Corrections

MEDICAL SCIENCES
Correction for “Regulation of bone remodeling by vasopressin explains the bone loss in hyponatremia,” by Roberto Tamma, Li Sun, Concetta Cuscuto, Ping Lu, Michelangelo Corcelli, Jianhua Li, Graziana Colaianni, Surinder S. Moonga, Adriana Di Benedetto, Maria Grano, Silvia Colucci, Tony Yuen, Maria I. New, Alberta Zallone, and Mone Zaidi, which appeared in issue 46, November 12, 2013, of Proc Natl Acad Sci USA (110:18644-18649; first published October 28, 2013; 10.1073/pnas.1318257110).

The authors note that Fig. 1 appeared incorrectly. The corrected figure and its legend appear below.

Fig. 1. Bone cells express Avprs. Immunofluorescence micrographs (A) and Western immunoblotting (B) show the expression of Avpr1α in osteoblasts and osteoclasts, and as a function of osteoblast (mineralization) and osteoclast (with Rankl) differentiation. The expression of Avp (ligand) and Avpr1α (receptor) in osteoblasts is regulated by 17β-estradiol, as determined by quantitative PCR (C) and Western immunoblotting (D). (Magnification: A, 63x.) Because Avp is a small peptide, its precursor neurophysin II is measured. Statistics: Student t test, P values shown compared with 0 h. Stimulation of Erk phosphorylation (p-Erk) as a function of total Erk (t-Erk) by Avp (10⁻⁸ M) in osteoclast precursors (preosteoclasts), osteoclasts (OC), and osteoblasts establishes functionality of the Avpr1α in the presence or absence of the receptor inhibitor SR49059 (10⁻⁸ M) (E). Western immunoblotting showing the expression of Avpr2 in preosteoclasts, OCs (F), and osteoblasts (G) isolated from Avpr1α⁻/⁻ mice, as well as in MC3T3.E1 osteoblast precursors (G). Functionality of Avpr2 was confirmed by the demonstration that cells from Avpr1α⁻/⁻ mice remained responsive to AVP in reducing the expression of osteoblast differentiation genes, namely Runx2, Osx, Bsp, Atf4, Opn, and Osteocalcin (quantitative PCR, P values shown) (H). Only relevant bands from Western blots are shown, with gaps introduced where empty lanes are excised to conserve space.

www.pnas.org/cgi/doi/10.1073/pnas.1415306111
PHARMACOLOGY

The authors note that an additional affiliation should be listed for Torsten Christ and Alberto Kaumann. The new affiliation should appear as Department of Experimental Pharmacology and Toxicology, University Medical Center Hamburg-Eppendorf. The corrected author and affiliation lines appear below. The online version has been corrected.

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www.pnas.org/cgi/doi/10.1073/pnas.1416216111

PHYSIOLOGY

The authors note that the author name Timo Myöhännen should instead appear as Timo Myöhänen. The corrected author line appears below. The online version has been corrected.

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www.pnas.org/cgi/doi/10.1073/pnas.1416210111
Hypothalamic prolyl endopeptidase (PREP) regulates pancreatic insulin and glucagon secretion in mice

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Prolyl endopeptidase (PREP) has been implicated in neuronal functions. Here we report that hypothalamic PREP is predominantly expressed in the ventromedial nucleus (VMH), where it regulates glucose-induced neuronal activation. PREP knockdown mice (Prepgt/gt) exhibited glucose intolerance, decreased fasting insulin, increased fasting glucagon levels, and reduced glucose-induced insulin secretion compared with wild-type controls. Consistent with this, central infusion of a specific PREP inhibitor, S17092, impaired glucose tolerance and decreased insulin levels in wild-type mice. Arguing further for a central mode of action of PREP, isolated pancreatic islets showed no difference in glucose-induced insulin release between Prepgt/gt and wild-type mice. Furthermore, hyperinsulinemic euglycemic clamp studies showed no difference between Prepgt/gt and wild-type control mice. Central PREP regulation of insulin and glucagon secretion appears to be mediated by the autonomic nervous system because Prepgt/gt mice have elevated sympathetic outflow and norepinephrine levels in the pancreas, and propranolol treatment reversed glucose intolerance in these mice. Finally, re-expression of PREP by bilateral VMH injection of adeno-associated virus–PREP reversed the glucose-intolerant phenotype of the Prepgt/gt mice. Taken together, our results unmask a previously unknown player in central regulation of glucose metabolism and pancreatic function.

