P110β in the ventromedial hypothalamus regulates glucose and energy metabolism

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Abstract
Phosphoinositide 3-kinase (PI3K) signaling in hypothalamic neurons integrates peripheral metabolic cues, including leptin and insulin, to coordinate systemic glucose and energy homeostasis. PI3K is composed of different subunits, each of which has several unique isoforms. However, the role of the PI3K subunits and isoforms in the ventromedial hypothalamus (VMH), a prominent site for the regulation of glucose and energy homeostasis, is unclear. Here we investigated the role of subunit p110β in steroidogenic factor-1 (SF-1) neurons of the VMH in the regulation of metabolism. Our data demonstrate that the deletion of p110β in SF-1 neurons disrupts glucose metabolism, rendering the mice insulin resistant. In addition, the deletion of p110β in SF-1 neurons leads to the whitening of brown adipose tissues and increased susceptibility to diet-induced obesity due to blunted energy expenditure. These results highlight a critical role for p110β in the regulation of glucose and energy homeostasis via VMH neurons.

Introduction
Obesity and obesity-related metabolic diseases are major public health burdens1. The central nervous system (CNS) governs whole-body metabolism by sensing and responding to fluctuating levels of circulating cues, such as nutrients and hormones. Unraveling the neuronal mechanisms by which the CNS regulates metabolism is a fundamental step in the treatment of metabolic disease and recent scientific efforts in this area have led to a new class of Food and Drug Administration-approved anti-obesity drugs2.

The hypothalamus is an important region for the regulation of metabolism3. In particular, the ventral medial nucleus of the hypothalamus (VMH) has been known since the early 1940s, to play a critical role in the regulation of glucose and energy balance4,5. However, the molecular blueprint underlying the VMH regulation of glucose and energy homeostasis remains unclear. Phosphoinositide 3-kinase (PI3K) is critical for the integration of metabolic hormone cues. It is composed of the regulatory subunit p85 and the catalytic subunit p110, and each subunit comprised several variant forms. Previously, we demonstrated that mice lacking p110α in the VMH are more prone to high-fat diet (HFD)-induced obesity and obesity-related metabolic disturbances6. Recent studies have shown distinct metabolic roles for each subunit/variant in proopiomelanocortin (POMC) and agouti-related peptide (AgRP) neurons of the arcuate nucleus (ARC) of the hypothalamus7,8. These studies indicate that, at least in ARC neurons, p110β plays a greater role in the regulation of metabolism than does p110α. Although electrophysiological approaches suggest that p110β is required for leptin and insulin action in the VMH9, the specific metabolic roles of each of the PI3K subunits in VMH neurons are not well understood. Here we investigated the role of p110β in the VMH in the regulation of glucose and energy metabolism.

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Materials and methods

Animal care and generation of tissue-specific KO mice

All experimental procedures were approved by the Institutional Animal Care and Use Committees at UT Southwestern (Dallas, TX) and Yonsei University College of Medicine. Mice were kept at room temperature (22 °C–24 °C) with a 12 h light/dark cycle (lights on at 06:00 h) and fed a normal mouse chow diet (4% fat diet; 7001; Harlan Laboratories) or a HFD (Research Diet #D12331; 58% kcal from fat, 26% from sucrose, 5.56 kcal/g) with water provided ad libitum. To generate VMH-specific p110β knockout (KO) (p110β KOfl/fl) mice, males that were homozygous for the floxed (F) p110β allele and heterozygous for the Sf-1-Cre transgene were crossed with female mice homozygous for the floxed p110β allele. Littermate mice homozygous for the floxed p110β allele (p110βfl/fl) served as controls (Ctr). All experimental mice were on a mixed C57BL/6;129S6/SvEv background.

