The BBSome in POMC and AgRP Neurons is Necessary for Body Weight Regulation and Sorting of Metabolic Receptors

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ABSTRACT

The BBSome, a complex of eight Bardet-Biedl syndrome (BBS) proteins involved in cilia function, has emerged as an important regulator of energy balance, but the underlying cellular and molecular mechanisms are not fully understood. Here, we show that the control of energy homeostasis by the anorexigenic proopiomelanocortin (POMC) neurons and orexigenic agouti-related peptide (AgRP) neurons require intact BBSome. Targeted disruption of the BBSome by Bbs1 gene deletion in POMC or AgRP neurons increases body weight and adiposity. We demonstrate that obesity in mice lacking the Bbs1 gene in POMC neurons is associated with hyperphagia. Mechanistically, we present evidence implicating the BBSome in the trafficking of G protein coupled neuropeptide Y Y2 receptor (NPY2R) and serotonin 5-HT2C receptor (5-HT2cR) to cilia and plasma membrane, respectively. Consistent with this, loss of the BBSome reduced cell surface expression of the 5-HT2cR and interfered with serotonin-evoked increase in intracellular calcium and membrane potential and blunted the anorectic and weight-reducing responses evoked by the 5HT2cR agonist, lorcaserin. Finally, we show that disruption of the BBSome causes the 5-HT2cR to be stalled in the late endosome. Our results demonstrate the significance of the hypothalamic BBSome for the control of energy balance through regulation of trafficking of important metabolic receptors.
INTRODUCTION

Genetic studies have consistently pointed to the central nervous system as a key player in the regulation of body weight and the development of obesity and its adverse side effects (1, 2). This is consistent with the tremendous influence the brain exerts on the processes that control food intake and energy expenditure (3, 4). The brain receives afferent signals about the status of body energy reserves leading to autonomic, humoral and behavioral adaptations aimed at maintaining body weight and adiposity in a normal range. These neuroendocrine systems are tightly controlled, and their disruption leads to obesity.

The distribution and mechanism of action of the key signals that influence metabolic functions point to a significant role of the hypothalamus in integrating the external and internal signals and in enacting appropriate and consequential metabolic and behavioral responses to maintain energy balance (3, 4). Anorexigenic neurons expressing proopiomelanocortin (POMC) and orexigenic agouti-related peptide (AgRP)-expressing neurons located in the hypothalamic arcuate nucleus are critically involved in the control of feeding and energy expenditure. These neurons are considered as first-order neurons that mediate the effects of various signals such as leptin, insulin and serotonin (3, 5-7). Despite being the most extensively studied neuronal populations regarding energy balance our understanding of the molecular processes controlling their activity remains incomplete.

The BBSome is a complex composed of eight Bardet-Biedl syndrome (BBS) proteins (BBS1, BBS2, BBS4, BBS5, BBS7, BBS8, BBS9 and BBS18) (8, 9). The assembly of
the BBSome depends on another protein complex that contains three BBS proteins (BBS6, BBS10, BBS12) (10, 11). The discovery that various BBS proteins interact to form complexes that are critical is consistent with the overlapping phenotypes arising from mutations in different BBS genes (11). Indeed, mutation in BBS genes results in human diseases with multiple features including obesity. In addition, variants of several BBS genes were found to increase susceptibility to obesity in non-BBS individuals (12-14). Together, these findings highlight the importance of the BBSome for energy balance and the development of obesity, but the underlying mechanisms are not well understood.

The BBSome is a critical regulator of cilia function by targeting cargos to the ciliary membrane. Primary cilia are important signaling organelles present in virtually every cell type in the body including neurons (15). A number of G protein-coupled receptors including somatostatin receptor 3 (SSTR3) (16), neuropeptide Y Y2 receptor (NPY2R) (17) and melanocortin-4 receptor localize to cilia (18). In addition to its involvement in the trafficking of cargos to cilia the BBSome has emerged as a key mediator of many cellular processes including the delivery of receptors to the plasma membrane (19, 20).

Here, we reveal an important role of the BBSome in the control of energy balance by POMC and AgRP neurons. We show that selective disruption the BBSome through Bbs1 gene deletion in POMC or AgRP neurons led to a significant increase in body weight and adiposity. We also uncovered a critical role of the BBSome in the cellular trafficking of serotonin 5-HT2C receptor (5-HT2C-R), an important receptor for energy homeostasis and the target of anti-obesity drug lorcaserin. Our data demonstrate that
the BBSome is required for 5-HT$_{2C}$R trafficking to the plasma membrane providing a molecular explanation for the obesity phenotype evoked by disruption of the BBSome.

RESEARCH DESIGN AND METHODS

Animals

All animal testing was approved by The University of Iowa Animal Care and Use Committee. Mice were housed in groups of 3-5 per cage and maintained on 12-h light-dark cycle with lights on at 6 am. Room temperature was maintained at 22°C. Food and water were available ad libitum.

To obtain selective deletion of the Bbs1 gene in POMC or AgRP neurons, male transgenic POMC$^{Cre}$ or AgRP$^{Cre}$ mice (Jackson Laboratory, stock# 005965 and 012899, respectively) were crossed with Bbs1$^{fl/fl}$ female mice (20). POMC$^{Cre}$/Bbs1$^{fl/wt}$ and AgRP$^{Cre}$/Bbs1$^{fl/wt}$ offspring were subsequently crossed with Bbs1$^{fl/wt}$ mice to generate POMC$^{Cre}$/Bbs1$^{fl/fl}$ and AgRP$^{Cre}$/Bbs1$^{fl/fl}$ mice, respectively. The genotype of mice was determined by PCR analysis of tail DNA (20) using primer sequences provided in Supplementary Table 1. A group of diet-induced obese C57BL/6J mice fed a high fat diet (Research Diet, Inc. D12492) for 12 weeks and controls on normal chow were obtained from the Jackson Laboratory.

Metabolic studies

Mice were weighed once a week, beginning at weaning (4 weeks of age) until 25 weeks of age. Body composition was determined by Magnetic Resonance Imaging (MRI) under anesthesia (91 mg/kg ketamine and 9.1 mg/kg xylazine, i.p.) using a Varian
Unity/Inova 4.7 T small-bore MRI system (Varian Inc.). To measure food intake, mice were housed individually in regular cages and allowed to acclimate for at least 3 days prior to measurements of 24-hour food intake for 5 days. Resting metabolic rate was measured by push-pull respirometry at thermoneutrality during the day time as described previously (20).

Food intake and body weight response to lorcaserin were compared between control animals, POMC^{Cre}/Bbs1^{fl/fl} mice and diet-induced obese mice. Baseline body weight and food intake of individually housed mice were measured daily for 5 consecutive days before beginning treatment with vehicle or lorcaserin (1 \mu g/\mu l/g body weight i.p., twice per day at 8 am and 5 pm, for 4 days).

