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Hypothalamic PDE3B deficiency alters body weight and glucose homeostasis in mouse

Maitrayee Sahu,

Department of Obstetrics, Gynecology and Reproductive Sciences, Magee-Womens Research Institute, University of Pittsburgh School of Medicine, Pittsburgh, PA, USA

Prashanth Ananthmakula, and

Department of Obstetrics, Gynecology and Reproductive Sciences, Magee-Womens Research Institute, University of Pittsburgh School of Medicine, Pittsburgh, PA, USA

Abhiram Sahu

Department of Obstetrics, Gynecology and Reproductive Sciences, Magee-Womens Research Institute, University of Pittsburgh School of Medicine, Pittsburgh, PA, USA

Abstract

Pharmacological studies have suggested hypothalamic phosphodiesterase-3B to mediate leptin and insulin action in regulation of energy homeostasis. Whereas *Pde3b*-null mice show altered energy homeostasis, it is unknown whether this is due to ablation of *Pde3b* in the hypothalamus. Thus to address the functional significance of hypothalamic phosphodiesterase-3B, we used *Pde3b^{flox/flox}* and *Nkx2.1*-Cre mice to generate *Pde3b^{Nkx2.1}* KD mice that showed 50% reduction of phosphodiesterase-3B in the hypothalamus. To determine the effect of partial ablation of phosphodiesterase-3B in the hypothalamus on energy and glucose homeostasis, males and females were subjected to either a low- or high-fat diet for 19–21 weeks. Only female but not male *Pde3b^{Nkx2.1}* KD mice on the low-fat diet showed increased body weight from 13 weeks onward with increased food intake, decreased fat pad weights and hypoleptinemia. Glucose tolerance was improved in high-fat diet-fed male *Pde3b^{Nkx2.1}* KD mice in association with decreased *phosphoenolpyruvate carboxykinase-1* and *glucose-6-phosphatase* mRNA levels in the liver. Also, insulin sensitivity was increased in male *Pde3b^{Nkx2.1}* KD mice on the low-fat diet. Changes in body weight or in glucose homeostasis were not associated with any alteration in hypothalamic *proopiomelanocortin*, *neuropeptide Y* and *agouti-related peptide* mRNA levels. These results suggest that partial loss of phosphodiesterase-3B in the hypothalamus produces a sex-specific response in body weight and glucose homeostasis, and support a role, at least in part, for hypothalamic phosphodiesterase-3B in regulation of energy and glucose homeostasis in mice.

Keywords

PDE3B; hypothalamus; feeding; energy balance; glucose homeostasis

*Corresponding author: Abhiram Sahu, Ph.D., Department of Obstetrics, Gynecology and Reproductive Sciences, University of Pittsburgh School of Medicine, Magee-Womens Research Institute, 204 Craft Avenue, Pittsburgh, PA 15213, USA, (abhisahu15@gmail.com).

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Introduction

Hypothalamus is the critical site of integration for the central and peripheral signals in regulation of energy and glucose homeostasis. Leptin and insulin are the two most important peripheral signals that play obligatory role in body weight regulation and glucose homeostasis by acting at the level of the hypothalamus (Schwartz *et al.* 2000, Sahu 2003, Sahu 2004, Morton *et al.* 2006, Myers & Olson 2012, Timper & Bruning 2017). Thus, signaling molecules that mediate actions of leptin and/or insulin in the hypothalamus are expected to play a significant role in energy and glucose homeostasis. In this regard, phosphatidylinositol-3 kinase (PI3K)-Akt pathway (Niswender *et al.* 2003), and several signaling molecules downstream of Akt—including the mammalian target of rapamycin/S6 kinase (Avruch *et al.* 2006, Manning & Cantley 2007), and forkhead box O1 (FoxO1) (Accili & Arden 2004)—have been implicated for insulin signaling in the hypothalamus and energy homeostasis (Kim *et al.* 2006, Ono *et al.* 2008, Woods *et al.* 2008, Kitamura *et al.* 2006, Watterson *et al.* 2013). On the other hand, leptin action in the hypothalamus is mediated through several intracellular signaling pathways including Jak2-Stat3, PI3K, and mTOR-S6K pathways (Vaisse *et al.* 1996, Niswender *et al.* 2001, Zhao *et al.* 2002, Woods *et al.* 2008, Myers *et al.* 2008, Rahmouni *et al.* 2009). However, our laboratory has demonstrated that, as shown in peripheral tissues, particularly in the adipose tissue and liver (Zhao *et al.* 2000, Shakur *et al.* 2001, Bender & Beavo 2006, Conti & Beavo 2007, Degerman *et al.* 2011), leptin and insulin signaling in the hypothalamus is also mediated through an activation of phosphodiesterase-3B (PDE3B) and regulation of cAMP (Zhao *et al.* 2002, Sahu 2011, Sahu *et al.* 2017).

Cyclic nucleotide phosphodiesterases are a large superfamily of enzymes, encoded by at least 21 different genes sub-grouped into 11 families (Bender & Beavo 2006, Conti & Beavo 2007). PDE3B, one of the two members of the type 3 PDE genes, exhibits high affinities for both cAMP and cGMP, with the former being the preferred substrate. Thus increased PDE3B activity decreases cAMP levels by degradation of cAMP to 5'-AMP (Bender & Beavo 2006, Conti & Beavo 2007). Accordingly, we have shown that leptin- and insulin-induced PDE3B activity was associated with decreased cAMP levels in the hypothalamus (Zhao *et al.*, 2002, Sahu *et al.* 2017). PDE3B, initially identified in adipose tissue and liver, was subsequently found in a variety of tissues, including the hypothalamus. Specifically, we have demonstrated that (i) PDE3B is expressed in the hypothalamic arcuate nucleus, ventromedial nucleus, dorsomedial nucleus, lateral hypothalamic areas, paraventricular nucleus, and perifornical hypothalamic areas (Sahu 2003, Sahu *et al.* 2011) that are involved in energy and glucose homeostasis; (ii) PDE3B is expressed in proopiomelanocortin (*Pomc*)- and neuropeptide Y (*Npy*)/agouti-related peptide (*Agrp*)-expressing neurons (Sahu *et al.* 2011) that are the targets of both leptin and insulin signaling (Schwartz *et al.* 2000, Sahu 2003, Sahu 2004, Morton *et al.* 2006, Myers & Olson 2012, Timper & Bruning 2017); (iii) both leptin and insulin increase the activity of PDE3B in the hypothalamus (Zhao *et al.* 2002, Sahu 2011, Sahu *et al.* 2017); and (iv) prior administration of a specific PDE3 inhibitor cilostamide reverses the anorectic and body weight reducing effects of leptin and insulin (Zhao *et al.* 2002, Sahu 2011, Sahu *et al.* 2017). Whereas these findings suggest a potential role for the PDE3B in mediating leptin and insulin signaling in the hypothalamus

in the regulation of energy homeostasis, the physiological role of hypothalamic PDE3B in energy and glucose homeostasis is yet to be established.