Prolyl endopeptidase (PREP; EC 3.4.21.26) is a highly conserved enzyme (1). In humans and rodents it is highly expressed in the brain (2), including the cortex, striatum, hypothalamus, hippocampus, and amygdala (3–6). The physiological role of PREP remains elusive (7). Many studies have focused on the putative effect of PREP on neuropeptide levels because this enzyme could function to cleave virtually all neuropeptides shorter than 30 amino acids that contain an internal proline residue (8).

However, much of our understanding of this enzyme is based on in vitro data. Because PREP’s putative targets regulate a large number of signaling pathways, PREP has the capacity to regulate a variety of cellular tasks.

To gain a better understanding of the role of PREP in the hypothalamus, we analyzed the effect of PREP knockdown on hypothalamic mechanisms including glucose and energy metabolism.

Results

Hypothalamic PREP Expression. In the central nervous system, Prep mRNA was found in the hypothalamus (Fig. L1), hippocampus, and cortex (5, 6). Within the hypothalamus, higher expression of Prep mRNA was detected in the ventromedial nucleus (VMH) (Fig. L1 and Fig. S1A). In mice in which PREP was knocked down by the gene trap (Prepgt/gt mice) (9), β-gal expression showed an expression pattern similar to that seen by the in situ hybridization (Fig. LB and Fig. S1B). Prep mRNA and protein expression were also observed in peripheral organs (Fig. S1C and D). A significant decrease of PREP protein levels was found in all examined tissues of Prepgt/gt mice, including the VMH (Fig. S1E).

Analysis of the mRNA expression levels of two other serine proteases, dipetidyl peptidase-4 and alyclamid acid-releas- ing enzyme, in the hypothalamus and peripheral organs such as liver, brown adipose tissue (BAT), and pancreas showed no compensatory changes in their mRNA levels in Prepgt/gt mice compared with their WT controls (Fig. S2, Upper and Lower).

Metabolic Characterization of Prepgt/gt Mice. Prep gene-trap (Prepgt/gt) mice fed on a standard chow diet showed no difference in body weights and body composition compared with their WT controls (Fig. S3 A and B). Further analysis of food and water intake did not reveal any difference. (Fig. S3 C and D).

Impaired Glucose Metabolism in Prepgt/gt Mice. The VMH is considered an important center for glucose regulation (for review see ref. 10). Prepgt/gt mice had increased fasting blood glucose levels compared with their WT controls (time 0 in Fig. 1C). A glucose tolerance test (GTT) showed that Prepgt/gt mice had a marked increase in blood glucose levels compared with WTs (Fig. 1C). During GTT, significant lower insulin levels were found in Prepgt/gt mice compared with WT controls (Fig. 1D). On the other hand, fasting glucagon levels were elevated in Prepgt/gt mice compared with their WT controls (Fig. 1E). A pyruvate tolerance test (PTT) showed that Prepgt/gt mice had an enhanced glucose production (Fig. 1F) that was associated with increased mRNA expression of liver enzymes involved in gluconeogenesis.

Significance

The ventromedial nucleus of the hypothalamus (VMH) plays an important role in the regulation of glucose metabolism. Here we show that prolyl endopeptidase (PREP), a serine protease, is expressed in the VMH where it functions to regulate glucose-induced insulin secretion. Experimental knockdown of central PREP induced impairment of VMH glucose sensing, resulting in reduced insulin and increased glucagon secretion by the pancreas via altered sympathetic outflow. Our data reveal PREP as a new hypothalamic player in the control of glucose homeostasis.


The authors declare no conflict of interest.

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This article contains supporting information online at www.pnas.org/lookup/suppl/doi:10.1073/pnas.1406000111/-/DCSupplemental.

www.pnas.org/cgi/doi/10.1073/pnas.1406000111
such as glucose-6-phosphatase (G6Pase) and phosphoenolpyruvate carboxykinase (Pck1) (Fig. 1 G and H).