**Glucose and insulin tolerance tests and insulin measurements**

Glucose tolerance test (GTT) and insulin tolerance test (ITT) were performed as described previously. Mice were fasted overnight (GTT) or for 5 h (ITT) in a clean cage. Basal blood glucose measurements were taken before intraperitoneal injection of glucose (2 g/kg) or insulin (1 U/kg). Blood glucose measurements were assessed at multiple time points during the two-hour test. Plasma insulin levels were measured using an ELISA kit (RayBiotech).

**Generation of mouse IMCD3 Bbs1 knockout cell line**

The human codon-optimized Cas9 plasmid pX459 (Addgene # 48139) was obtained from Addgene. sgRNAs were designed and constructed as described previously. Briefly, target 20 bp sequences starting with guanine and preceding the PAM motif (5′-NGG-3′) was selected from mouse Bbs1 exon 4. Potential off-target effects of sgRNA
candidates were analyzed using the online tool CRISPR Design (http:// crispr.mit.edu/). One sgRNA in exon 4 (Supplemental Table 1) of the mouse Bbs1 gene was selected for transfection to create knockout cell lines. Clonal genomic DNA was extracted and used for PCR and Sanger sequencing with the forward primer and the reverse primer (Supplemental Table 1). This resulted in cell lines with homozygous 1 bp T insertions and homozygous 2bp deletions (del CT) in exon 4.

Mouse kidney epithelial cells (mIMCC-3, ATCC# CRL-2123) were cultured in DMEM/F12 (Life Technologies) supplemented with 10% FCS. Cells were seeded in a 6-well plate at 1 x 10^6 cells/well. Transfection was performed with Lipofectamine 2000 using recommended conditions. Briefly, 2 µg of plasmid containing a sgRNA targeting Bbs1 exon 4 and 6 µl Lipofectamine 2000 were diluted in 100 µl Opti-MEM, mixed 1:1 and added to cells after 5 min incubation. After 24 hours the cells were passed at low density (2000 cells/well) in 10 cm culture dishes and selected with puromycin at 1.5 µg/ml for clonal expansion. The media was changed every 2 days until colonies were harvested using cloning cylinders.

**Measurement of intracellular Ca^{2+}**

IMCD3 and IMCD3-Bbs1^{-/-} cells were transiently transfected with GFP-tagged 5-HT_{2C}R. Forty eight hours post transfection, the cells were loaded with fura-2 using the acetoxyethyl ester form of the indicator (fura-2/AM; 2 µM for 30 min) to monitor changes in intracellular Ca^{2+} concentration ([Ca^{2+}]_i) in response to 5 µM serotonin, and then to 10 µM ATP. The cells were placed in a chamber for flow through perfusion and mounted on an inverted IX-71 microscope (Olympus) and were perfused with Hepes-
buffered Hanks balanced salt solution (HBSS) composed of 140 mM NaCl, 5 mM KCl, 1.3 mM CaCl$_2$, 0.4 mM MgSO$_4$, 0.5 mM MgCl$_2$, 0.4 mM KH$_2$PO$_4$, 0.6 mM NaHPO$_4$, 3 mM NaHCO$_3$, 10 mM glucose and 10 mM Hepes, pH 7.4 with NaOH (310 mOsm/kg with sucrose). Fluorescence was excited sequentially at 340 nm (12 nm bandpass) and 380 nm (12 nm bandpass), using a Polychrome V monochromator (TILL Photonics) and focused on the cells via a 20X, NA=0.75 objective (Olympus). Fluorescence emission was measured at 540 nm (50 nm bandpass) using an IMAGO charge-coupled device camera (640 × 480 pixels; TILL Photonics). 2×2 binning was used for acquisition (1 pixel ∼500 nm). A series of 340 nm and 380 nm images was acquired every 2 seconds. ATP (10 μM) was also used as a positive control. [Ca$^{2+}$], data were analyzed using TILLvisiON (TILL Photonics) software and presented as $F_{340}/F_{380}$ ratio, where $F_{340}$ and $F_{380}$ correspond to the fluorescence intensity at excitation wavelength 340 and 380 nm, respectively. The $F_{340}$ and $F_{380}$ values were corrected for background, which was determined for each experiment by measuring fluorescence at 340 and 380 nm in an area free of cells.

**Electrophysiological recording**

HEK 293 cells were used to measure serotonin-induced membrane potential. Briefly, the cells were co-transfected with control shRNA or $Bbs1$-shRNA with GFP-tagged 5-HT$_{2c}$R. Seventy-two hours post-transfection, cells were placed in a perfusion chamber mounted on the stage of an upright Zeiss Axioskop FS2 microscope (Carl Zeiss) equipped with a Hamamatsu C2741-60 charged-coupled device camera (Hamamatsu Photonics). Epifluorescence was briefly used to target fluorescent cells, after which the
light source was switched to infrared differential interference contrast imaging to allow visualization while obtaining whole-cell patch clamp recordings. Membrane potentials were recorded in current clamp mode using an Axopatch 700B amplifier (Molecular Devices), low-pass filtered at 1 kHz, digitized at 10 kHz and analyzed offline on a PC with pCLAMP (Molecular Devices) and Origin software (OriginLab). Recording electrodes had resistances of 2.5–5 MΩ when filled with the K-gluconate pipette solution which contained 120 mM K-gluconate, 10 mM KCl, 10 mM HEPES, 5 mM EGTA, 1 mM CaCl$_2$, 1 mM MgCl$_2$, and 5 mM Mg-ATP adjusted to pH 7.3.

**Biochemical analysis**

To detect cell surface receptor expression, cell surface biotinylation assays were performed in HEK 293 cells transiently transfected with the pcDNA3-Flag-5-HT$_{2C}$R receptor (Flag-5-HT$_{2C}$R), with Bbs2-shRNA or control shRNA on an LKO1 plasmid with X-tremeGENE 9 transfection reagent (Roche). Silencing efficacy of Bbs2-shRNA was reported previously (21).

Forty-eight hours post transfection, the cells were incubated with cleavable EZ-Link Sulfo-NHS-SS-Biotin (Thermo Scientific) for 30 minutes on ice, and biotinylated proteins were recovered by using streptavidin-agarose beads (GE Healthcare). Beads were resuspended in Laemmli buffer, and bound proteins were separated by SDS-PAGE and detected by immunoblotting using mouse M2 anti-Flag (Sigma, F1804) to detect 5-HT$_{2C}$R and mouse anti-transferrin receptor (Invitrogen Inc.) monoclonal antibody.