Notably, alteration in regulation of energy homeostasis along with metabolic dysregulation, including systemic insulin resistance, has been reported in *Pde3b-null* mice (Choi *et al.* 2006). However, because PDE3B is expressed in variety of tissues including adipocytes, liver, pancreatic beta cells and the hypothalamus (Zhao *et al.* 2000, Shakur *et al.* 2001, Bender & Beavo 2006, Conti & Beavo 2007, Degerman *et al.* 2011, Sahu *et al.* 2011), that have been implicated in energy and glucose homeostasis, it is unknown whether the phenotype of *Pde3b-null* mice were due to *Pde3b* deficiency in specific tissues. Thus to begin to address the functional significance of *Pde3b* *in vivo* in a tissue-specific manner, in the present study we generated hypothalamus-specific PDE3B conditional knockout mice using the Cre-loxP system. First, we generated transgenic mice with a floxed PDE3B allele (*Pde3b^{fl/fl}*). Then we generated mice with knockdown of PDE3B specifically in the hypothalamus, referred to as *Pde3b^{fl/fl} Nkx2.1-cre* (*Pde3b^{Nkx2.1}KD*) mice. We have analyzed the phenotype of this model at the level of energy and glucose homeostasis and here, we report a sex-specific changes in these processes.

Materials and methods

Animals

Mice with loxP-flanked *Pde3b* alleles (*Pde3b^{fl/fl}*) were generated as described below. *Nkx2.1-Cre* mice, which are known to drive Cre expression in the hypothalamus and not in more caudal region of the brain (Xu *et al.* 2008), were obtained from the Jackson Laboratory (Bar Harbor, Maine, USA). Mice were housed in a light (lights on 0600 h to 1800 h) and temperature (22 °C)-controlled room. Mice were placed on standard laboratory chow (Prolab RMH 2000; TestDiet, St Louis, MO) at weaning and water was available *ad libitum*. Both males and females were used in this study unless indicated otherwise. All procedures were approved by the Institutional Animal Care and Use Committee of the University of Pittsburgh.

Generation of *Pde3b^{fl/fl}* mice and hypothalamus-specific *Pde3b*-KO mice.

—To address the question whether loss-of-function of PDE3B in hypothalamic neurons modifies energy balance and glucose homeostasis we used Cre-loxP technology, in which *Pde3b^{fl/fl}* mice when crossed with mice carrying the appropriate Cre gene deletes exon 4 of the *Pde3b* gene (Fig. 1A). The deletion of exon 4 removes 140 nucleotides from the transcript. To generate *Pde3b^{fl/fl}* mice, the *Pde3b*-targeting vector (PRPGS00 067 A D07 CSD) was purchased from CHORI (Children's Hospital Oakland Research Institute, Oakland, CA) and the Mouse Biology Program (MBP) at UC Davis made a targeting B6 ES clone (*Pde3b*-2A11). The UC Davis MBP facility performed blastocyst injection. One founder male was bred with B6 females and produced eight pups, of which two males were heterozygous (*Pde3b^{neo-flox/+}*). We bred *Pde3b^{neo-flox/+}* male mice with transgenic females carrying the *Actin-FLPe transgene* (*B6.CgTg(ACTFLPe) 9205Dym/J*; The Jackson Laboratory, Bar Harbor, ME) to remove the *frt*-flanked *Pgk-NEO* cassette. Subsequently, we bred *Pde3b^{fl/fl}* males and females and generated mice homozygous for a floxed *Pde3b*

allele (*Pde3b^{fl/fl}*). *Pde3b^{fl/fl}* mice were bred with C57Bl/6J mice (The Jackson Laboratory) for at least three generations before crossing with *Cre* expressing mice. We crossed *Pde3b^{fl/fl}* mice with the mice transgenic for *Nkx2.1-Cre* mice to generate *Pde3b^{fl/+}* and *Pde3b^{fl/+}Nkx2.1-cre* mice, which were subsequently crossed to generate *Pde3b^{fl/fl}* littermate controls and *Pde3b^{Nkx2.1}*KO mice for our studies.

We genotyped mice by PCR from DNA extracted from tail clips. Primer sequences used for genotyping of *Pde3b^{fl/fl}* and *Nkx2.1-Cre* mice were as follows: *Pde3b^{fl/fl}*, GTTGCAAGGAGC-TCTGAAGG (forward, PD5-fl-F); and AAAATGGTCGTGGACAGGAG (reverse, PD-w5-R); *Nkx2.1-Cre*, CCACAGGCACCCACAAAAATG (forward) and GCCTGGCGATCCC-TGAACAT (reverse). Amplicon sizes for *Pde3b^{fl/fl}*, wild-type and *Nkx2.1-Cre* were ~315 bp, ~218 bp and ~ 666 bp, respectively.

