	300
	LepreacR2	TAGGGCCAAACCCACATTTA	Wild type allele	450
	pDisRreac	CCCAAGGCCATACAAGTGTT	delta allele	550
Leprflox	mLepR-65-A	AGAATGAAAAAGTTGTTTTGGGACGA	Transgenic allele	200
	mLepR-105	TGAACAGGCTTGAGAACATGAACAC	Wild type allele	250
Leprflox for Δ	mLepR-105	TGAACAGGCTTGAGAACATGAACAC	delta allele	300
	mLepR-106	GGTGTCTGATTTGATAGATGGTCTT	All sample	600
POMC-Cre	N16	TGGCTCAATGTCCTTCCTGG	Transgenic allele	200
	AA03	GAGATATCTTTAACCCTGATC	All sample	600
	N57	CACTGTATATGACCCTTGCTCTTG		
SF1-Cre	M176	GGCAGCCTAATTAGCTGT	Transgenic allele	300
	M177	GATCTCCAGCTCCTCCTCTGTC	All sample	600
	M245	CTGAGCTGCAGCGCAGGGACAT		
	M247	TGCGAACCTCATCACTCGTTGCAT		
VGAT-Ires-Cre	vGAT-IRESSF	CTTCGTCATCGGCGGCATCTG	Transgenic allele	900
	CRE-FR	ATCGACCGGTAATGCAGGCAA	All sample	450
	PY6R	CCCTCCTCACCTTTCCCTTA		
	PY7F	CTGTCCTCAGAAAGCCTTGG		
tDTomatoflox	oIMR9020	AAGGGAGCTGCAGTGGAGTA	Transgenic allele	200
	oIMR9021	CCGAAAATCTGTGGGAAGTC	Wild type allele	300
	oIMR9103	GGCATTAAAGCAGCGTATCC		
	oIMR9105	CTGTTCCTGTACGGCATGG		
Lepr-Ires-Cre	M176	GGCAGCCTAATTAGCTGT	Transgenic allele	300
	M177	GATCTCCAGCTCCTCCTCTGTC	All sample	600
	M262	GCCCTGGAAGGGATTTTTGAAGCA		
	M263	ATGGCTAATCGCCATCTTCCAGCA		

Table S3. Sequences of primers for genotyping, Related to Supplemental Experimental Procedure. Tail DNA was extracted and PCR was performed by commercial available kit. A detailed method of generation of mice was described in Supplemental Experimental Procedures.

Supplemental Experimental Procedure

Generation of genetically-modified mice

RIP-DTR, *Pomc*-Cre, *Sf1*-Cre, *Lepr*^{flox}, *Vgat*-ires-Cre, *Lepr*^{TB}, *LepR*-ires-Cre, β-less transgenic mouse lines were generated as previously described (Bachman et al., 2002; Balthasar et al., 2004; Dhillon et al., 2006; Scott et al., 2009; Thorel et al., 2010; Tong et al., 2008; Vong et al., 2011). A *tdTomato*^{flox} mouse line (B6;129S6-*Gt(ROSA)26Sor*$^{tm14(CAG-tdTomato)Hze}$/J; Stock# 007908) and FVB/NJ (Stock# 001800) mice were purchased from Jackson Laboratory. All mice were housed with food (standard chow diet) and water available ad libitum in a light-controlled (12-h light/dark cycle: light cycle was from 6:00 am to 6:00 pm) and temperature-controlled (23 °C) 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 at Dallas. *Pomc*-Cre;*Lepr*$^{flox/flox}$ (Balthasar et al., 2004), *Sf1*-Cre;*Lepr*$^{flox/flox}$ (Dhillon et al., 2006), and *LepR*-ires-Cre*;tdTomato*$^{flox/flox}$ (Scott et al., 2009) were generated as previously described. To generate *Pomc*-Cre*;Lepr*$^{flox/flox}$;*RIP-DTR* mice, *Pomc*-Cre;*Lepr*$^{flox/flox}$ male mice were mated with *Lepr*$^{flox/flox}$;homozygous *RIP-DTR* (Thorel et al., 2010) female mice. *Pomc*-Cre (Balthasar et al., 2004) and *Vgat*-ires-Cre (Vong et al., 2011) mice were mated with mice carrying a *lox*P-flanked transcriptional blocking sequences in between exons 16 and 17 of the LEPR gene (*Lepr*$^{TB/+}$) as previously described (Berglund et al., 2012). To generate *Pomc*-Cre*;Lepr*$^{TB/TB}$;*RIP-DTR*, *Vgat*-ires-Cre*;Lepr*$^{TB/TB}$;*RIP-DTR* and *Vgat*-ires-Cre*;Pomc*-Cre *Lepr*$^{TB/TB}$;*RIP-DTR*, *Pomc*-Cre;*Lepr*$^{TB/+}$, *Vgat*-ires-Cre;*Lepr*$^{TB/+}$ and *Vgat*-ires-Cre;*Pomc*-Cre*; Lepr*$^{TB/+}$ male mice were mated with *Lepr*$^{TB/+}$;homozygous *RIP-DTR* female mice. Our Cre lines can sporadically lead to ectopic expression of Cre-recombinase. Thus, tail DNA samples were assessed by PCR for the presence of Cre-mediated recombination of loxP-flanked alleles. All mice positive for this test were excluded from our studies.