To detect interaction between BBS1 or other BBSome proteins and CD63, HEK 293 cells were transiently transfected with HA-tagged BBS1, BBS2, BBS4, BBS5, BBS7,
BBS8 or BBS9 plasmids with control shRNA or Bbs2-shRNA. Forty-eight hours post-transfection, the cells were lysed with lysate buffer (50 mM HEPES, pH 7.5, 150 mM NaCl, 1 mM MgCl₂, 1 mM CaCl₂, 10 mM NaF, 5 mM EDTA, 1% Triton, 2 mM sodium orthovanadate and Roche cocktail protease inhibitor tablet). Cellular protein samples (800 µg) were subjected to immunoprecipitation assay with 5 µg anti-HA (Roche Applied Science, 11815016001) antibody. Immunocomplex was separated on a 9% SDS-PAGE gel, transferred to PVDF membrane, then probed with anti-CD63 antibody (SBI system Biosciences, EXOAB-CD63A-1, 1:1000), followed by either a secondary anti-rabbit HRP goat antibody (Cell Signaling, 7074, 1:10000) or a goat anti-mouse HRP (Cell Signaling, 7076, 1:10000). Protein expression was visualized with ECL detection kit (GE healthcare) and exposed to film.

Gene expression analysis

Mice were sacrificed by CO₂ asphyxiation and brain quickly isolated and put on ice. The mediobasal hypothalamus was carefully dissected and total RNA isolated using RNeasy Plus Mini Kit from Qiagen. Total RNA (2 µg in final volume of 40 µl) was used to synthesize first-strand cDNAs with the Super-Script pre-amplification system. Then, 10 µl of cDNA and 0.4 mmol/L of primers were added in a final volume of 25 µl PCR mixture (iQ SYBR Green supermix, Bio-Rad), and amplified in a iQ5 Multicolor Real Time PCR Detection System (Bio-Rad). The PCR conditions for all genes were as follow: denaturation for 5 min at 95°C, then 40 cycles for 30 seconds at 95°C and 30 seconds at 60°C. S18 ribosomal RNA expression was used as internal control to normalize mRNA expression of these genes. Primer sequences are provided in Supplementary Table 1.
**RNAscope**

To detect Bbs1 and POMC mRNA, the brain was freshly harvested and put into OCT solution. The brain was sectioned with cryostat and the brain sections were hybridized with specific probes purchased from Advanced Cell Diagnostics (491121 for Bbs1 and 314081-C2 for POMC). Processed brain sections were mounted using VectaShield® mounting medium with DAPI. Images were visualized using confocal microscopy (Zeiss LSM710) and analyzed using Image J software.

**Immunofluorescence**

Mice anesthetized with ketamine and xylazine were perfused with PBS (5 ml/min; 50 ml) followed by 4% paraformaldehyde/HistoChoice Tissue Fixative (Amresco) in PBS (2.5 ml/min; 50 ml) using Harvard PHD 22/2000 Syringe Pump. Entire brain was excised and incubated in the same fixative overnight at 4°C. Fixed brains were washed 3 times with PBS and incubated in 30% sucrose/PBS overnight with one change of solution after 4-6 hrs of initial incubation. Brains were vibratome-sectioned with 30 µm thickness. Immunochemistry was performed on brain sections using antibodies against POMC (Phoenix Pharmaceuticals, H-029-30, 1:250), phosphorylated Stat3 (p-Stat3, Cell Signaling #9131S, 1:1000), 5-HT_{2C}R (Neuromics, RA24505, 1:250), NPY_{2}R (Neuromics, 14112, 1:250) or ACIII (Santa Cruz, sc-32113(N-14), 1:250 dilution) as described previously (20, 22). For p-Stat3 staining, mice were fasted overnight and treated with leptin (2 mg/kg, intraperitoneally) 1 hr before sacrifice. The anti-5-HT_{2C}R antibody was validated using cells expressing a GFP-tagged 5-HT_{2C}R and brains of 5-HT_{2C}R^{-/-} mice (provided by Dr. Joel Elmquist, University of Texas Southwestern Medical Center).
Processed brain sections were mounted using VectaShield® mounting medium with DAPI. Images were visualized using confocal microscopy (Zeiss LSM710) and analyzed using Image J software.

IMCD3 and IMCD3-\(Bbs1^{-/-}\) cells were transfected with GFP-tagged 5-HT\(\text{2C}\)R, NPY\(\text{2}\)R or SSTR\(\text{3}\) and cilia were stained with acetylated \(\alpha\)–tubulin (Santa Cruz Biotechnology, sc-23950). To detect 5-HT\(\text{2C}\)R or BBS1 subcellular localization in IMCD3-\(Bbs1^{-/-}\) cells, the cells were transfected with GFP-tagged-5-HT\(\text{2C}\)R or co-transfected with RFP-\(Bbs1\) with GFP-Rab5, GFP-Rab7, GFP-Golgi, then stained with early endosome marker EEA1 (Cell Signaling, 3288), Lysosomal marker Lamp1 (Cell Signaling, 9091), endosome marker Cav1 (Cell Signaling, 3267), Golgi marker RCAS1 (Cell Signaling, 12290) or late endosome marker CD63 (SBI system Biosciences, EXOAB-CD63A-1). HEK 293 cells were transiently co-transfected with GFP-tagged 5-HT\(\text{2C}\)R with control shRNA or \(Bbs2\)-shRNA, and the receptor was visualized after 48 hours of transfection.

**Data analysis**

The data are expressed as means ± SEM and analyzed using Student’s t-test or 1- or 2-way analysis of variance (ANOVA). When ANOVA reached significance, a post-hoc comparison was made using Fisher test. A \(P<0.05\) value was considered statistically significant. Univariate regression modeling with body weight considered a covariate was used to assess resting metabolic rate data using SPSS.
RESULTS

Selective deletion of the Bbs1 gene in POMC neurons cause obesity

In silico re-analysis of available single-cell RNA sequencing data sets from dissociated mouse hypothalamic cells (23) revealed that BBS genes are expressed in both POMC and AgRP neurons (Supplemental Fig. 1A-B). We have confirmed Bbs1 gene expression in POMC neurons using RNAscope (Supplemental Fig. 1C). Strikingly, fasting was found to cause contrasting changes in the expression of several BBS genes in POMC vs AgRP neurons (Supplemental Fig. 1A-B). To determine the relevance of the BBSome in these neuronal populations for energy homeostasis, we generated mice bearing selective deletion of the Bbs1 gene in POMC neurons (POMC\textsuperscript{Cre}/Bbs1\textsuperscript{fl/fl} mice) by breeding the conditional Bbs1\textsuperscript{fl/fl} mice with POMC\textsuperscript{Cre} mice. Further crossing with a reporter mouse model expressing fluorescent td-Tomato (ROSA), in a Cre-dependent manner, confirmed the Cre-mediated recombination in POMC neurons (Supplemental Fig. 2A-B). It should be noted that absence of the BBS1 protein disrupts BBSome formation and function (21). Furthermore and consistent with previous reports implicating the BBSome in leptin sensitivity and leptin receptor (LepRb) trafficking (20, 24), we found that leptin-induced Stat3 activation was blunted in td-Tomato positive (td-Tomato\textsuperscript{+}) cells of the arcuate nucleus of POMC\textsuperscript{cre}/Bbs1\textsuperscript{fl/fl} mice (Supplemental Fig. 2C).