Mouse studies.—At 4 weeks of age, both male and female KO mice and their WT littermates were placed on either a diet of standard mouse chow (low-fat diet; LFD) (6% kcal as fat; F6 rodent diet, #8664; Harlan Teklad, Madison, WI, USA) or a HFD (58% kcal as fat; #D12331; Research Diets Inc., New Brunswick, NJ, USA) for 19-21 weeks. Body weights were measured twice per week. Glucose and insulin tolerance tests were carried out between 13 to 16 weeks of dieting. Mice at 15 to 17 weeks of dieting were fasted overnight and blood was collected from tail bleeding to measure glucose levels (using Precision Xtra Strips, Abbott Diabetes are Inc., Alameda, CA, USA) and plasma levels of insulin. Food intake was measured during the last week of the study in animals that were individually housed for at least 1 week before the study. At the end of the study, fed glucose level was measured in blood collected from the tail using Precision Xtra Strips, and then the mice were killed by decapitation. The brains were removed immediately and medial basal hypothalamus (MBH) tissue was dissected out, frozen in liquid nitrogen and kept at -80°C until processed. Trunk blood was collected to determine circulating leptin levels. Epididymal fat (WAT), gonadal fat-pad (in female), retroperitoneal fat (RP-fat), brown adipose tissue (BAT) and liver were dissected out, weighed, frozen in liquid nitrogen and kept at -80°C until processed.

In another cohort of mice, overnight fasted males that were on a standard laboratory chow (Prolab RMH 2000) were killed by decapitation. The MBH and liver were dissected out, frozen in liquid nitrogen and kept at -80°C until processed for RNA and protein extraction as appropriate.

Metabolic measurements

Glucose was measured in blood collected from the tail using Precision Xtra Strips. Leptin and insulin levels were determined with enzyme-linked immunosorbent assay (ELISA) kits (mouse insulin ELISA kit, Mercodia AB, Uppsala, Sweden; mouse/rat leptin Quantikine ELISA kit, R&D Systems Inc., Minneapolis, MN, USA). To measure glucose and insulin in fasting condition, blood was collected by tail bleeding in animals that were fasted for 16 hours. For the fed animals, glucose was measured in the blood collected from the tail, and leptin was measured in the trunk blood. For glucose tolerance test (GTT), overnight-fasted

mice were injected with D-glucose (2 g/kg body weight, i.p.) and glucose was measured before and 15, 30, 45, 60, and 120 min after injection. For insulin tolerance test (ITT), mice were fasted for 2 h and injected with human regular insulin (Humulin, 0.75mIU/g body weight, i.p.; Eli Lilly and Company, Indianapolis, IN, USA). Blood glucose levels were measured before and at 15, 30, 45, 60, and 120 min after injection.

RNA extraction and real-time PCR

RNAs from the MBH, liver, and BAT were extracted from male and female *Pde3b*^{Nkx2.1KO} and control mice for *Pomc*, *Npy*, and *Agrp* gene expression in the MBH, uncoupling protein 1 (*Ucp1*) gene expression in the BAT, and phosphoenolpyruvate carboxykinase 1 (*Pck1*) and glucose-6-phosphatase (*G6pc*) gene expression in the liver. In addition, *Pde3b* gene expression was examined in all tissues. Total RNA was extracted using TRIzol reagent (Life Technologies) and subjected to DNase treatment using RNase-Free DNase (Promega, Madison, WI) according to the manufacturer's protocol and re-extracted with TRIzol. Total RNA (1 µg) was reverse transcribed using a High Capacity cDNA RT Kit (Life Technologies). Real-time PCR with diluted cDNA (50 ng) was performed in duplicate using 2× Power SYBR Green PCR master mix (Life Technologies) containing 500 nm each primer and run on the Applied Biosystems Prism 7900HT real-time PCR machine at 95 °C for 10 min followed by 40 cycles of 95 °C (15 s) and 60 °C (60 s). The primer sequences are as follows: *Pomc* (forward, atgccgagattctgtacagtc; reverse, ttcattccgttgccaggaaacac); *Npy* (forward, cagaaaacccccagaa; reverse, aaaagtcggggaacaagttt-catt); *Agrp*: (forward, cggaggtgctagatccacaga; reverse, aggactcgtgcagcctta-cac); *Ucp1*(forward, aactgtacagcggtctgcct; reverse, taagccggctgagatcttgtt); *Pck1* (forward, atgacaactgttgctggct; reverse, ctgaggccagttttgggg-at); *G6pc* (forward, gctggagtctgtcaggcat; reverse, atccaagcgcgaaacc-aaac); *Pde3b* (forward, atcgagcagtggtgaagagg; reverse, aaagggcc-atttagtgga); and *Cyclophilin* (forward, aagtgaaagaaggcatgaac; reverse, agctgtccacagtcggaaatg). The relative quantification of mRNA levels (fold change) normalized to cyclophilin was calculated using the CT method from the Ct (threshold cycle) values obtained from ABI SDS software package.

Tissue RT-PCR

To demonstrate *Pde3b* deletion in specific tissue, various tissues (hypothalamus, cortex, cerebellum, brainstem, liver and gonadal fat (WAT) were dissected out from age-matched male *Pde3b*^{fl/fl} and *Pde3b*^{Nkx2.1KO} mice. Total RNA was isolated using TRIzol according to manufacturer instruction (Life Technologies). RNA was digested with RQ1-DNase (Promega) followed by re-extraction with TRIzol. cDNA was synthesized from total RNA using a High Capacity RT-PCR kit (Life Technologies). cDNA (100 ng) was used for PCR to detect *delta* and *fl/fl* alleles using *Pde3b*^{fl/fl} reverse primer (CATGCATCTGAAAACCCACA, PD-w3-R) in combination with two other *Pde3b*^{fl/fl} primers (PD5-fl-F, PD-w5-R) as mentioned above.

Protein extraction and Western blotting

The tissues were lysed with a 1 × lysis buffer (Cell Signaling, Danvers, MA, USA) supplemented with phenyl-methylsulfonyl fluoride and a cocktail of protease and phosphatase inhibitors (Roche Diagnostics, Indianapolis, IN, USA). Total protein

concentration was determined using a BCA Protein Assay Kit (Pierce, Rockford, IL, USA). Fifty μ g of protein, unless indicated otherwise, was separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (8% gel) and transferred to polyscreen polyvinylidene difluoride membranes. The membranes were then incubated overnight with anti-rabbit PDE3B antibody (3B N-T, dilution 1:1000, provided by Dr. V. C. Manganiello, NIH, Choi *et al.* 2006) at 4°C followed by incubation for 60 min with horseradish peroxidase-conjugated secondary antibodies (Santa Cruz Biotechnology, Santa Cruz, CA, USA) at room temperature. Immunoreactive bands were visualized by Western Lightning chemoluminescence Reagent Plus-ECL as described by the manufacturer (Perkin Elmer, Boston, MA, USA). The membranes were stripped and then blotted with a monoclonal anti- β -actin antibody (dilution 1:40,000, Sigma). Images of the bands were scanned and analyzed using NIH IMAGE software (NIH, Bethesda, MD, USA). PDE3B levels were normalized to β -actin.