β-adrenergic receptor blocker administration

Timolol maleate salt (Sigma) was dissolved in sterile 0.9% saline solution. Timolol (1 mg/kg B.W.) and control (0.9% saline) solution were ip administered 2 times (8:00 am and 6:00 pm) per day as described in Figure 6B.

Assessment of energy substrates and hormones levels

Tail vein blood was collected between 2 and 4 PM from mice that were fed *ad libitum* but did not have access to food for the 2 hours prior to the blood collection procedure. Daily glycemia was measured by commercially available glucometer. Plasma samples (using EDTA) were collected after centrifugation (3000 g × 20 min) and stored at −80ºC. To prevent degradation of glucagon, 0.1 M p-hydroxymercuribenzoate was added into plasma sample (1:100) immediately after sample collection. Insulin was measured using ELISA kits (Crystal Chem. Inc, IL, USA). Glucagon was measured by Vanderbilt hormone assay Core. Pancreatic insulin level was determined as previously described (Fujikawa et al., 2010). Hepatic glycogen contents were measured as described before (Miyaki et al., 2011).

Assessment of mRNA

Mice were sacrificed two hours before the onset of the dark cycle (i.e.: 4 PM) and hypothalamus was quickly removed, frozen in liquid nitrogen and subsequently stored at –80ºC. Pancreas was quickly removed and kept in RNAlater (Qiagen, CA, USA) for 24 hours at room temperature, then stored at –80ºC. Hypothalamic RNAs were extracted using Trizol reagent (Invitrogen) and pancreatic RNAs were extracted by Qiagen mRNA extract kits (RNeasy plus). Complementary DNA was generated by Superscript II (Invitrogen) and used with SYBR Green PCR master mix (Applied Biosystem, CA, USA) for quantitative real time PCR analysis. Sequences of deoxy-oligonucleotides primers are outlined here; *Pomc* (5'gaggccactgaacatctttgtc and 5'gcagaggcaaacaagattgg), *preproinsulin* (5'ggggagcgtggcttcttcta and 5'ggggacagaattcagtggca), *Dtr* (5'gggctagggaagaagaggga and 5'agcgattttccactgggagg) and *36B4* (5'cactggtctaggacccgagaag and 5'ggtgcctctgaagattttcg).