Body weight was comparable between young (4-7 weeks old) POMC\textsuperscript{Cre}/Bbs1\textsuperscript{fl/fl} mice and littermate controls (Fig. 1A-B). However, at around 8 weeks of age body weight of both males and females begin to diverge with POMC\textsuperscript{Cre}/Bbs1\textsuperscript{fl/fl} mice gaining more weight than the controls. The divergence in body weight continued over time with
POMC^{Cre}\text{}/Bbs1^{fl/ff} mice weighing 10-17 g more than the control mice at 25 weeks of age (Fig. 1A-B). The increase in body weight in POMC^{Cre}/Bbs1^{fl/ff} mice was due to an increase in adiposity as indicated by the increased fat mass (Fig. 1C-D and Supplemental Fig. 2D-E). Liver mass was elevated in POMC^{Cre}/Bbs1^{fl/ff} mice relative to controls (females: 1.74 ±0.15 vs 1.12±0.09 g, P<0.05; males 2.94±0.3 vs 1.71±0.08 g, P<0.05). However, there was no difference in lean mass between POMC^{Cre}/Bbs1^{fl/ff} mice and controls (Fig. 1E).

Consistent with the obesity phenotype, POMC^{Cre}/Bbs1^{fl/ff} mice exhibited elevated food intake (Fig. 1F-G). In contrast, resting metabolic rate, corrected for body weight by univariate regression modeling (model p<0.001, body weight p<0.001), was comparable between POMC^{Cre}/Bbs1^{fl/ff} mice and controls (Fig. 1H; genotype p=0.912, sex p=0.501, interaction p=0.453). These findings indicate that obesity in mice lacking the Bbs1 gene in POMC neurons is driven by hyperphagia.

Circulating insulin levels were elevated in POMC^{Cre}/Bbs1^{fl/ff} mice (Fig. 1I-J). A significant increase in fasting blood glucose was also noted in POMC^{Cre}/Bbs1^{fl/ff} mice (114.7 ±7.7 mg/dL) compared to controls (77.1 ±6.8 mg/dL, P<0.05). Moreover, POMC^{Cre}/Bbs1^{fl/ff} mice displayed glucose intolerance (Fig. 1K-L) and tended to be insulin resistant (Supplemental Fig. 2F-G).

Interestingly, disruption of the BBSome in POMC neurons led to a significant decrease in the expression of the hypothalamic anorexigenic Pomc gene without altering the expression of orexigenic Agrp and Npy genes (Fig. 1M). Of note, there was no difference in the expression pattern of these genes between and male and female
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POMC$^{\text{Cre/Bbs1}^{\text{fl/fl}}}$ mice. These findings highlight the relevance of the BBSome in POMC neurons for the mechanisms that underlie metabolic regulation.

**Mice lacking the $Bbs1$ gene in AgRP neurons are obese**

We previously reported that global BBS mice as well as mice lacking the $Bbs1$ gene in the nervous system or LepRb-expressing cells have normal expression of hypothalamic $Agrp$ and $Npy$ genes. This led us to hypothesize that loss of the BBSome in AgRP neurons does not contribute to the obesity phenotype observed in these mice. To test this directly, we generated mice lacking the $Bbs1$ gene selectively in AgRP neurons by breeding the $Bbs1^{\text{fl/fl}}$ mice with AgRP$^{\text{Cre}}$ mice. As above, we used the td-Tomato (ROSA) reporter mouse model to confirm that AgRP$^{\text{Cre}}$ drives Cre-recombinase, indicated by td-Tomato protein expression (Supplemental Fig. 3A).

Surprisingly and in contrast to our expectation, mice lacking expression of the $Bbs1$ gene in AgRP neurons (AgRP$^{\text{Cre/Bbs1}^{\text{fl/fl}}}$) developed obesity as indicated by the significantly increased body weight, starting at ~6 weeks of age (Fig. 2A-B). Notably, this increase in body weight was more pronounced in the females relative to the males suggesting that loss of the BBSome in AgRP neurons may contribute to the sex difference in the development of obesity in BBS mice (25, 26). Fat mass was increased in female, but not male, AgRP$^{\text{Cre/Bbs1}^{\text{fl/fl}}}$ mice (Fig. 2C-D and Supplemental Fig. 3B-C) without alteration in lean mass (Fig. 2C-D). Consistent with body weight, the increase in liver mass was more noticeable in female than male AgRP$^{\text{Cre/Bbs1}^{\text{fl/fl}}}$ mice (females: $2.74 \pm 0.46$ vs $1.58 \pm 0.12$ g, $P<0.05$; males $3.41 \pm 0.58$ vs $2.45 \pm 0.22$ g, $P=0.06$). Of note,
plasma insulin levels were elevated in female and male AgRP\textsuperscript{cre}/Bbs1\textsuperscript{fl/fl} mice relative to control littermates (Fig. 3E-F)

There was no significant change in the expression of hypothalamic anorexigenic and orexigenic genes in AgRP\textsuperscript{cre}/Bbs1\textsuperscript{fl/fl} mice compared to control littermates although expression of AgRP tend to be lower in AgRP\textsuperscript{cre}/Bbs1\textsuperscript{fl/fl} mice (Fig. 2G). These findings indicate that different molecular mechanisms may underlies the obesity phenotype of AgRP\textsuperscript{cre}/Bbs1\textsuperscript{fl/fl} mice versus POMC\textsuperscript{cre}/Bbs1\textsuperscript{fl/fl} mice.

In addition and consistent with the previous report (17), localization of NPY\textsubscript{2}R in primary cilia of td-Tomato\textsuperscript{+} cells was substantially reduced in AgRP\textsuperscript{cre}/Bbs1\textsuperscript{fl/fl} mice (Fig. 2H). Of note, there was no difference in Npy2r gene expression between AgRP\textsuperscript{cre}/Bbs1\textsuperscript{fl/fl} mice (1.4±0.4 AU) and controls (1.0±0.3 AU). These findings confirm the importance of the BBSome for the trafficking of key metabolic receptors in hypothalamic neurons.