Glucose Responsiveness of VMH Neurons Is Impaired in Prep<sup>gt/gt</sup> Mice. To determine the effect of glucose on VMH neuronal activation, we assessed the effect of peripheral glucose administration on c-fos staining in WT and Prep<sup>gt/gt</sup> mice (Fig. 2 A–C). Glucose administration to WT mice induced a significant increase of c-fos immunolabeling in the VMH compared with baseline WT saline-treated mice (Fig. 2 A and C; 210.00 ± 17.41%). However, glucose injection of Prep<sup>gt/gt</sup> mice induced a significantly smaller increase in c-fos immunoreactivity (Fig. 2 B and C; 138.80 ± 5.13%; P < 0.05), suggesting that Prep<sup>gt/gt</sup> mice have impaired glucose sensitivity in the VMH. No difference in c-fos staining was found in the hypothalamic arcuate (Fig. S4A) and dorsomedial nuclei (Fig. S4B).

Decreased Glucose-Induced Insulin Receptor Phosphorylation in the Hypothalamus of Prep<sup>gt/gt</sup> Mice. Because reduction in the activation of insulin receptors in the VMH induced glucose intolerance in the absence of weight gain (11), we analyzed insulin receptor (IR) phosphorylation levels in fed and fasted states and 5 min after the systemic saline or glucose administration. In WT mice, feeding induced a significant increase in the phosphorylated-insulin receptor (pIR)/insulin receptor (IR) ratio in the VMH compared to fasting (Fig. 2D). Although in Prep<sup>gt/gt</sup> mice feeding also induced a significant increase of the pIR/IR ratio in the VMH, this increase was significantly lower than that of WT mice (Fig. 2D). Similarly, 5-min glucose-treated fasted WT and Prep<sup>gt/gt</sup> mice showed significant increases in the pIR/IR ratio of the VMH compared with their saline-treated fasted control (Fig. 2E). However, the increase in the Prep<sup>gt/gt</sup> mice was significantly lower than that of WT (Fig. 2E), suggesting impairment in

Fig. 1. (A and B) VMH PREP and glucose metabolism. Light microscopic photograph of representative coronal sections after in situ hybridization (A) or LacZ staining (B). (C) Results from the GTT in Prep<sup>gt/gt</sup> and WT. The area AUC analysis showed a significant glucose intolerance in Prep<sup>gt/gt</sup> mice compared with the WT controls (n = 10 for each group). (D) Circulating insulin levels in WT (n = 6) and Prep<sup>gt/gt</sup> mice (n = 4) during the GTT. (E) Glucagon levels during GTT (WT: n = 6; Prep<sup>gt/gt</sup>, n = 4). (F) The results of PTT in Prep<sup>gt/gt</sup> and WT mice (n = 6/group). (6 and H) Increased G6pase and Pepck mRNA expression in the Prep<sup>gt/gt</sup> mice (n = 6) compared with their WT controls (n = 6). All data represent the mean ± SEM. *P < 0.05; **P < 0.01; ***P < 0.001. [Scale bar in A (also applies to B), 2 mm.] ARC, arcuate nucleus; LH, lateral hypothalamus; 3v, third ventricle; VMH, ventromedial nucleus.