Statistical analyses

Values are given as means $\pm$ S.E.M. Statistical significance in body weight, GTT and ITT data was determined using repeated measures two-way ANOVA with post-hoc testing using Fisher's least significant difference (LSD) (protected t-tests). All other data were analyzed by randomized one-way ANOVA followed by Fisher's LSD (protected t-tests) to compare three or more groups or Student's *t* test to compare two groups. All statistical analyses were done using GB-Stat software for the Macintosh (Dynamic Microsystems, Inc., Silver Spring, MD). A *P* value of < 0.05 was considered statistically significant.

Results

Generation of mice with specific disruption of *Pde3b* in *Nkx2.1* neurons

To clarify the role of PDE3B in the hypothalamus in energy homeostasis and glucose homeostasis, we generated a conditional null allele by crossing *Pde3b^{fl/fl}* with *Nkx2.1-Cre* mice. The resulting *Pde3b^{fl/+}Nkx2.1-cre* mice were crossed to *Pde3b^{fl/fl}* mice, yielding *Pde3b^{Nkx2.1}KO*, *Pde3b^{fl/+}Nkx2.1-cre* (heterozygous) and *Pde3b^{fl/fl}* (control, hereafter termed wild-type or WT) mice. Finally, *Pde3b^{Nkx2.1}KO* and *Pde3b^{fl/fl}* mice that were used in the study were obtained by crossing each other. In mice, *Nkx2.1-Cre* has been shown to express mostly in the hypothalamus and very less in the cortex and lung (Xu *et al.* 2008). Although we did not include the lung in our screening, reverse transcription of RNA isolated from central and peripheral tissues followed by PCR amplification of the resulting cDNA revealed *Nkx2.1-Cre* expression mostly in the hypothalamus and some in cortex but not in other parts of the brain (cerebellum, brain stem) or in liver or WAT (Fig 1). qPCR data showed ~50% decrease in *Pde3b* gene expression in the hypothalamus of *Pde3b^{Nkx2.1}KO* mice as compared to control mice (Fig 2A), but there was no change in *Pde3b* mRNA levels in the liver (Fig 2B). Similarly, western blotting with PDE3B specific antibody showed ~50% decrease in PDE3B protein levels in the hypothalamus of *Pde3b^{Nkx2.1}KO* mice (Fig 2C and D). These results suggest a partial (50%) deficiency of *Pde3b* in the hypothalamus of *Pde3b^{Nkx2.1}KO* mice, therefore these mice were termed as *Pde3b^{Nkx2.1}KD* (KD) mice indicating *Pde3b* knockdown instead of *Pde3b* knockout in the hypothalamus.

Changes in body weight (BW), tissue weight, and metabolic parameters

Analyses with two-way ANOVA with repeated measures showed that in male *Pde3b*^{Nkx2.1}KD mice on a LFD, BW was significantly higher than that of WT mice at 4-6 weeks of age but not thereafter (Fig. 3A). However, on a HFD, there was no significant difference in BW between the genotypes at any time points during the 18 weeks of dieting (Fig. 3B). In addition, we did not observe any difference in food intake or in fat or liver weights between the genotypes on a LFD or HFD (Fig 4).

In contrast, female *Pde3b*^{Nkx2.1}KD mice on the LFD had significantly higher BW from 13 weeks of age onward (Fig. 3C), and although this was accompanied by increased food intake (Fig 5A), the weights of fat, particularly that of WAT and BAT, but not the liver, were significantly decreased in these mice as compared to WT mice (Figs 5C, 5D, 5F, 5G). In addition, *Pde3b*^{Nkx2.1}KD mice on the HFD had significantly higher BW from 15 weeks of age onward (i.e. after approximately 11 weeks on the HFD) as compared to WT mice (Fig. 3D); which was however not associated with any change in food intake or tissue weights (Fig 5, J-N).

Because *Pde3b*-null mice show impaired glucose homeostasis (Choi *et al.* 2006), we performed a number of *in vivo* studies in *Pde3b*^{Nkx2.1}KD mice and in control WT mice to assess whether partial ablation of PDE3B in hypothalamic neurons could lead to abnormalities in glucose homeostasis. First, we measured fed and fasted (16 hours) glucose levels in the mice that were on either a LFD or HFD. Results showed that in males on the LFD, fed glucose levels were significantly increased ($p = 0.0468$) in *Pde3b*^{Nkx2.1}KD mice as compared to WT mice (Fig 4F). In females on the LFD, however, *ad libitum*-fed glucose levels were significantly decreased ($p = 0.0481$) in *Pde3b*^{Nkx2.1}KD mice as compared to WT mice (Fig 5I). In the HFD-fed animals, there was no change in fed glucose levels between the groups of either sex (Figs. 4L and 5O). Also, there was no change in fasting glucose levels between genotypes of either sex on the LFD or HFD (Fig 6). Second, we performed GTT and ITT in males and females. In males on the LFD, clearance of glucose during GTT was similar between genotypes (Fig. 7A), and those on the HFD, clearance of glucose was increased in the *Pde3b*^{Nkx2.1}KD mice at 45, 60 and 120 min of i.p. glucose injection (Fig 7D), which was confirmed with area under the curve analysis of the GTT (GTT-AUC) showing significant decrease ($p = 0.0356$) in GTT-AUC in these mice (Fig 7E). In addition, in LFD-fed male mice, ITT showed that i.p. injection of insulin was more effective in reducing blood glucose levels at 15 to 60 min post-injection in *Pde3b*^{Nkx2.1}KD than in control WT mice (Fig 7B). However, although area under the curve analysis for ITT (ITT-AUC) showed a trend towards a decrease in ITT-AUC (Fig 7C), it was not statistically significant ($p = 0.0734$). In HFD-fed male mice, ITT did not reveal a significant difference between genotypes (Fig. 7 F and G), except at 15 minutes post-insulin injection when decrease in blood glucose level was significantly more in *Pde3b*^{Nkx2.1}KD mice ($p < 0.05$) than in control mice (Fig 7F). In female mice on the LFD or HFD, there was no change in either GTT or ITT between genotypes (Fig 8).

