

BRS3 in both MC4R- and SIM1-expressing neurons regulates energy homeostasis in mice



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ABSTRACT

Objective: Bombesin-like receptor 3 (BRS3) is an orphan receptor and *Brs3* knockout mice develop obesity with increased food intake and reduced resting metabolic rate and body temperature. The neuronal populations contributing to these effects were examined.

Methods: We studied energy metabolism in mice with Cre-mediated recombination causing 1) loss of BRS3 selectively in SIM1- or MC4R-expressing neurons or 2) selective re-expression of BRS3 from a null background in these neurons.

Results: The deletion of BRS3 in MC4R neurons increased body weight/adiposity, metabolic efficiency, and food intake, and reduced insulin sensitivity. BRS3 re-expression in these neurons caused partial or no reversal of these traits. However, these observations were confounded by an obesity phenotype caused by the *Mc4r-Cre* allele, independent of its recombinase activity. The deletion of BRS3 in SIM1 neurons increased body weight/adiposity and food intake, but not to the levels of the global null. The re-expression of BRS3 in SIM1 neurons reduced body weight/adiposity and food intake, but not to wild type levels. The deletion of BRS3 in either MC4R- or SIM1-expressing neurons affected body temperature, with re-expression in either population reversing the null phenotype. MK-5046, a BRS3 agonist, increases light phase body temperature in wild type, but not *Brs3* null, mice and BRS3 re-expression in either population restored response to MK-5046.

Conclusions: BRS3 in both MC4R- and SIM1-expressing neurons contributes to regulation of body weight/adiposity, insulin sensitivity, food intake, and body temperature.

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Keywords Bombesin-like receptor 3; BRS3; Obesity; MC4R neurons; SIM1 neurons; Body temperature; Food intake; Energy homeostasis

1. INTRODUCTION

Bombesin-like receptor 3 (BRS3, bombesin receptor subtype 3, BB₃) is a G protein-coupled receptor regulating energy homeostasis. It belongs to a subfamily including the neuromedin B (NMB) and gastrin-releasing peptide (GRP) receptors [1]. An endogenous ligand for BRS3 has not been detected in placental mammals [2–4], although NMB has modest efficacy in a subset of assays [5] and both NMB and GRP are high affinity ligands in nonplacental vertebrates [3,6]. BRS3 may also function through receptor crosstalk [7]. The BRS3 null mouse has a decreased resting metabolic rate and light phase core body temperature (Tb), increased food intake, and develops obesity [8–12]. While obesity occurs on a chow diet [8], it is amplified by a high fat diet (HFD) [13]. BRS3 in glutamatergic, but not GABAergic, neurons is responsible for the metabolic changes of the BRS3 global knockout mice [14]. Stimulation of BRS3 neurons in the dorsomedial hypothalamus (DMH^{Brs3}) increases energy expenditure and Tb, and activates brown adipose tissue, but does not alter food intake or physical activity, whereas stimulation of BRS3 neurons in the paraventricular hypothalamus (PVH^{Brs3}) reduces food intake, with no effect on energy expenditure, Tb, or physical activity [15]. Rat studies also support a

role for PVH^{Brs3} neurons in food intake and DMH^{Brs3} neurons in energy expenditure [16].

Mutations in melanocortin receptor 4 (MC4R) cause obesity in humans and mice. Mouse *Mc4r* ablation causes hyperphagia, reduced energy expenditure, reduced sympathetic tone, increased fat mass, and impaired glucose homeostasis, leading to overt early-onset obesity [17–19]. MC4R is localized in the thalamus, hypothalamus, and hippocampus among other brain and peripheral sites [20,21].

Single-minded homolog 1 (SIM1) is a member of the bHLH-PAS (basic helix-loop-helix Per-Arnt-Sim) family of transcription factors and is required for the proper formation of the basal forebrain [22,23]. SIM1 homozygous knockout mice fail to properly form at least the paraventricular (PVH), supraoptic (SON), and anterior periventricular (aPV) hypothalamic nuclei and die perinatally [24]. SIM1 heterozygous (*Sim1*^{+/-}) mice survive and develop early-onset obesity with increased linear growth, hyperinsulinemia, and hyperleptinemia. *Sim1*^{+/-} mice are hyperphagic but their energy expenditure is not significantly decreased [25]. In humans, germline haploinsufficiency of SIM1 causes hyperphagic obesity, sometimes with Prader-Willi-like features [26,27].