Fig. 2. PREP effect on glucose-induced VMH neuronal and insulin receptor activation. (A and B) Representative microphotographs showing c-fos immunolabeling in the hypothalami of a WT (A) and a Prep<sup>gt/gt</sup> mouse (B) injected with glucose. (C) Results of the quantification of c-fos immunolabeling in the medial VMH of WT and Prep<sup>gt/gt</sup> mice injected with glucose (n = 3 per group). Data are expressed as percentage of WT mice treated with saline. (D and F) Western blot images and graphs showing the analysis of phosphorylated and total insulin receptor (pIR and IR, respectively) and β-actin in the VMH of fed and fasted WT and Prep<sup>gt/gt</sup> mice (A: n = 3/group) and in the VMH of fasted WT and Prep<sup>gt/gt</sup> mice 5 min after either saline or glucose administration (B: n = 3/group). (F) pIR/IR ratio in the hypothalamus of fed Prep<sup>gt/gt</sup> mice (n = 3) compared with fed WT controls after saline or insulin administration (n = 3–4/group). All data represent the mean ± SEM. *P < 0.05; **P < 0.01; ***P < 0.001. ns, not statistically significant. [Scale bar in B (also applies to A), 100 μm.]
glucose-induced phosphorylation of insulin receptors. Although no difference in pIR/IR ratio was found in the VMH of fasted WT compared with fasted Prep<sup>gt/gt</sup> (Fig. 2 D and E), in the fed state Prep<sup>gt/gt</sup> mice showed a significant lower pIR/IR ratio in the hypothalamus compared with fast WT controls (P < 0.01; Fig. 2F). Glucose administration to fed WT mice significantly increased the pIR/IR ratio (Fig. 2F), which was absent in fed Prep<sup>gt/gt</sup> mice (Fig. 2G). In contrast to glucose, systemic insulin injection efficiently induced the phosphorylation of IR in fed Prep<sup>gt/gt</sup> mice (Fig. 2G) to a level that was not significantly different from that of fasted WT controls (Fig. 2G).

**Prep<sup>gt/gt</sup> Mice Have Normal Glucose Uptake.** An insulin tolerance test (ITT) showed similar insulin sensitivity between WT and Prep<sup>gt/gt</sup> mice (Fig. 3A). Consistent with this, during the hyperinsulinemic-euglycemic clamps (Fig. 3B; circulating insulin levels: 1.87 ± 0.28 ng/mL in WT and 1.72 ± 1.18 ng/mL in Prep<sup>gt/gt</sup> mice; P = 0.68), Prep<sup>gt/gt</sup> mice required similar exogenous glucose infusions to maintain euglycemia (24.44 ± 1.82 mg/kg/min in WT mice vs. 20.83 ± 3.31 mg/kg/min in Prep<sup>gt/gt</sup> mice; P = 0.32; Fig. 3C). Furthermore, whole-body glucose uptake was not significantly different between WT and Prep<sup>gt/gt</sup> mice (P = 0.81; Fig. 3D), indicating a normal insulin sensitivity of Prep<sup>gt/gt</sup> mice. Baseline hepatic glucose production was significantly increased in Prep<sup>gt/gt</sup> mice compared with WT controls (Fig. 3E). However, during the clamp, hepatic glucose production significantly decreased in Prep<sup>gt/gt</sup> mice, reaching a level no significantly different from that of WT controls (Fig. 3E), showing that the hepatic insulin resistance seen at baseline condition disappeared after insulin infusion in Prep<sup>gt/gt</sup> mice. Finally, plasma glucagon levels during clamp were measured to assess whether differences in glucagon secretion could have an indirect impact on peripheral insulin sensitivity. No differences in glucagon levels were observed between WT controls and Prep<sup>gt/gt</sup> mice (24.00 ± 1.47 pg/mL; n = 5 WT and 26.40 ± 4.16 pg/mL; n = 3 Prep<sup>gt/gt</sup> mice; P = 0.53), suggesting that insulin infusion strongly suppressed glucagon secretion in both experimental groups.

**Intact Pancreatic Islet Function in Prep<sup>gt/gt</sup> Mice.** Because PREP is also expressed in the pancreas (Fig. S1 A and B), we next assessed islets morphology and function (Fig. 3 F–I). No differences in islet staining for either insulin or glucagon were observed (Fig. 3F). Analysis of the density of the islets (Fig. 3G; P = 0.54) and the ratio of the area of the islets to the area of the pancreas (Fig. 3H; P = 0.105) showed no difference between the two groups. We then assessed glucose-stimulated insulin secretion (GSIS) in isolated pancreatic islets. No difference in GSIS was observed between WT and Prep<sup>gt/gt</sup> mice (Fig. 3I). These results indicate that Prep<sup>gt/gt</sup> mice have functionally intact pancreatic islets.