**Plasma membrane localization of 5-HT\textsubscript{2C}R in POMC neurons require the BBSome**

Next, we asked whether the BBSome is involved in the trafficking of other receptors that are important for energy homeostasis such as the serotonin 5-HT\textsubscript{2C}R which is present in POMC neurons (7, 27). Using an antibody that recognizes specifically the 5-HT\textsubscript{2C}R, as confirmed by Western blot and immunohistochemistry in cells and arcuate nucleus of 5-HT\textsubscript{2C}R\textsuperscript{-/-} null mice (Supplemental Fig. 4A-C), we found that in the arcuate nucleus of control mice 5-HT\textsubscript{2C}R is predominantly present in the plasma membrane of td-Tomato\textsuperscript{+} cells as well as in some td-Tomato\textsuperscript{-} cells (Fig. 3A). Notably, in POMC\textsuperscript{cre}/Bbs1\textsuperscript{fl/fl} mice the localization of 5-HT\textsubscript{2C}R is altered selectively in td-Tomato\textsuperscript{+} cells, appearing stalled in the cytoplasm (Fig. 3A). Of note, 5-HT\textsubscript{2C}R does not localize in cilia of either td-Tomato\textsuperscript{+} or
td-Tomato⁺ cells of the hypothalamic arcuate nucleus of control and POMC<sup>cre</sup>/Bbs1<sup>fl/fl</sup> mice. In contrast, the NPY<sub>2</sub>R localizes predominantly in neuronal cilia and POMC<sup>cre</sup>/Bbs1<sup>fl/fl</sup> mice displayed reduced ciliary localization of this receptor in td-Tomato⁺ cells of the arcuate nucleus (Supplemental Fig. 5A-B). Hypothalamic mRNA levels of 5-HT<sub>2C</sub>R and NPY<sub>2</sub>R were not altered in POMC<sup>cre</sup>/Bbs1<sup>fl/fl</sup> mice (0.9±0.1 and 1.2±0.3 AU, respectively) relative to littermate controls (1.0±0.1 AU for both). AgRP<sup>cre</sup>/Bbs1<sup>fl/fl</sup> mice also exhibit normal hypothalamic mRNA levels of 5-HT<sub>2C</sub>R (0.9±0.1) compared to controls (1.0±0.2 AU). Interestingly, deletion of the Bbs1 gene does not appear to affect cilia formation as indicated by the relatively normal cilia, stained with adenylyl cyclase type III (ACIII), in the arcuate nucleus td-Tomato⁺ cells of POMC<sup>cre</sup>/Bbs1<sup>fl/fl</sup> mice (Supplemental Fig. 5B).

Based on the evidence above, we predicted that the metabolic effects evoked by activation of 5-HT<sub>2C</sub>R would be blunted in POMC<sup>cre</sup>/Bbs1<sup>fl/fl</sup> mice. Thus, we assessed the anorectic and weight-reducing responses induced by the 5-HT<sub>2C</sub>R agonist, lorcaserin. In control mice, lorcaserin decreases food intake and body weight (Fig. 3B-C). However, the responses evoked by lorcaserin are significantly blunted in POMC<sup>cre</sup>/Bbs1<sup>fl/fl</sup> mice. The reduced responses to lorcaserin in POMC<sup>cre</sup>/Bbs1<sup>fl/fl</sup> mice does not appear due to obesity, but rather caused by loss of the BBSome. This is supported by the decrease in food intake and body weight in diet-induced obese mice treated with lorcaserin (Fig. 3D-E). Taken together, these data demonstrate the importance of the BBSome in POMC neurons in underlying serotonin-mediated control of energy homeostasis.

**BBSome deficiency hinders serotonin-evoked cellular responses**
To facilitate the analysis of the trafficking of the 5-HT$_{2C}$R in vitro, we generated a green fluorescent protein tagged receptor (5-HT$_{2C}$R-GFP). First, we used confocal microscopy to investigate the membrane localization of the 5-HT$_{2C}$R in the IMCD3 cell line. While 5-HT$_{2C}$R-GFP was present in the plasma membrane we were unable to detect 5-HT$_{2C}$R-GFP in cilia (labeled with acetylated α-tubulin, Fig. 4A) indicating that the 5HT$_{2C}$R is not targeted to the cilium. In contrast, we found that NPY$_2$R and SSTR$_3$ localize almost exclusively to cilia in IMCD3 cells (Supplemental Fig. 6A-B).

Next, we tested whether loss of the BBSome interferes with the membrane localization of 5-HT$_{2C}$R in IMCD3 cells. Interestingly, Bbs1 gene disruption using CRISPR/Cas9 reduced 5-HT$_{2C}$R-GFP in the plasma membrane (Fig. 4B) confirming the importance of the BBSome for membrane localization of the 5-HT$_{2C}$R. In addition, both the NPY$_2$R and SSTR$_3$ failed to localize to cilia in IMCD3-Bbs1$^{-/-}$ cells (Supplemental Fig. 6A-B) confirming the importance of the BBSome for the trafficking of these receptors to cilia. Consistent with the observation in POMC neurons, in the IMCD3-Bbs1$^{-/-}$ cells the 5-HT$_{2C}$R was predominantly localized in unusual large cytoplasmic vesicles (Fig. 4B).

Like CRISPR/Cas9-mediated deletion in IMCD3 cells, shRNA-induced silencing of Bbs1 gene expression reduces the membrane localization of the 5-HT$_{2C}$R in HEK 293 cells (Fig. 4C). The majority of GFP-tagged 5-HT$_{2C}$R localize to the plasma membrane in the control cells (transfected with scrambled shRNA) whereas the localization of this receptor is mostly in vesicles in the Bbs1-shRNA transfected HEK 293 cells. The reduced amount of 5-HT$_{2C}$R in the plasma membrane of HEK 293 cells transfected with Bbs1-shRNA was further confirmed using a cell surface biotin labeling assay followed by Western blotting (Fig. 4D). Importantly, total 5-HT$_{2C}$R expression is not changed.
when the \textit{Bbs1} gene is silenced. In addition, surface level of the transferrin receptor is not affected by \textit{Bbs1} gene silencing (Fig. 4D) demonstrating the specificity of the effects evoked by BBS1 loss on receptor trafficking to the plasma membrane.

We studied the functional consequence of altered 5-HT$_{2C}$R trafficking in absence of the BBSome by assessing the response of intracellular Ca$^{2+}$ [Ca$^{2+}$]$_{i}$ to serotonin. Compared to control cells, serotonin-induced increase in [Ca$^{2+}$]$_{i}$ is substantially decreased in IMCD3-\textit{Bbs1}/\textminus cells (Fig. 4E-F) and HEK 293 cells transfected with \textit{Bbs1}-shRNA (Fig. 4G). Leptin-evoked increase in [Ca$^{2+}$]$_{i}$ is also blunted after BBSome disruption (Supplemental Fig. 7). However, the increase in [Ca$^{2+}$]$_{i}$ evoked by ATP was not altered by disruption of the BBSome (Fig. 4E,H). We also examined the effects of silencing the \textit{Bbs1} gene on serotonin-induced membrane potential using patch-clamp recording. Membrane potential response to serotonin is dramatically reduced in HEK 293 cells transfected with \textit{Bbs1}-shRNA compared to control cells (Fig. 4I). These results demonstrate that reduction in 5-HT$_{2C}$R trafficking to the plasma membrane caused by disruption of the BBSome is associated with attenuated cellular responses evoked by serotonin.