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Abbreviations: DMH, dorsomedial hypothalamus; HFD, high fat diet; PVH, paraventricular nucleus of the hypothalamus; Tb, core body temperature; MeA, medial amygdala; MC4R, melanocortin receptor 4; SIM1, single-minded homolog 1

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To further define the neurons relevant for BRS3 function in energy intake and expenditure [28], we assessed the effects of the loss and re-expression of BRS3 in MC4R- and SIM1-expressing neurons. We report a role for BRS3 in these neurons in regulating body weight/adiposity, insulin sensitivity, and Tb.

2. MATERIALS AND METHODS

2.1. Animals

Male mice were singly housed on a 12:12-h dark–light cycle (lights on at 0600), at $\sim 22^\circ\text{C}$, with Teklad bedding (7090, Envigo Inc.) and ad libitum access to food and water. At 8 weeks of age, they were singly housed and placed on an HFD (D12492, 60% kcal fat, 5.24 metabolizable kcal/g; Research Diets, New Brunswick, NJ). Procedures were approved by the NIDDK Animal Care and Use Committee (protocol K016-DE0B-17).

Mc4r^{tm3.1(Cre)Lowl} (JAX 030759 [29], hereafter Mc4r-Cre) mice were supplied by Dr. Michael Krashes, NIDDK. Tg(Sim1-cre)1Lowl/J (hereafter Sim1-Cre) mice were purchased from the Jackson laboratory (JAX 006395 [18]). *Brs3* inactivation studies used littermate male progeny of female *Brs3^{flox/flox}* (JAX 031353 [14]) \times male *Brs3^{+/-};Cre/+* matings, on a congenic C57BL/6J background. *Brs3* re-expression studies used littermate male progeny of female *Brs3^{loxTB/loxTB}* (JAX 032580 [14]) \times male *Brs3^{+/-};Cre/+* matings, also on a congenic C57BL/6J background. Littermate mice differing only by *Cre* status were compared.

2.2. Phenotyping

Body weight and food intake were measured weekly and body composition was assessed every two weeks by EchoMRI (EchoMRI LLC, Houston, TX) [14]. Mice were studied on an HFD (rather than chow) because it amplifies the obesity caused by the loss of BRS3 [13].

2.3. Glucose and insulin tolerance tests, hormone and metabolite profiles

Intraperitoneal glucose (1 g/kg, with AUC calculated from the baseline) tolerance tests were performed at 0930, following an overnight (16 h) fast. Glucose was measured with a Glucometer Contour (Bayer, Mishawaka, IN). Insulin (0.75 unit/kg, i.p.) tolerance tests were performed at 0930, in nonfasted mice, with AUC calculated from 0 mg/dl. Blood was collected at 0930 by tail bleed at 17 weeks of age for measurements of fed glucose and insulin, triglycerides, free fatty acids, cholesterol, leptin, and adiponectin. Free fatty acids (Fujifilm Waco Diagnostics, Mountain View, CA, reagents # 999-34691, 995-34791, 991-34891, 993-35191), triglycerides (Pointe Scientific Inc., Canton, MI, # T7532-120), and cholesterol (Thermo Scientific, Middletown, VA, # TR13421) were measured using the indicated colorimetric assays. Leptin (R&D Systems, Minneapolis, MN, # MOB00), insulin (Crystal Chem, Downers Grove, IL, # 90010 using mouse insulin standard # 90020), and adiponectin (Alpco, Salem, NH, #47-ADPMS-E01) were measured by ELISA.

2.4. Telemetric monitoring of body temperature (Tb)

Tb and physical activity were measured continuously by telemetry using intraperitoneally implanted G2 E-mitters, ER4000 energizer/receivers, and VitalView software (Starr Life Sciences, Oakmont, PA) with data collected each minute. Tb span is defined as the difference between the Tb 95th and 5th percentiles from 24-h measurement intervals (i.e., 24, 48, or 72 h).

2.5. Effects of MK-5046 on food intake and Tb

For food intake response, mice were fasted overnight (1700–0930), dosed, and HFD was resupplied at 1000 with food intake measured over the next 6 h. Mice were treated with MK-5046 (10 mg/kg, i.p., MedChemExpress, Monmouth Junction, NJ) [30] or vehicle (10% Tween 80 in 0.25% methylcellulose in saline) in random order two days apart. The food intake response to MK-5046 (ΔFI) is the 6-h food intake after MK-5046 minus the food intake after vehicle, within mouse.