Protein and mRNA analyses

All samples were collected between 1300 and 1500 h for quantitative PCR (Q-PCR) analysis. Total RNA was isolated using Trizol reagent (Invitrogen, Carlsbad, CA) and reverse transcribed with a SuperScript First-Strand Synthesis System (Invitrogen) for reverse transcriptase PCR (RT-PCR). Real-time PCR (Q-PCR) was performed using an ABI 7900 HT Sequence Detection System (Applied Biosystems, Foster City, CA). The Q-PCR primers used for the Taqman method (Applied Biosystems) are as follows: 18S (ABI, Hs99999901), pik3ca (ABI, Mm00435673_m1), pik3cb (ABI, Mm00659576_m1), pik3r1 (ABI, Mm00808818_s1), pik3r2a (ABI, Mm00478162_m1), β-adrenergic receptor 3 (β3-AR) (ABI, Mm00260181_g1), Cidea (ABI, Mm00432554_m1), PGC1α (ABI, Mm01208835_m1), PPARy (ABI, Mm01184322_m1), PRDM16 (ABI, Mm01266507_g1), uncoupling protein 1 (UCP1) (ABI, Mm01244861), and UCP3 (ABI, Mm01163394_m1).

For protein analysis, tissues from control and p110β KOfl/fl mice were homogenized in lysis buffer [20 mM Tris, 5 mM EDTA, and NP40 1% (v/v)] containing protease inhibitors (P2714 Sigma, St. Louis, MO), resolved by SDS-polyacrylamide gel electrophoresis and transferred to a nitrocellulose membrane. After blocking the membrane with 5% non-fat milk, proteins were detected using infrared beams. To assess diet-induced thermogenesis, chow-fed mice with matched body weights were acclimatized in the TSE metabolic chambers as described above, followed by continuous monitoring of the metabolic rate. Chow was
provided from day 1 to day 4 and replaced with a HFD at 17:00 h of day 4. Metabolic parameters were measured for 3 additional days. The ΔVO₂ was calculated by the VO₂ difference before and after the HFD.

Hormone measurement
Corticosterone levels were measured as previously described. Briefly, psychosocial stress was given to male mice by housing for 30 min in groups of four animals after 3 days of isolation. Trunk blood for corticosterone measurements was taken by decapitation at the indicated times (Supplementary Table 1). For follicle-stimulating hormone (FSH), luteinizing hormone (LH), testosterone, epinephrine, and norepinephrine measurements, serum and/or plasma were obtained between 14:00 and 15:30 h. The blood samples for corticosterone, FSH, LH, testosterone, epinephrine, and norepinephrine levels were sent for analysis to either the Ligand Assay & Analysis Core at the University of Virginia or the Hormone Assay & Analytical Services Core, Vanderbilt Diabetes Research and Training Center.

VMH dissection for western blotting and Q-PCR analyses
To assess leptin-mediated AKT and forkhead box-containing protein of the O subfamily-1 (FoxO1) phosphorylation, body weight-matched 9- to 13-week-old male mice were fasted for 18 h and given murine leptin (5 mg/kg body weight, Sigma, St. Louis, MO) or pyrogen-free saline (Sigma, St. Louis, MO). After 40 min, the animals were transcardially perfused with 10% formalin. A coronal slice between bregma −1.22 mm and −2.06 mm was made, and then the VMH was microdissected with a scalpel under a microscope. All samples were immediately frozen on dry ice. Protein lysate was prepared from the VMH sample and used for western blotting analysis as described above.

To measure mRNA levels in the VMH of control and p110β KO mice, mice were decapitated after deep anesthesia. The VMH was microdissected with a scalpel under a microscope. All samples were immediately frozen on dry ice. Total mRNA was extracted and used for Q-PCR analyses.

Histology
All tissues were fixed in 10% neutral buffered formalin and either transferred to 1× phosphate-buffered saline followed by paraffin embedding or cryoembedded after sucrose infiltration for hematoxylin and eosin (H&E), Nissl, pSTAT3, or Oil Red O staining.

Body weight and composition
The body weight of control and p110β KO mice was determined using a Bruker Minispec mq10 nuclear magnetic resonance analyzer (The Woodlands, TX).