**Enhanced Uncoupling Protein 1 Expression in Prep<sup>gt/gt</sup> Mice.** The secretory activity of pancreatic islet cells is in part controlled by the autonomic nervous system (ANS) (12). Thus, we analyzed Enhanced Uncoupling Protein 1 (UCP1) mRNA expression in the BAT of WT and Prep<sup>gt/gt</sup> mice (13, 14). A significant increase of UCP1 mRNA levels was observed in Prep<sup>gt/gt</sup> mice (P = 0.009; Fig. 4A), whereas no differences in circulating free T4 (2.15 ± 0.11 ng/dL in n = 14 WT and 2.14 ± 0.12 ng/dL in n = 11 Prep<sup>gt/gt</sup> mice) and free T3 levels (3.52 ± 0.34 ng/dL in n = 12 WT and 4.34 ± 0.38 ng/dL in n = 11 Prep<sup>gt/gt</sup>) were observed, suggesting that the sympathetic tone is increased in Prep<sup>gt/gt</sup> mice compared with WT mice. In support of this, when norepinephrine levels (NE) were assessed (Fig. 3I), no differences were observed between WT controls and Prep<sup>gt/gt</sup> mice (13, 14). A significant increase of circulating glucagon secretion in both experimental groups.

**Effect of Central Infusion of S17092, a PREP Inhibitor, on GTT.** To determine the role of central PREP on glucose metabolism, next we infused i.c.v. a specific PREP inhibitor, S17092 (15), in WT mice and performed a GTT. Thirty minutes of infusion of S17092 in WT mice significantly increased glucose levels (Fig. 4E). Although WT mice treated with S17092 had a slight increase of the area under the curve (AUC) (Fig. 4E) compared with WT treated with saline (Fig. 4E), this difference was not significant. Analysis of circulating insulin levels during the GTT showed that infusion of S17092 significantly lowered insulin levels in WT mice (Fig. 4F).
Re-expression of PREP in the VMH of Prep\(^{-/-}\) Mice. To assess whether re-expression of PREP in the VMH could rescue glucose intolerance and pancreatic function in Prep\(^{-/-}\) mice, we cloned a cDNA encoding mouse prolyl endopeptidase into aden-associated virus (AAV) expression vector carrying E1a promoter and bilaterally injected in the VMH of WT and Prep\(^{-/-}\) mice (Fig. S5 A–C). An AAV vector carrying EGFP was used as control (Fig. S5 A–C). Two weeks after the intracranial injections, fasting glucose levels (time 0) in Prep\(^{-/-}\) mice infected with AAV–PREP (110.5 ± 4.25 mg/dL; Fig. 4G) were significantly lower than those of Prep\(^{-/-}\) mice (140.5 ± 5.42 mg/dL; Fig. 4G). No significant differences in fasting glucose levels were found between WT mice infected with AAV–EGFP and Prep\(^{-/-}\) mice infected with AAV–PREP (Fig. 4G). Furthermore, Prep\(^{-/-}\) mice infected with AAV–PREP showed significantly lower glucose levels compared with controls (Fig. 4G). Analysis of the AUC showed that both WT and Prep\(^{-/-}\) mice infected with AAV–PREP had a significantly lower AUC compared with either WT or Prep\(^{-/-}\) controls (Fig. 4H). Circulating insulin level measurements showed a significant increase in insulin levels in treated Prep\(^{-/-}\) mice compared with controls (Fig. 4I). Furthermore, WT–AAV–PREP showed significantly higher insulin levels at time 15 compared with WT–AAV–EGFP and Prep\(^{-/-}\)–AAV–PREP (Fig. 4I). Glucagon levels at time 0 were significantly higher in Prep\(^{-/-}\) mice infected with AAV–EGFP compared with all other groups (Fig. 4J).