\textbf{Stalled 5-HT$_{2C}$R in the late endosome in the absence of the BBSome}

The reduced membrane localization of the 5-HT$_{2C}$R without alteration in its total expression when the BBSome is disrupted indicate that the receptor is stuck in the secretory pathway. To understand the fate of the 5-HT$_{2C}$R in cells lacking the \textit{Bbs1} gene, we first studied the localization of the receptor in the secretory pathway using markers of key organelles including Caveolin-1 (Cav-1, endosome), LAMP1 (lysosome),
RSAC1 (Golgi) and EEA1 (early endosome) using IMCD3-Bbs1-/- cells. Interestingly, we observed that 5-HT₂C-R-GFP co-localizes predominantly with the endosome marker, Cav-1, when the 5-HT₂C-R-GFP expression is low (Fig. 5A). This localization of 5-HT₂C-R-GFP and Cav-1 is only partial when the expression of 5-HT₂C-R-GFP is high (Fig. 5B).

We next analyzed the cellular localization of BBS proteins. For this, HEK 293 cells were transiently co-transfected with RFP-BBS1 along with GFP tagged marker proteins of early endosome (Rab5 constitutive active form, Rab5-GTP), late endosome (Rab7 constitutive active form Rab7-GTP or CD63), Golgi or early endosome (EEA1) and localization visualized with confocal microscopy. We found that RFP-BBS1 co-localized with the late endosome marker proteins, Rab7-GTP and CD63, and partially co-localized with Rab5-GTP, but not with Golgi or early endosome markers (Fig. 5C). Similarly, RFP-BBS7 co-localized with late endosome proteins, Rab7-GTP and CD63 (Supplemental Fig. 8A).

To test whether the BBSome proteins physically interacts with CD63, HEK 293 cells were transiently transfected with HA-tagged BBS1 protein. BBS1 protein was then immunoprecipitated with HA-agarose beads and the endogenous CD63 protein was detected with CD63 antibody by Western blot. As shown in Fig. 6A, CD63 protein is detected in a BBS-immunoprecipitated protein complex. Furthermore, silencing BBS2 increased BBS1 interaction with the CD63 compared to control shRNA (Fig. 6A), indicating the specificity of their interaction. We then tested whether other subunits of the BBSome also interact with the CD63 in HEK 293 cells. We found that in addition to BBS1, other BBSome proteins including BBS2, BBS4, BBS5, BBS7, BBS8 and BBS9 interact with the endogenous CD63 (Fig. 6B). In HEK 293 cells overexpressing BBS1 or
BBS7 proteins, endogenous CD63 protein co-localizes with BBS1 or BBS7 whereas 5-HT$_{2C}$R is mostly present in the plasma membrane (Fig. 6C and Supplemental Fig. 8B). On the other hand, disruption of the BBSome by silencing BBS2 in HEK 293 cells causes 5-HT$_{2C}$R to co-localize with BBS1 or BBS7 with the endogenous CD63 protein. In contrast, 5-HT$_{2C}$R appears in the plasma membrane when the BBSome complex is intact (Fig. 6C and Supplemental Fig. 8B). These results indicate that loss of the BBSome causes 5-HT$_{2C}$R to stall in the late endosome possibly forming a complex with CD63 and the remaining BBS proteins.

**DISCUSSION**

There are several new findings in the current study. First, we show that targeted disruption of the BBSome by deleting the Bbs1 gene in POMC neurons leads to obesity. We demonstrate that obesity in these mice is associated with an increase in food intake. Second, we provide evidence that mice lacking the BBSome in AgRP neurons also display an increase in body weight and fat mass. Third, we identified an important role for the BBSome in mediating the trafficking of the 5-HT$_{2C}$R in POMC neurons. We demonstrate that loss of the BBSome reduces surface expression of the 5-HT$_{2C}$R interfering with the ability of serotonin to modulate cellular processes. In absence of the BBSome, 5-HT$_{2C}$R becomes stalled in the late endosome of the secretory pathway. Together, these findings provide mechanistic insights into the contribution of the hypothalamic BBSome to the control of energy balance and handling of metabolic receptors.
Substantial evidence points to the importance of POMC and AgRP neurons as first-order neurons in the neurocircuit that controls metabolic functions (3, 6). These neurons are equipped with receptors for various metabolic signals including serotonin and regulate the downstream neurocircuitry to enact appropriate and consequential metabolic and behavioral responses to maintain energy balance. Several cellular processes within POMC and/or AgRP neurons have previously been shown to have important implications for metabolic control. This includes gene expression (23), protein translation and processing (28, 29), endoplasmic reticulum stress (30, 31) and mitochondria function (32-35). The changes in the trafficking of metabolic receptors evoked by disruption of the BBSome in POMC and AgRP neurons extend the cellular processes within these neurons that influence energy homeostasis.

We demonstrate that absence of the BBSome substantially reduce surfaces expression of the 5-HT$_{2C}$R in cultured cells and POMC neurons. In BBSome-deficient cells the 5-HT$_{2C}$R is held up in large vesicles in the cytoplasm. We previously demonstrated that the LepRb is also stalled in large cellular vesicles when the BBSome is disrupted (24). To determine the identity of these vesicles we analyzed the localization of the BBSome proteins and 5-HT$_{2C}$R in key organelles of the secretary pathway. We found that BBS proteins co-localize with the late endosome markers and partially co-localize with the early endosome marker. Importantly, we have identified the late endosome as the vesicle where the 5-HT$_{2C}$R is held up when the BBSome is disrupted. Future studies will determine what makes the receptors stall in this stage of the secretory pathway after loss of the BBSome. Additional studies are also needed to assess the possibility of overcoming the mistrafficking of metabolic receptors when the BBSome is disrupted.
Obesity is a major phenotype criterion in patients with BBS (15, 36). Mice carrying deletion or mutations in BBS genes also display an obesity phenotype that is driven by hyperphagia and a decrease in energy expenditure (24, 37). These findings point to a fundamental role of BBS proteins in the regulation of appetite and energy balance. This has triggered tremendous interest in understanding the mechanisms underlying the control of energy homeostasis by the BBSome. We previously demonstrated that loss of the BBSome in the nervous system or LepRb-containing cells, but not in adipocytes, largely recapitulated the increase in body weight and food intake associated with BBS (21). Our findings that BBSome deficiency in POMC neurons or AgRP neurons is sufficient to increase body weight and adiposity demonstrate the importance of the BBSome in these neuronal populations. The decreased hypothalamic POMC mRNA levels in mice lacking BBS gene globally or in defined neuronal populations such as POMC neurons is consistent with the defective signaling of receptors that regulate pomc gene expression including the LepRb and NPY$_2$R (38, 39). This reduction in pomc gene expression may contribute to the hyperphagia and obesity associated with BBS. Thus, the obesity associated with BBS may emanate from loss of the BBSome in hypothalamic neurons.