Fasting plasma insulin levels measured from tail blood did not show any difference between *Pde3b*^{Nkx2.1}KD and control mice of either sex on the LFD or HFD (Fig 9). Fed plasma leptin levels, measured in trunk blood, were significantly decreased ($p < 0.01$) in LFD-fed

female *Pde3b*^{Nkx2.1}KD mice than in control WT mice (Fig 10A). However, there was no change between genotypes in plasma leptin levels in females on the HFD (Fig. 10B) or in males on the LFD (Fig 10C) or HFD (Fig 10D).

Changes in gene expression of hypothalamic *Npy*, *Agrp* and *Pomc*, liver *Pck1* and *G6pc*, and in BAT *Ucp1*

Because PDE3B is expressed in hypothalamic NPY/AgRP and POMC neurons that are known to mediate leptin and insulin action in regulation of energy and glucose homeostasis (Schwartz *et al.* 1992, Benoit *et al.* 2002, Morton *et al.* 2006, Sahu *et al.* 2011, Myers & Olson 2012, Timper & Bruning 2017), we measured gene expression of these neuropeptides. There was no change in hypothalamic *Npy*, *Agrp* or *Pomc* mRNA levels between genotypes of either sex on the LFD or HFD (Figs. 11A, 11C, 12A, 12C). Although there was a tendency of an increase in *Pomc* mRNA levels in female *Pde3b*^{Nkx2.1}KD mice fed with the HFD, it did not reach significant ($p = 0.0798$, Fig 12C). There was also no change in *Npy* or *Pomc* gene expression between genotypes in the hypothalamus of standard chow fed males under fasting condition (Fig 13A). However, *Agrp* gene expression was increased ($p = 0.0567$) in standard chow fed male *Pde3b*^{Nkx2.1}KD mice under fasting condition compared with WT mice (Fig 13A).

To address if gluconeogenic gene expression was altered following partial ablation of *Pde3b* in the hypothalamus, we examined *Pck1* and *G6pc* gene expression in the liver. In male LFD-fed group, there was no change in *Pck1* or *G6pc* mRNA levels between genotypes (Fig 11B). In male HFD-fed group, however, both *Pck1* ($p = 0.0086$) and *G6pc* ($p = 0.03$) mRNA levels were significantly decreased in *Pde3b*^{Nkx2.1}KD liver as compared to control liver (Fig. 11D). In females, there was no change in either *Pck1* or *G6pc* mRNA levels in the liver between genotypes (Fig. 12 B and D). Since UCP1 is involved in thermogenesis and energy expenditure (Inokuma *et al.* 2006, Sugimoto *et al.* 2014), we examined if *Ucp1* gene expression was altered in BAT of female *Pde3b*^{Nkx2.1}KD mice, which could explain increased body weight (Fig 3 C and D) and food intake (Fig 5 A and B) seen in these animals. There was, however, no difference in *Ucp1* mRNA levels in the BAT between genotypes (Fig 12 B and D).

Discussion

The PDE3B pathway has been demonstrated to mediate both leptin and insulin signaling in the hypothalamus (Zhao *et al.* 2002, Sahu 2011, Sahu *et al.* 2017), yet the physiological role of this pathway in regulation of energy homeostasis is completely unknown. To this end, in the present study we aimed to unravel the *in vivo* metabolic consequences of disrupting PDE3B signaling in the hypothalamus. For this purpose, we employed the *Cre-loxP* strategy and crossed *Pde3b*^{fl/fl} mice, which have *loxP* sites flanking exon 3 of the *Pde3b* gene, with *Nkx2.1-Cre* mice that mainly express Cre recombinase in the hypothalamic neurons (Xu *et al.* 2008). Thus, resulting *Pde3b*^{Nkx2.1}KO mice should specifically lack *Pde3b* in the hypothalamus. Unexpectedly, PCR and western blot analysis of *Pde3b*^{Nkx2.1}KO hypothalamus showed only ~50% decrease in *Pde3b* gene expression and PDE3B protein levels in the hypothalamus, suggesting a partial ablation of *Pde3b* in these mice and

therefore we named these mice as *Pde3b*^{Nkx2.1}KD mice indicating *Pde3b* knockdown in the hypothalamus. Notably, a previous study using *Nkx2.1-Cre* mice reported ~75% decrease in FoxO1 in *FoxO1*^{fl/fl} *Nkx2.1-cre* mice (Heinrich *et al.* 2014). The reason of different degree of Cre recombination between the studies is unknown but it could be due to strain differences. Also, the partial PDE3B knockdown could be due to the possibility that there are non-Nkx2.1-cre expressing PDE3B neurons or PDE3B is expressed non-neuronal population such as glia in the hypothalamus. The later possibility is unlikely, because glial population in the hypothalamus do not express PDE3B (Sahu & Sahu, 2015). Notably, *Nkx2.1-Cre* mice have been used in combination with specific *floxed* mice to knock out various signals such as leptin receptor (Ring & Zeltser, 2010) and FoxO1 (Heinrich *et al.* 2014) in the hypothalamus.