Discussion
The present study reveals an unexpected role of prolyl endopeptidase in glucose homeostasis. We have shown that Prep is expressed in the VMH. Prep knockdown mice (Prep\(^{-/-}\)) showed a significant reduction of glucose-induced neuronal activation in the VMH together with glucose intolerance but normal insulin sensitivity. Prep\(^{-/-}\) mice showed reduced fasting insulin and increased fasting glucagon levels together with an impaired in vivo glucose-induced insulin secretion. Analyses of islets morphology and insulin secretion revealed no significant differences between Prep\(^{-/-}\) and WT mice, and in vitro islet function was similar between the two groups, indicating that the reduced pancreatic PREP expression in Prep\(^{-/-}\) mice may not be the cause of the altered in vivo pancreatic function. Prep\(^{-/-}\) mice showed increased sympathetic tone, and treatment of Prep\(^{-/-}\) mice with a sympathetic nervous system antagonist significantly improved their glucose intolerance to a level similar to that of WT controls. In addition, WT mice centrally infused with a specific PREP inhibitor, S17092 (15), showed reduced glucose levels compared with vehicle infused mice, with a concomitant decrease in circulating insulin levels, suggesting that central PREP may play a role in the regulation of glucose metabolism and pancreatic secretion. In support of this, re-expression of PREP in the VMH of Prep\(^{-/-}\) mice normalized glucose, insulin, and glucagon levels.

Although reported for the first time in 1971 (16), a physiological role of PREP as a cleaving enzyme until now remained elusive (17). Previous in vitro studies have indicated that PREP functions to inactivate short peptides with internal Pro-Xaa bond (8). However, several in vivo studies could not confirm these findings (8). Other putative functions have been hypothesized (18–21). Due to the lack of any transmembrane region or a lipid anchor domain (1) in PREP’s sequence, these functions include participation in the inositol phosphate signaling (18, 19), protein interaction (21), protein secretion, and/or axonal transport (20) and protein–protein interaction (21).

Our study unmasked a functional role for PREP in glucose metabolism in vivo. PREP expression in the VMH together with a decreased glucose-induced neuronal activation in the VMH, an impaired glucose tolerance, and glucose-induced pancreatic secretion in Prep\(^{-/-}\) mice suggest a novel function of PREP in the hypothalamic regulation of glucose and pancreatic functions. Our data show that PREP affects circulating insulin and glucagon levels but not their sensitivity in peripheral tissues. In support of this, although at baseline condition Prep\(^{-/-}\) mice showed significantly greater hepatic glucose production (HGP),
hyperinsulinemic significantly suppressed HGP in Prep<sup>gt/gt</sup> mice, indicating that hepatic insulin sensitivity is not affected in these mice. Because both fasting insulin and glucagon levels and glucose-induced insulin secretion were affected in Prep<sup>gt/gt</sup> mice, we investigated pancreas morphology and function. Histological analysis of the pancreas revealed no differences between Prep<sup>gt/gt</sup> and WT mice. Moreover, perfusion experiments of isolated pancreatic islets showed no difference in GSIS. These data were further supported by the experiment in which acute central inhibition of PREP activity increases circulating glucose and decreases insulin levels. Finally, the effect of PREP re-expression selectively in the VMH of Prep<sup>gt/gt</sup> mice argues that central PREP action plays an important role in glucose-induced insulin secretion.

The mechanism by which hypothalamic PREP controls glucose-induced insulin secretion may involve the ANS. Indeed, we found that the sympathetic tone of Prep<sup>gt/gt</sup> mice was increased compared with their WT controls as shown by the increased norepinephrine levels in the pancreas of fasted Prep<sup>gt/gt</sup> mice compared with fasted WT controls. Furthermore, when Prep<sup>gt/gt</sup> mice were treated with a β-adrenergic receptor antagonist, propranolol, at a dose that did not affect the GTT of WT mice, a significant improvement in glucose tolerance was observed in Prep<sup>gt/gt</sup> mice, showing now a glycemic profile similar to that of WT controls. The VMH has been shown to directly project to a number of autonomic centers in the brainstem regions, including the rostral ventrolateral medulla, a primary regulator of the sympatho-adrenomedullary system projecting into the sympathetic preganglionic neurons in the spinal cord (22). Furthermore, by projecting into other hypothalamic and extrahypothalamic areas, VMH neurons may also indirectly influence sympathetic activities (23) to regulate glucose homeostasis.