GTT and ITT
The glucose tolerance test (GTT) was performed as previously described. Male p110β KO mice and control littermates between the ages of 20–23 weeks were fasted for 18 h with water provided ad libitum. After fasted glucose levels were measured, glucose was administered via intraperitoneal (i.p.) injection (1.5 g/kg body weight). Blood glucose levels were measured from blood sampled from tail nicks at 20, 40, 60, 90, and 120 min after injection. Blood glucose levels were determined by the glucose oxidase method using a commercial glucometer (Ascensia Contour; Bayer HealthCare, Mishawaka, IN). For the insulin tolerance test (ITT), male mice between the ages of 20–23 weeks were fasted for 2 h with water provided ad libitum. After measurements of basal glucose levels, insulin (0.8 U/kg, Eli Lilly and Company, HI-210, Indianapolis, IN) was administered via i.p. injection. Blood glucose levels were monitored as described above.

Data analysis
The data are presented as the mean ± SEM, as indicated in each figure legend. Statistical significance was determined by Student’s t-test or two-way analysis of variance. GraphPad Prism, version 5.0a (GraphPad, San Diego, CA), was used for all statistical analyses and P < 0.05 was considered a statistically significant difference.

Results
Generation of SF-1 neuron (VMH)-specific p110β KO mice
p110β is ubiquitously expressed and mice lacking p110β in the VMH were generated by crossing floxed p110β mice with steroidogenic factor-1 (SF-1) Cre mice (p110β KOfl)12, which in the CNS, express Cre recombinase exclusively in the VMH. Histological analyses confirmed that the deletion of p110β was confined to the VMH (Fig. 1a–d) without disturbing VMH cytoarchitecture (Supplementary Fig. 1). Q-PCR analysis of RNA isolated from the VMH showed that p110β was significantly reduced, and that the expression of the remaining isoforms and subunits was unchanged (Fig. 1e). Peripherally, SF-1 is also expressed in the pituitary, adrenal glands, and gonads, which are important tissues for the regulation of metabolism. We therefore examined these tissues for morphological changes and measured the circulating levels of corticosterone (normal and stressed), testosterone, FSH, and LH. We found similar tissue morphology and hormone parameters between the two genotypes, indicating that the hypothalamic–pituitary–adrenal and hypothalamic–pituitary–gonadal axes were intact (Supplementary Fig. 2 and Supplementary Table 1). These data
suggest that the metabolic phenotype of the p110β KOsf1 mice described in this study is not secondary to disruptions in these hormones.

To determine whether the deletion of p110β in SF-1 neurons altered PI3K signaling in the VMH, we measured the pAKT and FoxO1 in the VMH after intraperitoneal leptin administration (5 mg/kg). Although leptin administration activated pAKT and FoxO1 in the VMH of control mice, this effect was significantly blunted in mice that lacked p110β (Fig. 1f). In contrast, the activation of pSTAT3 by leptin was comparable (Supplementary Fig. 3). These results indicate that p110β in SF-1 neurons of the VMH is necessary for the normal activation of the PI3K pathway.

**p110β isoform in the VMH is required for normal glucose homeostasis**

To investigate the role of p110β in the regulation of energy homeostasis, we first examined several metabolic parameters in mice fed a NCD. No differences were observed in body weight, body composition, leptin and insulin levels, food intake, oxygen consumption (VO2), locomotor activity, or respiratory exchange ratio (RER) compared with control littermates (Fig. 2g–j). These results suggest that the VMH is a key brain site for the regulation of glucose homeostasis through the modulation of the autonomic nervous system (SNS).

Although we found no significant differences in the glucose levels of mice fed a NCD, glucose levels during the refeeding period following a 24 h fast were significantly elevated in p110β KOsf1 mice compared with control mice (Fig. 2a). Furthermore, p110β KOsf1 mice exhibited blunted glucose and insulin sensitivity in response to both i.p. GTTs and ITTs (Fig. 2b–f). Notably, previous studies have shown that the deletion of p110α in the VMH does not affect glucose metabolism in NCD-fed mice. Our data suggest that glucose homeostasis by SF-1 neurons in the VMH is uniquely mediated by the p110β subunit.