Our pharmacological studies with a specific PDE3 inhibitor, cilostamide, demonstrated a role for the PDE3B pathway in mediating anorectic and body weight-reducing effects of both leptin and insulin (Zhao *et al.* 2002, Sahu 2011, Sahu *et al.* 2017). In addition, *Pde3b-null* mice show increased bodyweight and impaired glucose homeostasis (Choi *et al.* 2006). Thus, despite having partial conditional knockout, we assessed whether *Pde3b*^{Nkx2.1}KD mice might exhibit altered food intake, body weight and glucose homeostasis. Our results demonstrate a sex-specific alteration in body weight in that in males on the LFD, but not on the HFD, a small increase in body weight was evident only at 4-6 weeks of age and there was no difference in the body weight thereafter. The mechanisms behind this early increase in body weight in male *Pde3b*^{Nkx2.1}KD mice are currently unknown, but it could be related to increased growth during this period. In females, however body weight was significantly increased approximately at 13 weeks and 15 weeks of age in the LFD-fed and HFD-fed groups, respectively. Interestingly, although increased in body weight in LFD-fed female *Pde3b*^{Nkx2.1}KD mice was associated with an increase in food intake, fat pad weights were decreased in association with reduced circulating leptin levels. These results suggest the possibility of an increased lean mass in these mice, which however require further investigation. Other possibility of increased body weight in female *Pde3b*^{Nkx2.1}KD mice could be due to decreased energy expenditure in association with increased food intake. Since *Ucp1* gene expression in the BAT of females on the LFD was not different between genotypes, it is likely that there was no change in energy expenditure, which needs to be confirmed using indirect calorimetry. It also remains to be determined if UCP1 protein level in the BAT was increased in the mutant, because UCP1 mRNA and protein levels may not always corroborate each other (Nedergaard & Cannon 2013). Notably, female *Pde3b-null* mice fed regular chow also show increased body weight in association with decreased fat pads weight but without any change in circulating leptin levels (Choi *et al.* 2006). Thus, it is likely that changes in body weight and fat pad weights seen in *Pde3b-null* mice could be, at least partly, due to a loss of *Pde3b* in the hypothalamus. On the other hand, increased body weight in female *Pde3b*^{Nkx2.1}KD mice fed HFD was not associated with any change in food intake, fat pad weight, or circulating leptin levels. Because PDE3B pathway plays a significant role in mediating the actions of both leptin and insulin in the hypothalamus in regulation of body weight and food intake (Zhao *et al.* 2002, Sahu 2011, Sahu *et al.* 2017), it is most likely that increased body weight seen in females could be due to reduced leptin and/or insulin signaling through this pathway. Overall, increased body weight in females is

consistent with the notion that hypothalamic PDE3B pathway plays an important role in energy homeostasis. Our results also show a sex- and diet-specific response in body weight changes in *Pde3b*^{Nkx2.1}KD mice. Since estrogen is one of the major factors that act in the hypothalamus to regulate food intake and body weight (Lopez & Tena-sempere 2015), we speculate that, like leptin and insulin, estrogen could also use PDE3B pathway to exert its effect on food intake and body weight, and therefore, decrease in PDE3B could reduce the action of estrogen resulting in increased bodyweight and food intake seen specifically in female *Pde3b*^{Nkx2.1}KD mice. Although both leptin and estrogen use hypothalamic STAT3 pathway to reduce food intake and body weight (Gao et al. 2007, Gao & Horvath 2008), it remains to be determined if, estrogen, like leptin, also uses hypothalamic PDE3B-cAMP pathway to regulate energy homeostasis; and whether ovariectomy modifies energy balance in *Pde3b*^{Nkx2.1}KD mice.

A role of PDE3B in glucose homeostasis has been evident *in vivo* studies using *Pde3b*-null mice (Choi *et al.* 2006). The question of whether hypothalamic PDE3B plays any role in this process has not been addressed before. In this study, we attempted to address this issue in mice with partial ablation of *Pde3b* specifically in the hypothalamus. Our data suggest a sex specific changes in glucose homeostasis in that *ad libitum*-fed glucose levels were increased in male but decreased in female *Pde3b*^{Nkx2.1}KD mice as compared to their respective WT mice. These changes were only evident when the mice were on the LFD but not HFD. It is also complicated by the fact that decreased glucose levels were associated with increased body weight in females and increased glucose levels were associated with no change in body weight in male *Pde3b*^{Nkx2.1}KD mice. However, it remains to be determined whether a possible increase in lean mass due to association of increased body weight and decreased fat mass in female *Pde3b*^{Nkx2.1}KD mice could be involved in lowering glucose levels in *ad-lib* fed animals. Nevertheless, the effect of PDE3B knockdown in the hypothalamus on glucose homeostasis was more apparent from GTT and ITT. Thus, clearance of glucose during GTT was significantly increased only in HFD-fed but not in LFD-fed male *Pde3b*^{Nkx2.1}KD mice. Also, insulin's effectiveness in reducing blood glucose levels during ITT was increased mainly in LFD-fed male *Pde3b*^{Nkx2.1}KD mice. In male *Pde3b*-null mice, in contrast, insulin is reported to be much less effective in reducing blood glucose levels during ITT (Choi *et al.* 2006). The differences in insulin's effectiveness in reducing blood glucose levels between *Pde3b*^{Nkx2.1}KD and *Pde3b*-null mice are most likely due to the level of *Pde3b* deficiency with one specific to the hypothalamus and the other is whole body knockout including the liver and adipose tissue. On the other hand, female mice did not show any changes in glucose clearance during GTT or ITT. These results collectively suggest an improved glucose homeostasis in male but not in female *Pde3b*^{Nkx2.1}KD mice. Specifically, glucose tolerance and insulin sensitivity were improved in male *Pde3b*^{Nkx2.1}KD mice. This was also supported by decreased expression of gluconeogenic genes, *Pck1* and *G6pc*, in the liver of HFD-fed male *Pde3b*^{Nkx2.1}KD mice, without any change in female mice. Although hepatic insulin sensitivity was not directly examined, decreased *Pck1* and *G6pc* mRNA levels suggests increased insulin sensitivity in the liver of HFD-fed male mice. Notably, LFD-fed male *Pde3b*^{Nkx2.1}KD mice had no change in expression of either *Pck1* or *G6pc* gene. Nevertheless, improved glucose homeostasis particularly in HFD-fed male *Pde3b*^{Nkx2.1}KD mice is quite significant, because it suggests that glucose homeostasis could be improved in

HFD-fed obese mice by manipulation of PDE3B in hypothalamic neurons, and provides evidence in support of an important role for hypothalamic PDE3B in glucose homeostasis.