Because both parasympathetic and sympathetic nervous systems contribute significantly to the insulin and glucagon secretory responses (24, 25), it is conceivable that central PREP, via direct or indirect projections to the ANS, may affect pancreatic function. In support of our study, two recent reports (11, 26) showed that hypothalamic glucose sensors play an important role in the control of insulin secretion (26) and that specific reduction of insulin receptor levels in the VMH produces glucose intolerance and islet dysfunction (11). Similar to this latter work, we found that Prep<sup>gt/gt</sup> mice have impaired glucose sensing and activation of insulin receptors in the VMH. Finally, re-expression of PREP in the VMH of Prep<sup>gt/gt</sup> mice normalized glucose sensitivity and insulin and glucagon secretion.

In summary, our study shows that central PREP plays an important role in the regulation of glucose sensing and insulin and glucagon secretion. Future studies are warranted to define the mechanism(s) of action of central PREP in the regulation of glucose sensing and pancreatic function.

**Experimental Procedures**

**Animal Care.** All animal care and experimental procedures were approved by the Yale University Institutional Animal Care and Use Committee. All mice were housed in a temperature-controlled environment (25 °C) with a 12-hr light and a 12-hr dark photoperiod. All animals (age 4–6 mo) were provided regular chow diet and water ad libitum unless otherwise stated.

**Generation of Gene-Trap PREP (Prep<sup>gt/gt</sup>) Mice.** BayGenomics clone RRM213 (Mutant Mouse Regional Resource Centers, www.mmrrc.org) was identified as having an insertion in the second intron of the PREP gene in a 129-strain ES cell (9). As protein coding begins in the first exon, the resulting protein in the gene-trap mice would include the first 40 amino acids of this 710-residue protein before the inserted β-galactosidase, as previously reported (9). Founder chimeric mice were bred to B6 for at least 10 generations.

**Metabolic Measurements.** Adult male mice for Prep<sup>gt/gt</sup> and WT were acclimated in metabolic chambers (TSE System) for 4 d before the start of the recordings as previously published (27, 28). For body composition, adult males were scanned in an EchoMRI machine (Echo Medical Systems), and their body composition was calculated according to body weight.

**Glucose, Insulin, and PTTs.** Glucose tolerance tests were performed in 16-h fasted animals as previously reported (27, 28). Each animal received an i.p. injection of 2 g/kg body weight (BW) glucose (DeltaSelect) in sterile saline. Blood glucose and insulin levels were measured after 15, 30, 60, and 120 min. An ITT was performed in ad-libitum-fed mice (27, 28). Each animal received an i.p. injection of 1 U/kg of insulin (Actrapid, Novo Nordisk). A PTT was performed in 16-h fasted animals. Animals received an i.p. injection of 2 g/kg BW sodium pyruvate (Sigma-Aldrich, catalog #P5280).

**In Situ Hybridization.** In situ hybridization was based on our previously published protocol (27, 28). A 500-bp sequence between 1531 and 2030 was selected from the mouse PREP (GenBank accession no. NM_011156), synthesized (Biomatik), incorporated into pBluescript vector, and used as template (27, 28). 35S-labeled riboprobes were then purified using sephadex columns (ProbeQuant G-50 Micro Columns, Pharmacia Biotech) following the manufacturer’s protocol, and 5 × 10<sup>6</sup> cpm per section was used for the hybridization (27, 28).

**X-Gal Staining.** Prep<sup>gt/gt</sup> male mice were perfused with 4% paraformaldehyde. Brains were sectioned (50 μm), washed in PBS, and incubated overnight at 37 °C in the staining solution containing 25 mM K<sub>3</sub>Fe(CN)<sub>6</sub>, 25 mM K<sub>3</sub>Fe(CN)<sub>6</sub>·3H<sub>2</sub>O, 2 mM MgCl<sub>2</sub> in PBS, and 1 mg/mL of X-Gal.

**Real-Time RT-PCR.** Real-time RT-PCR was performed as previously described (29) using the Light Cycler 480 (Roche) and Taqman Gene Expression Assay Primers (Applied Biosystems) in a 10-μL reaction volume in triplicates. All Taqman Gene Expression Assay primers used in our study are commercially available at Applied Biosystems (Prep: Mn 00448777_m1; Pck1: Mn01247059_g1; G6Pase: Mn00839363_m1; Ucp1: Mn00494069_m1; Gapdh: Mn99999915_g1; 18S: Mm03928990_g1).