We also speculate that our findings may have implications for understanding the abnormalities associated with common obesity. This is supported by the demonstration that variants of BBS genes increase susceptibility to common human obesity (12-14). Of note, a reduction in surface expression of hypothalamic receptors was reported in animals with dietary obesity (40). A possible contribution of the BBSome to the abnormalities associated with common obesity warrant further investigation.
One limitation of our study relates to the use of mice expressing constitutive POMC\textsuperscript{Cre} and AgRP\textsuperscript{Cre} to delete the \textit{Bbs1} gene in these neuronal populations since embryonic expression of Cre may have deleted the \textit{Bbs1} gene in peripheral tissues and cells that do not necessarily differentiate to POMC or AgRP neurons. Indeed, it is known that a subset of POMC-expressing progenitor cells differentiates to other cell types including AgRP neurons (41) which can result in misleading feeding responses when constitutive POMC\textsuperscript{Cre} mice are used (42). It should be noted, however, that constitutive POMC\textsuperscript{Cre} and AgRP\textsuperscript{Cre} were used successfully to dissociate the contribution of POMC vs AgRP neurons to leptin regulation of regional autonomic nerve activity (22). Nonetheless, the recent development of inducible POMC\textsuperscript{Cre} (27) and AgRP\textsuperscript{Cre} mice (43) that allow circumventing the issues related to constitutive Cre activation will be useful to test the effects of \textit{Bbs1} gene deletion in POMC and AgRP neurons of adult mice. The use of \textit{in vitro} systems including a kidney cell line (HEK 293) to understand the neuronal mechanisms of obesity in our conditional mouse models can also be viewed as limitation to our studies. However, \textit{in vitro} studies are very useful to examine the biology of the BBSome and gain mechanistic insights into its function. Importantly, we provide evidence showing that the \textit{in vitro} data are recapitulated \textit{in vivo} as indicated by the similar defects in the trafficking of NP\textsubscript{2}R and 5-HT\textsubscript{2C}R evoked by loss of the BBSome in cultured cells and hypothalamic neurons.

In conclusion, our findings point to the BBSome in POMC and AgRP neurons as a critical regulator of energy homeostasis. Our results enhance our understanding of the cellular and molecular processes that underlie obesity in BBS with potential implications for common obesity.
AUTHOR’S CONTRIBUTIONS

D-F.G. designed and performed the experiments and wrote the manuscript; Z.L performed the calcium experiments; Y.W. performed the electrophysiology experiments, C.S created the knockout cell line, D.R.T measured body composition by MRI; J.L.G measured energy expenditure, G.B.R., Y.M.U., J.L.G and V.C.S. helped with experimental design and data interpretation, and K.R. conceptualized, analyzed and interpreted studies and wrote the manuscript.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

ACKNOWLEDGEMENTS

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K.R. serves as the guarantor.
FIGURE LEGENDS

**Figure 1.** Mice lacking the *Bbs1* gene in POMC neurons develop obesity. (A and B) Weekly body weights of female (A) and male (B) mice (n = 12-16/group: 12 males and 15 females for control mice, 16 males and 16 females for POMC<sup>cre</sup>/Bbs1<sup>fl/fl</sup> mice). (C-E) Fat mass and lean mass, measured by MRI (n=7/group: 3 males and 4 females in each group). (F-G) Cumulative daily food intake (n=6-13/group: 6 males and 12 females for control and 6 males and 13 females for POMC<sup>cre</sup>/Bbs1<sup>fl/fl</sup> mice). (H) Resting metabolic rate (RMR, n=4-6/group: 6 males and 4 females for controls and 6 males and 4 females for POMC<sup>cre</sup>/Bbs1<sup>fl/fl</sup> mice), analyzed by univariate regression modeling (model p<0.001, body mass p<0.001, genotype p=0.912, sex p=0.501, interaction p=0.453; body mass evaluated at 34.263 g; $R^2$=0.772). (I-J) Plasma insulin levels. (K-L) Glucose tolerance test (n=10-12/group: 5 males and 5 females for controls and 6 males and 6 females for POMC<sup>cre</sup>/Bbs1<sup>fl/fl</sup> mice). (M) Hypothalamic mRNA levels of metabolic neuropeptides (n=6/group: 3 males and 3 females in each group). *P< 0.05 vs. control group.

**Figure 2.** *Bbs1* gene deletion in AgRP neurons causes obesity. (A-B) Weekly body weights of female (A) and male (B) mice (n=10-23/group: 19 males and 23 females for controls and 15 males and 10 females for AgRP<sup>cre</sup>/Bbs1<sup>fl/fl</sup> mice). (C-D) Fat and lean masses. (E-F) Plasma insulin levels. (G-H) Hypothalamic mRNA levels of metabolic neuropeptides (n=6/group). (I) Selective loss of ciliary localization of NPY<sub>2</sub>R in td-Tomado<sup>+</sup> neurons of the hypothalamic arcuate nucleus of AgRP<sup>cre</sup>/Bbs1<sup>fl/fl</sup> mice. *P<0.05 vs. control group.
Figure 3. The BBSome mediates surface expression of the 5-HT<sub>2C</sub>R in POMC neurons. (A) Reduced membrane localization of the 5-HT<sub>2C</sub>R in td-Tomato<sup>+</sup> cells of POMC<sup>Cre</sup>/Bbs1<sup>fl/fl</sup> mice. (B and C) Blunted lorcaserin-induced decrease in food intake and body weight in POMC<sup>Cre</sup>/Bbs1<sup>fl/fl</sup> mice. (D and E) Lorcaserin decreases food intake (D) and body weight (E) in high fat diet (HFD)-induced obese mice. *P<0.05 and **P<0.01 vs. control group.

Figure 4. The BBSome is required for the cellular responses evoked by the 5-HT<sub>2C</sub>R. (A) Lack of localization of 5-HT<sub>2C</sub>R in cilia (labeled with acetylated α-tubulin) in IMCD3 cells. (B) Reduced membrane localization of 5-HT<sub>2C</sub>R in IMCD3-Bbs1<sup>−/−</sup> cells. (C-D) Bbs1 gene silencing reduces the surface levels of 5-HT<sub>2C</sub>R, but not transferrin receptor, in HEK 293 cells by immunostaining (C) and biotin labeling assay followed by Western blotting (D). (E-F) Reduced [Ca<sup>2+</sup>]i response to serotonin in IMCD3-Bbs1<sup>−/−</sup> cells. (G-I) Bbs1 gene silencing decrease [Ca<sup>2+</sup>]i response to serotonin (G), but not ATP (H), and reduce the membrane potential evoked by serotonin (I) in HEK 293 cells. *P<0.01, and **P<0.0001 vs. control group. Scale bar: 10 μm.

Figure 5. Localization of 5-HT<sub>2C</sub>R and BBS1 in the secretory pathway. (A) GFP tagged 5-HT<sub>2C</sub>R localizes in the endosome (Cav-1), but not in the lysosome (LAMP1), Golgi (RSAC1) or early endosome (EEA1) in IMCD3-Bbs1<sup>−/−</sup> cells. (B) Co-localization of 5-HT<sub>2C</sub>R-GFP and Cav-1 is only partial when the expression of 5-HT<sub>2C</sub>R-GFP is high. (C) RFP tagged BBS1 co-localizes with GFP tagged Rab5-GTP, Rab7-GTP and CD63
(markers of the late endosome), but not with markers of Golgi or early endosome (EEA1) in HEK 293 cells. Scale bar: 10 µm.