Among various signals that regulate energy and glucose homeostasis, AMP-activated protein kinase (AMPK) appears to be the most important sensor of cellular energy in various tissues including the hypothalamus (Lopez et al. 2016). Thus, increase in hypothalamic AMPK activity by orexigenic signals is associated with increased feeding and weight gain; and the signals, such as leptin, insulin, GLP-1, estradiol, etc., that decrease feeding and cause weight loss also decrease hypothalamic AMPK activity (Lopez et al. 2016). Recently, it has been reported that AMPK activity was increased in the adipocytes of *Pde3b*-null mice (Chung et al. 2017). Thus, it is tempting to speculate that decrease in PDE3B in *Pde3b*^{Nkx2.1KD} mice could increase hypothalamic AMPK activity leading to increased food intake and body weight seen in female *Pde3b*^{Nkx2.1KD} mice and improved glucose tolerance and insulin sensitivity in male *Pde3b*^{Nkx2.1KD} mice. Future studies should address if there is a connection between PDE3B and AMPK pathways in the hypothalamus in regulation of energy and glucose homeostasis.

A role of hypothalamic NPY, AgRP and POMC neurons, that also co-express PDE3B (Sahu et al. 2011), in energy homeostasis is well established (Schwartz et al. 2000, Sahu 2003, Morton et al. 2006, Myers & Olson 2012, Timper & Bruning 2017). Therefore, we investigated if there was any change in expression of *Npy*, *Agrp* and *Pomc* genes in the hypothalamus of *Pde3b*^{Nkx2.1KD} mice. Our demonstration of no changes in expression of these genes between the genotypes of either sex when they were on the LFD or HFD suggest that changes in food intake and body weight seen specifically in female *Pde3b*^{Nkx2.1KD} mice were not due to changes in hypothalamic *Npy*, *Agrp* or *Pomc* gene expression. Under fasting condition however there was an increase in *Agrp* gene expression in the male *Pde3b*^{Nkx2.1KD} mice that were on standard chow diet suggesting a role of PDE3B in AgRP neuronal activity. Because *Pde3b* was deleted only partially in the hypothalamus, future study should address if greater deficiency of *Pde3b* in the hypothalamus could produce significant alteration in *Npy*, *Agrp* or *Pomc* gene expression resulting in different phenotypes, or if deletion of *Pde3b* specifically in NPY/AgRP or POMC neurons could alter energy and glucose homeostasis differently than seen after partial ablation of *Pde3b* in the hypothalamus. Thus, to address further the role of hypothalamic PDE3B in energy and glucose homeostasis, it will be necessary to delete *Pde3b* in specific neurons such as NPY/AgRP, POMC or Steroidogenic Factor 1-positive neurons in the hypothalamus using *Pde3b*^{fl/fl} and specific Cre-expressing mice or to generate conditional *Pde3b* knockdown in adult hypothalamus using tamoxifen-inducible Cre system or viral approaches to rule out any compensatory mechanism that might develop during the developmental period using the Cre-expressing animals. Availability of *Pde3b*^{fl/fl} mice is therefore extremely valuable to pursue these future studies.

In summary, our study shows a sex-specific response in body weight and glucose homeostasis following *Pde3b* knockdown in the hypothalamus. Specifically, glucose homeostasis was improved in male and body weight was increased in female mutants. Overall, these findings support a role, at least in part, for the hypothalamic PDE3B in regulation of energy and glucose homeostasis, and warrant future investigation with animals

having complete knock-out or greater deficiency in *Pde3b* specifically in the hypothalamus. Because *Pde3b*^{Nkx2.1}KD mice are partial knockdown models of hypothalamic PDE3B, it could potentially underestimate the physiological role of hypothalamic PDE3B in energy and glucose homeostasis. Nevertheless, improved glucose homeostasis specifically in HFD-fed obese male *Pde3b*^{Nkx2.1}KD mice signifies that targeting hypothalamic PDE3B could be a viable therapeutic approach to improve glucose homeostasis in diet-induced male obese individuals.

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Figure 1.

Strategy for conditional inactivation of the *Pde3b* gene in specific neurons. A. Schematic representation of the *Pde3b*-targeted ES clone with conditional *Pde3b* allele (*Pde3b^{fllox-neo}*) (a), the conditional *Pde3b* allele after excision of the En2SA-Neo cassette by FLP recombinase (b), and the null *Pde3b* allele after Cre-mediated deletion (c). Legend: Boxes (E): coding exons. B. Confirmation of the floxed *Pde3b* allele in DNA prepared from tail clips of pups obtained from breeding *Pde3b^{fl/+}* mice. MW: 100-bp ladder; WT: wild-type (~218 bp); *fl/+*: *Pde3b^{fl/+}* (~315 bp); *fl/fl*: *Pde3b^{fl/fl}* littermates. C. *Pde3b* deletion by Nkx2.1-cre: Ethidium bromide staining of 1% agarose gel showing delta band (~405 bp) and loxP (*fl/fl*; ~315 bp) bands in *Pde3b^{Nkx2.1KD}* (Cre +) and *fl/fl* (Cre -) mice. Total RNA was isolated from tissues followed by reverse transcription and 40 cycles of PCR followed by electrophoresis in agarose gel. Delta bands were only seen in the medial basal hypothalamus (MBH) and cortex (Cort, very faint band), but not in other tissues. Cereb: cerebellum; BStem: brain stem; WAT: gonadal fat; MW: 100-bp ladder. 100 ng of cDNA was used for PCR.

Figure 2.

Partial *Pde3b* deletion by *Nkx2.1-cre* in the medial basal hypothalamus (MBH, A-C) but not in the Liver (D). A, Representative PDE3B and β -actin Western blots obtained from the hypothalamic extract. B, Densitometric analysis of the immunoreactive bands for PDE3B. The values for PDE3B were expressed as ratio of β -actin. C and D, Changes in *Pde3b* mRNA levels in the MBH (C) and liver (D) of male WT (*fl/fl*) and KD (*Pde3b*^{Nkx2.1}KD) mice as determined by qPCR. Values represent the mean $\pm$ SEM for the number animals per group as indicated in parentheses. * $p = 0.0073$, ** $p < 0.0001$ vs WT control group.

Figure 3.