Serum insulin levels obtained during the course of the GTT were unaltered (Fig. 2d), suggesting an impairment in insulin sensitivity rather than impaired insulin secretion from pancreatic β-cells. Therefore, we measured insulin sensitivity in peripheral tissues, including the liver, interscapular brown adipose tissue (iBAT), heart, and muscle, by monitoring the activation of pAKT after i.p. injection of insulin. The insulin-mediated activation of pAKT was decreased in p110β KOsf1 mice in all tissues examined, including the iBAT, heart, and muscle, compared with control littermates (Fig. 2g–j). These results strongly suggest that the blunted insulin sensitivity in these peripheral tissues contributes to altered whole-body glucose homeostasis in p110β KOsf1 mice.

**Increased whitening of iBAT and decreased energy expenditure in p110β KOsf1 mice**

The VMH is a critical brain site mediating sympathetic tone to the iBAT. A disruption in β-adrenergic
signaling causes iBAT lipid accumulation\textsuperscript{30,31}, a process known as “whitening”\textsuperscript{31}. Notably, the activation of pAKT in the iBAT after insulin administration was significantly blunted in p110\textsubscript{β} KO\textsuperscript{sf1} mice (Fig. 2h). H&E staining revealed an increase in lipid droplets in p110\textsubscript{β} KO\textsuperscript{sf1} mice (Fig. 3a–c). In addition, the RNA levels of \(\beta\)3-AR and UCP1, and the protein levels of UCP1 were significantly reduced in the iBAT of p110\textsubscript{β} KO\textsuperscript{sf1} mice (Fig. 3d–f).

Moreover, plasma norepinephrine, a neurotransmitter released by sympathetic nerve terminals, was decreased in p110\textsubscript{β} KO\textsuperscript{sf1} mice (Fig. 3g). Our study demonstrates that the deletion of p110\textsubscript{β} in SF-1 neurons hampers sympathetic activity and leads to the whitening of iBAT. Collectively, these data suggest that p110\textsubscript{β} expression in the VMH is a key module to maintain BAT programming.

As p110\textsubscript{β} KO\textsuperscript{sf1} mice displayed changes in sympathetic tone, we postulated that metabolic stress would alter metabolic homeostasis in p110\textsubscript{β} KO\textsuperscript{sf1} mice. Of note, a HFD decreases UCP1, PGC1\textalpha, and other genes, which are important for maintaining BAT programming\textsuperscript{31}. To address this hypothesis, metabolic stress was induced by challenging mice with a HFD and assessing the metabolic response of p110\textsubscript{β} KO\textsuperscript{sf1} mice. The body weight of p110\textsubscript{β} KO\textsuperscript{sf1} mice began to diverge from that of control mice after 6 weeks of HFD feeding (Fig. 4a). The increased body weight was caused by increased fat mass but not lean mass (Fig. 4b, c). Indirect calorimetry studies revealed significantly decreased oxygen consumption in p110\textsubscript{β} KO\textsuperscript{sf1} mice, without changes in food intake, movement, or the RER during HFD feeding (Fig. 4d–h). These data imply that PI3K activity in SF-1 neurons of the VMH might be necessary for the regulation of energy expenditure, especially under high-calorie conditions. Serum analysis showed elevated levels of leptin, insulin, fasted glucose, triglyceride (TG), and free fatty acid in HFD-fed p110\textsubscript{β} KO\textsuperscript{sf1} mice (Fig. 4i–m). In addition, HFD-fed p110\textsubscript{β} KO\textsuperscript{sf1} mice exhibited increased liver TG (Fig. 4n) but not serum or liver cholesterol (Fig. 4o, p). These results indicate that the p110\textsubscript{β} subunit in the VMH might be involved in the regulation of metabolic homeostasis.