Assessment of protein content

Liver samples from mice were weighed and put into tubes that contain 1.4 mm ceramic spheres (Lysing Matrix D; MP biomedical, CA, USA). Lysis buffer was composed of RIPA buffer (Sigma), 1% (v/v) of protease inhibitors cocktail (P8340-5ML, Sigma), phosphatase inhibitors cocktail 2 and 3 (P5726-5ML and P0044-5ML from Sigma). Lysis buffer was added into each sample at 10 times volume (μL) of tissue weight (mg). Samples were homogenized for 30 seconds x 6,000 rpm. After homogenization, samples were kept on the ice for 1 hour with occasional shakings. Samples were centrifuged for 20 minutes x 12,000 *g* at 4ºC. Supernatant was carefully extracted and protein concentration was measured. An equal volume of laemmle sample buffer (2X concentrated; 4% SDS, 10% 2-mercaptoethanol, 20% glycerol, 0.004% bromophenol blue and 0.125 M pH6.8 Tris-HCl) was added into extracted supernatant and boiled for 5 minutes at 95ºC. These boiled samples were kept at –20ºC until experiment. The samples (15 μg of protein) were resolved by SDS-PAGE (10% EZ-run, Fisher Scientific, PA, USA) and finally transferred to a nitrocellulose membrane by electroblotting (Trans-blot turbo; Bio-Rad, CA, USA). The following primary antibodies were used for Western blot assays; rabbit polyclonal antisera against PEPCK (Cayman Chemical Co., MI, USA), rabbit monoclonal antisera against cAMP response element binding protein (CREB; 1:1,000; Cell signaling, MA, USA), rabbit monoclonal antisera against Ser^{133}-phosphorylated CREB (pCREB; 1:1,000; Cell signaling). Mouse polyclonal antisera against β-actin was used as loading control (1:5000; Abcam, MA, USA). The following secondary antibodies were used: IRDye 680 Goat anti-Mouse IgG (1:5,000; Li-Cor Bioscience, NE, USA) and IRDye 800CW Goat anti-Rabbit IgG (1:5,000; Li-Cor Bioscience). Restore Plus Western Blot Stripping Buffer (Fisher Scientific) was used to remove bound antibodies from membranes. All primary antibodies were diluted by ODYSSEY blocking buffer (Li-Cor Bioscience) contains 0.1 % of Tween 20 (Sigma) and 0.01% of SDS (Life technologies, NY, USA). All secondary antibodies were diluted by ODYSSEY blocking buffer contains 0.01% of SDS (Life technologies). The ODYSSEY infrared Imaging System (Li-Cor Bioscience) was used to detect signals and obtained signals were analyzed by the ODYSSEY software according to the manufacturer manuals.

Immunohistochemistry

Mice were deeply anesthetized with an ip injection of chloral hydrate and perfused as previously described (Scott et al., 2009). Phosphorylation of STAT3 in the brain was determined as previously described (Scott et al., 2009). Figurers of anatomical location were generated using digital data from "The Mouse Brain in Stereotaxic Coordinates" (Franklin and Paxinos, 2008). Pancreatic glucagon and insulin distribution was determined as previously described (Fujikawa et al., 2010).

Microarray and Metabolomics

Mice were sacrificed as described above and the liver was quickly removed, frozen in liquid nitrogen and subsequently stored at –80ºC. Hepatic RNAs were extracted by Qiagen mRNA extract kits (RNeasy plus). Mircoarray was performed by UTSW microarray Core facility (http://microarray.swmed.edu/) using illumina Chip Mouse WG-6 v2.0 (illumina, CA, USA). A measurement of metabolites was performed by Metabolon Inc (NC, USA). The differential analysis of the microarray and metabolomics data were performed using CyberT (Baldi and Long, 2001; Kayala and Baldi, 2012). Briefly, the average signal intensity was transformed using a log base 2 normalization and a regularized t-test was then performed using Cyber-T (http://cybert.ics.uci.edu/) with a window size of 51 and a Bayesian confidence value of 5. A p-value of 0.001 and 0.01 were considered significant for microarray and metabolomics analysis, respectively. Pathway and functional enrichment was carried out using DAVID (Huang da et al., 2009).

Supplementary References

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