Changes in body weight in male and female WT (*fl/fl*) and KD (*Pde3b*^{Nkx2.1}KD) mice fed with a low-fat F6 diet (LFD) or a high-fat diet (HFD) for 17–18 weeks. Mice were exposed to LFD or HFD at the age of 4 weeks. Values represent the mean $\pm$ SEM for the number of animals per group as indicated in parentheses. * $p < 0.05$, ** $p < 0.01$ vs WT control group.

Figure 4.

Changes in food intake and plasma glucose levels, and in fat pad and liver weights in male WT (*fl/fl*) and KD (*Pde3b*^{Nkx2.1}KD) mice fed with a LFD or HFD for 17–18 weeks. Mice were exposed to a LFD or HFD at the age of 4 weeks. A, Food intake; B, epididymal fat (WAT) weights; C, retroperitoneal (RP)-fat weights, D, brown adipose tissue (BAT) weight; E, liver weights, and F, fed plasma glucose levels in LFD-fed mice (n = 8 for WT and 10 for KD mice per group). G, Food intake; H, WAT weights; I, RP-fat weights, J, BAT weights; K, liver weights, and L, fed plasma glucose levels in HFD-fed mice (n = 8 for WT and 5 for KD mice per group). Values represent the mean ± SEM.

Figure 5.

Changes in food intake and plasma glucose levels, and in fat pad and liver weights in female WT (*fl/fl*) and KD (*Pde3b*^{Nkx2.1} KD) mice fed with a LFD or HFD for 17–18 weeks. Mice were exposed to a LFD or HFD at the age of 4 weeks. A, Food intake; B, Food intake per gram body weight (BW); C, gonadal fat pad weights; D, gonadal fat pad weight per gram BW; E, RP-fat weights, F, BAT weight; G, BAT weight per gram BW; H, liver weights, and I, fed plasma glucose levels in LFD-fed mice (n = 11–12 for WT and 13 for KD mice per group). J, Food intake; K, gonadal fat pad weights; L, RP-fat weights, M, BAT weight; N, liver weights, and O, fed plasma glucose levels in HFD-fed mice (n = 8 for WT and 7 for KD mice per group). Values represent the mean $\pm$ SEM.

Figure 6.

Changes in blood glucose levels in overnight fasted WT (*fl/fl*) and KD (*Pde3b^{Nkx2.1}*KD) mice on a LFD or HFD. A and B, glucose levels in males on the LFD (16 wk) or HFD (16.5 wk), respectively. C and D, glucose levels in females on the LFD (17 wk) or HFD (15 wk), respectively. Values represent the mean $\pm$ SEM for the number of animals per group as indicated in parentheses.

Figure 7.

Changes in glucose tolerance (GTT) and insulin tolerance (ITT) in male WT (*fl/fl*) and KD (*Pde3b^{Nkx2.1}*KD) mice fed with a on a LFD or HFD. A, GTT in overnight fasted mice at 13 wk on the diet. B, ITTs in 2-h fasted mice at 14 wk on the diet. C, Area under the curve (AUC) for ITT in animals on LFD. D, GTTs in overnight fasted mice that were on the HFD for 15 wk. E, Area under the curve (AUC) for GTT in animals on the HFD. F, ITTs in 2-h fasted mice at 14 wk on the diet. G, AUC for the ITT in the HFD-fed mice. Values represent the mean $\pm$ SEM for the number of animals per group as indicated in parentheses. * $p < 0.05$, ** $p < 0.01$ vs KD group.

Figure 8.

Changes in glucose tolerance (GTT) and insulin tolerance (ITT) in female WT (*fl/fl*) and KD (*Pde3b*^{Nkx2.1}KD) mice fed with a LFD or HFD. A, GTTs in overnight fasted mice at 15 wk on the diet. B, ITTs in 2-h fasted mice at 16 wk on the diet. C, GTTs in overnight fasted mice that were on the HFD for 13 wk. D, ITTs in 2-h fasted mice at 14 wk on the diet.

Values represent the mean $\pm$ SEM for the number of animals per group as indicated in parentheses.

Figure 9. Changes in circulating insulin levels in overnight fasted WT (*fl/fl*) and KD (*Pde3b^{Nkx2.1}*KD) mice on a LFD or HFD. A and B, Plasma insulin levels in females on a LFD (17 wk) or HFD (15 wk), respectively. C and D, Plasma insulin levels in males on a LFD (16 wk) or HFD (16.5 wk), respectively. Values represent the mean $\pm$ SEM for the number of animals per group as indicated in parentheses.

Figure 10.

Changes in circulating leptin levels in *ad-libitum* fed WT (*fl/fl*) and KD (*Pde3b*^{Nkx2.1}KD) mice on a LFD or HFD. A and B, Plasma leptin levels in females on a LFD or HFD, respectively. C and D, Plasma leptin levels in males on a LFD or HFD, respectively. Values represent the mean $\pm$ SEM for the number of animals per group as indicated in parentheses.

Figure 11.

Changes in hypothalamic *AgRP*, *NPY* and *POMC* mRNA levels (A, C) and in liver *PCK1* and *G6PC* mRNA levels (B, D) in male *ad-libitum* fed WT (*fl/fl*) and KD (*Pde3b*^{NKx2.1}KD) mice on a LFD (A, B) or HFD (C, D) as determined by qPCR. Values represent the mean $\pm$ SEM for the number of animals per group as indicated in parentheses.

Figure 12.

Changes in hypothalamic *Agrp*, *Npy* and *Pomc* mRNA levels (A) and in liver *Pck1* and *G6pc* mRNA levels (B) in female *ad-libitum* fed WT (*fl/fl*) and KD (*Pde3b*^{Nkx2.1} KD) mice on a LFD (A, B) or HFD (C, D) as determined by qPCR. Values represent the mean $\pm$ SEM for the number of animals per group as indicated in parentheses.

Figure 13.

Changes in hypothalamic *Agrp*, *Npy* and *Pomc* mRNA levels (A) and in liver *Pck1* and *G6pc* mRNA levels (B) in male *ad-libitum* fed WT (*fl/fl*) and KD (*Pde3b*^{Nkx2.1}KD) mice on a standard chow RMH diet as determined by qPCR. Values represent the mean $\pm$ SEM for the number of animals per group as indicated in parentheses.