**Histochemical Analysis.** Pancreas from WT and Prep<sup>gt/gt</sup> mice were collected and fixed in 4% (wt/vol) paraformaldehyde, in phosphate buffer 0.2% fatty acid-free BSA). After a 1.5-h perfusion, islet DNA was isolated and quantified using a QIAquick gel extraction kit (Qiagen). Islets were loaded into a perifusion chamber with acrylamide gel (27, 28). For body composition, adult male mice were scanned in an EchoMRI machine (Echo Medical Systems), and their body composition was calculated according to body weight.

**Western Blot Analysis.** VMH and hypothalamus samples were collected from mice that were fed, overnight-fasted, or fasted and injected with saline, glucose (2 g/kg BW), or insulin (0.75 U/kg BW; Actrapid; Novo Nordisk) and killed 5 or 30 min after the injection. Protein lysates from all of the tissues from WT and Prep<sup>gt/gt</sup> mice were prepared as previously described (30). Membranes were incubated overnight with anti-insulin receptor (Cell Signaling, catalog #2025) and antiphospho insulin receptor (Invitrogen, catalog #44800G). Membranes were re-used to detect β-actin (Sigma, catalog #A5441).

**Hyperinsulinemic-Euglycemic Clamp.** Hyperinsulinemic-euglycemic clamp studies were performed as previously reported (11). Insulin infusion rate was 2.5 mU/kg·min<sup>−1</sup>, and a variable-rate glucose infusion was used to maintain plasma glucose levels between 120 and 130 mg/dL for 120 min. Blood samples were collected at regular intervals for measurement of plasma glucose, hormones, and tracer. Red blood cells collected from a donor animal and resuspended in heparinized saline were constantly reinfused back into each animal to prevent volume depletion and anemia.

**Pancreas and Islet Studies.** Isolated islet studies were conducted as previously described (31). Islets were loaded into a perfusion chamber with acylamide gel column beads (Bio-Gel P4G; Bio-Rad) and basal perfusion buffer (Krebs-Ringer buffer with 2.5 mMglucose and 0.2% fatty acid-free BSA). After a 1.5-h equilibration period, islets were perfused and samples were collected at regular intervals. At the end of the perfusion, islet DNA was isolated and quantified for normalization of insulin data using a Picogreen dsDNA quantitation kit (Invitrogen) according to the manufacturer’s instructions.

**Pancreas NE Measurement.** Each frozen pancreas was quickly weighed before it was sonicated in 0.1 M of cold perchloric acid containing dihydroxybenzylamine (DHBA) as internal standard. Following centrifugation at 30,000 <i>x</i>g for 15 min at 4 °C, the catechols in a portion of the supernatant were extracted on alumina at pH 8.6, washed with water, and eluted in 0.1 M perchloric acid.
Propranolol Treatment. Propranolol (Sigma; nonselective mice i.p. for 3 d at a dose of 10 mg/kg BW (33). A group of Propranolol mice were used as controls and injected with an equal volume of saline. Male mice were fasted overnight, and propranolol was last injected 2 h before the GTT was performed. After determination of fasting blood glucose levels using a glucometer (Lifescan), mice were injected with 2 g/kg glucose (ΔGlu7) in sterile saline. Blood glucose levels were measured.

Statistical Analysis. Two-way ANOVA was used to determine the effect of the genotype and treatment with the Prism 4.0 software (GraphPad Software). For repeated measures analysis, ANOVA was used when values over different times were analyzed. Significant effects were evaluated with Fisher’s protected least significant difference post hoc test with Bonferroni’s correction. When two groups were analyzed, statistical significance was determined by an unpaired Student t test. A value of P < 0.05 was considered statistically significant. All data are shown as mean ± SEM unless stated otherwise.

Acknowledgments. We thank the Yale Diabetes Research Center for partial support of the clamp studies (Grant P30 DK-45735). This work was supported by National Institutes of Health Grants DK084065 and DK075756 (to S.D.) and by American Diabetes Association Research Award 7-11-B8-33 (to S.D.).