**Figure 6.** BBSome proteins interact with CD63. (A) Co-immunoprecipitation of the HA-tagged BBS1 with the endogenous CD63 (late endosome marker) is enhanced after Bbs2 gene silencing in HEK 293 cells. (B) BBSome proteins interact with CD63 in a co-immunoprecipitation assay. (C) Bbs2 gene silencing promotes localization of BBS1 with 5-HT2CR in the late endosome. Scale bar: 10 µm.
REFERENCES


**A**

5-HT$_2c$R | Acyt α-Tub | Merged
--- | --- | ---

**B**

IMCD3-Control | IMCD3-Bbs1$^{-/-}$

**C**

5-HT$_2c$R/DAPI | 5-HT$_2c$R/DAPI

**D**

Control-shRNA | Bbs1-shRNA

Surface | Total
--- | ---

5-HT$_2c$R | Transferrin receptor

**E**

IMCD3-Control | IMCD3-Bbs1$^{-/-}$

Serotonin (5 μM) | ATP (10 μM)

**F**

5-HT$_2c$R (340/380 ratio)

**G**

5-HT$_2c$R (340/380 ratio)

**H**

5-HT$_2c$R (340/380 ratio)

**I**

Control shRNA | Bbs1-shRNA

Membrane potential induced by serotonin

---

Diabetes
**A**

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**B**

**IP: HA**

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**C**

**Control-shRNA**

- **BBS1**
- **5HT2cR**
- **CD63**
- **Merged**

**Bbs2-shRNA**

- **BBS1**
- **5HT2cR**
- **CD63**
- **Merged**
Online Supplemental Material

The BBSome in POMC and AgRP Neurons is Necessary for Body Weight Regulation and Sorting of Metabolic Receptors

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Department of Pharmacology¹, Department of Neurology², Department of Pediatrics³, Department of Radiology⁴, Department of Internal Medicine⁵, Obesity Education and Research Initiative⁶ and Fraternal Order of Eagles Diabetes Research Center⁷, University of Iowa, Iowa City, Iowa

Short title: Neuronal BBSome and Energy Homeostasis.

Contain:
Supplemental figures 1-8
Supplemental table 1

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Tel: 319 353 5256
Fax: 319 353 5350
Supplemental Figure 1. Expression of BBS genes in POMC and AgRP neurons is modulated by energy status. (A-B) In silico re-analysis of available data (ref 23) showing BBS gene expression in POMC and AgRP neurons in fed versus fasted mice (cells obtained from 6 fed mice and 5 fasted mice were used for single-cell RNA sequencing). (C) Co-localization of Bbs1 mRNA and POMC mRNA by RNAscope. *P< 0.05, vs. the fed group.
**Supplemental Figure 2.** Evidence of Cre recombinase (presence of td-Tomado) (A) and its co-localization with POMC (B) in the arcuate nucleus of POMC\textsuperscript{cre}/Td-Tomato mice. (C) Intraperitoneal leptin increased p-Stat3 in POMC neurons of control mice, but not POMC\textsuperscript{cre}/Bbs1\textsuperscript{fl/fl} mice. (D-E) Weight of fat pads of POMC\textsuperscript{cre}/Bbs1\textsuperscript{fl/fl} mice compared to control littermates (n=6-8 per group: 7 males and 7 females for controls and 8 males and 6 females for POMC\textsuperscript{cre}/Bbs1\textsuperscript{fl/fl} mice). (F-G) Insulin tolerance test (n=10-12/group: 5 males and 5 females for controls and 6 males and 6 females for POMC\textsuperscript{cre}/Bbs1\textsuperscript{fl/fl} mice). BAT: brown adipose tissue, RP: retroperitoneal fat, Ing: inguinal fat, PG: perigonadal fat. ***P<0.001 and ****P<0.0001 vs. control group.
Supplemental Figure 3. BBSome disruption in AgRP neurons causes obesity. (A) Evidence of Cre recombinase (presence of td-Tomato) in the arcuate nucleus of the hypothalamus (where AgRP neurons reside) in AgRPPcre/td-Tomato mice. (B-C) Weight of fat pads in female (B) and male (C) AgRPPcre/Bbs1fl/fl mice compared to control littermates (n=6-8 per group: 7 males and 9 females for control and 7 males and 8 females for AgRPPcre/Bbs1fl/fl mice). **P < 0.01 and ***P < 0.001 vs. control group. Scale bar: 100 mm.
Supplemental Figure 4. Validation of 5-HT$_2c$R antibody. (A) The anti-5-HT$_2c$R antibody detects monomer, dimer and oligomer forms of the Flag-tagged 5-HT$_2c$R in HEK 293 cells. (B) Staining with the anti-5-HT$_2c$R antibody co-localized with GFP in HEK 293 cells transfected with GFP-tagged 5-HT$_2c$R. (C) The anti-5-HT$_2c$R antibody detects the endogenous 5-HT$_2c$R in the hypothalamic arcuate nucleus of control mice. This staining is absent in the arcuate nucleus of 5-HT$_2c$R knockout mice. Scale bar: 10 mm
Supplemental Figure 5. The BBSome is required for the ciliary localization of NPY2R in POMC neurons. (A-B) Loss of NPY2R in cilia of td-Tomato+ (red, arrow head), but not in td-Tomato- (non-red, arrow), cells in the arcuate nucleus of POMCCre/Bbs1fl/fl mice (n=3) vs controls (n=4). (C) Presence of cilia in td-Tomato+ (arrow head) and td-Tomato- (arrow) cells of POMCcre/Bbs1fl/fl mice. *P<0.05 vs Control. Scale bar: 10 µm.
Supplemental Figure 6. The BBSome is required for the ciliary localization of NPY$_2$R and SSTR$_3$. (A-B) Loss of localization in cilia (stained with Acetylated $\alpha$-Tub) of NPY$_2$R (A) and SSTR$_3$ (B) in IMCD3-$Bbs1^{{-/-}}$ cells. Scale bar: 10 µm.
Supplemental Figure 7. Representative tracings showing reduced [Ca^{2+}]_i response to leptin in control and Bbs1^{-/-} IMCD3 cells stably expressing the LepRb.
Supplemental Figure 8. BBSome proteins interacts with 5-HT$_2$C-R in the late endosome. (A) BBS7 (red) co-localizes with markers of the late endosome: CD63 (green) or Rab7-GTP (green). (B) Silencing Bbs2 gene expression promotes BBS7 (red) and 5-HT$_2$C-R (green) interaction in late endosome marked with CD63 (blue) in HEK 293 cells. Scale bar: 10 µm.
**SUPPLEMENTAL TABLE**  
**Supplemental Table 1:** Primer sequences used.

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