**Discussion**

Although the metabolic importance of PI3K has been shown in several tissues, little is known about its function in the hypothalamus\textsuperscript{9,11}. In this study, we specifically deleted the p110\textsubscript{β} isoform of PI3K from SF-1 neurons of the VMH. We found that p110\textsubscript{β} in the VMH, possibly through actions on the autonomic nervous system, is required for energy homeostasis and the maintenance of normal glucose and insulin sensitivity. p110\textalpha and p110\textsubscript{β} are class IA PI3K isoforms, and studies using global KO mice have suggested that each isoform has distinct metabolic functions\textsuperscript{32,33}. Notably, the deletion of class I PI3K isoforms in ARC POMC or AgRP neurons revealed that p110\textsubscript{β} has a greater contribution than does p110\textalpha to metabolic parameters, such as body weight, food intake, and leptin-mediated neuronal excitability\textsuperscript{9,34}. Our studies have extended these findings to the VMH. We previously
showed that p110α deletions in the VMH affect diet-induced obesity but not the basal metabolic rate6. Our current study shows that p110β in SF-1 neurons of the VMH plays a much broader role, affecting glucose and insulin homeostasis and BAT function.

The VMH is well known to regulate many physiological processes, including energy expenditure, reproduction, defensive behavior, food intake, carbohydrate and fat metabolism, and metabolic adaptation6,12,18,23,29,35–57. In 1966, Shimazu et al.35 demonstrated that electric stimulation of the VMH remarkably increased blood glucose and suggested the important role of the VMH in the regulation of glucose metabolism56,57. Previous reports have indicated that microinjection of leptin into the VMH can stimulate glucose uptake into the peripheral tissues, including skeletal muscle25. We recently found that the p110β subunit is required for leptin-induced depolarization in SF-1 neurons of the VMH10. Collectively, these studies suggest that the deletion of p110β in SF-1 neurons may compromise leptin’s glucoregulatory actions, leading to refractory responses to the GTT. Interestingly, we found that p110β KOsf1 mice exhibited glucose intolerance under refeed conditions, with no significant body weight change, and exhibited diet-induced obesity, with significantly increased fasted glucose levels. p110β KOsf1 mice exhibited insulin insensitivity in the iBAT, heart, and gastrocnemius muscle. These results highly imply that the higher glucose level in p110β KOsf1 mice might be the result of decreased glucose uptake and insulin sensitivity mediated by decreased sympathetic tone.

A recent paper showed that SNS input is necessary for maintaining the thermogenic properties of BAT31. Disruption of the SNS signaling pathway leads to a whitening of BAT accompanied by a reduction in mitochondrial activity and the accumulation of lipid droplets51. In fact, ob/ob58 and DIO31 mice show impaired SNS and BAT whitening. The VMH regulates BAT function via the SNS24,25,36,41. Lesions in the VMH have been shown to cause mitochondrial dysfunction and to reduce fatty acid oxidation59–61, indicating that an intact VMH is important for maintaining BAT function. Lower levels of noradrenaline together with increased iBAT whitening in p110β KOsf1 mice suggest that p110β in the VMH might be a critical component for the SNS-mediated BAT pathway, while further analyses including the direct visualization of sympathetic nerve fibers are necessary.

Our study supports the notion that the VMH plays a critical role in regulating metabolic adaptations under
conditions requiring high-energy expenditure, such as a HFD and exercise. The regulation of energy expenditure by the VMH is known to be mediated by the SNS; however, the precise neuronal pathway linking the SNS and the VMH has not yet been precisely determined. Genetic tracing experiments revealed that SF-1 neurons project to several brain nuclei that regulate SNS function; thus, future studies using emerging techniques such as channel rhodopsin-assisted neurocircuit mapping may provide further insights into the functional pathways linking the SNS and the VMH. In summary, the current study suggests that pharmaceutical therapies that target PI3K in a tissue- and isoform-specific manner may prove beneficial toward ameliorating metabolic syndrome, especially diabetes.

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