For Tb response, mice in their home cages with bedding at usual vivarium temperature ($\sim 22^\circ\text{C}$) were started on telemetric monitoring of Tb and activity, then fasted overnight and dosed as detailed for food intake response. The baseline- and vehicle-corrected Tb response to MK-5046 (ΔTb) was calculated within the mice as: effect of MK-5046 (mean Tb of 60–180 min minus mean Tb of -150 to -30 min) minus the effect of vehicle (mean Tb of 60–180 min minus mean Tb of -150 to -30 min), with all times relative to drug/vehicle dosing.

2.6. Dissection of paraventricular hypothalamic nuclei (PVH)

Briefly, brains were rapidly removed and sectioned (300 μm) with a Leica VT1000 vibratome (Wetzlar, Germany) using ice-cold 194 mM sucrose, 20 mM NaCl, 4.4 mM KCl, 2 mM CaCl_2 , 1 mM MgCl_2 , 1.2 mM NaH_2PO_4 , 10 mM glucose, and 26 mM NaHCO_3 saturated with 95% O_2 /5% CO_2 . A triangle around the PVH was dissected from a slice on a microscope slide using an Axioskop 2 microscope (Zeiss), transferred to a tube on dry ice, and stored at -80°C until processed for RNA.

2.7. Quantitative RT-PCR

RNA from whole hypothalamus (for Mc4r-Cre cohorts) or micro-dissected PVH (for Sim1-Cre cohorts) was extracted and processed as reported previously [31]. *Brs3* mRNA was quantitated by RT-PCR using primers x573 (5'-CTGCTGACTTGTGTGCCTGT) and x574 (5'-AGTGGCTTACGACTGCTTT). *Brs3* mRNA from the unrecombined *Brs3^{flox}* allele was present at $61 \pm 2\%$ of the level of the wild type allele. *Mc4r* mRNA was quantitated by RT-PCR using primers x581 (5'-ATCTGTAGCTCCTTGCTCGC) and x582 (5'-TGCAAGCTGCCAGATA-CAA), using QuantStudio™ 7 Flex Real-Time PCR System (Applied Biosystems, Waltham, MA). Expression was normalized using 18S RNA levels.

2.8. Statistical analysis

Data are presented as mean \pm SEM. Since mice with different *Brs3* background genotypes were not studied contemporaneously, statistical testing was limited to the effect of $\pm\text{Cre}$ within *Brs3* background genotype. Statistical significance was determined by two-tailed t-test at $P < 0.05$ using Prism v8.2.1 (GraphPad, San Diego, CA) or Excel.

3. RESULTS

3.1. BRS3 in MC4R-expressing neurons regulates body weight/adiposity

BRS3 and MC4R are expressed in overlapping locations in the hypothalamus. Mc4r-Cre-mediated deletion of *Brs3* caused a 10% reduction in hypothalamic BRS3 mRNA (Figure 1A). In *Brs3^{loxTB};Mc4r-Cre* mice the BRS3 mRNA recovered to 31% of wild type levels (Figure 1A). Thus Mc4r-Cre is expressed in a small subset of BRS3-expressing hypothalamic neurons.

Mc4r mRNA levels in heterozygous Mc4r-Cre (*Mc4r^{Cre/+}*) mice averaged $76 \pm 3\%$ of wild type (*Mc4r^{+/+}*) mice (Figure 1B). Thus, *Mc4r*

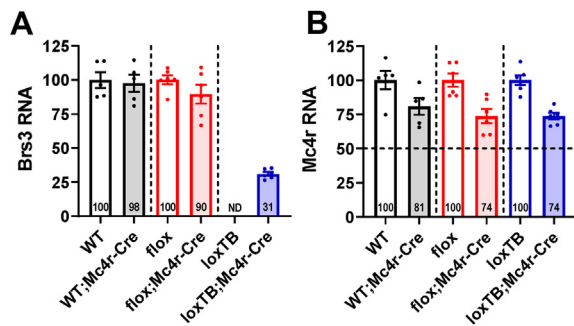


Figure 1: *Brs3* and *Mc4r* expression in *Mc4r-Cre* cohorts of mice. Hypothalamic *Brs3* (A) and *Mc4r* (B) mRNA were quantified by RT-PCR. Within each background genotype pair (indicated by dashed lines), the relative mRNA expression is reported with the higher value at 100 (except that *Brs3* mRNA in *loxTB;Mc4r-Cre* is normalized to the mean of WT, WT;*Mc4r-Cre*, and *flox*). Data are mean \pm SEM, $N = 5-6$ /group. ND indicates not detected, with a limit of detection of $\sim 1\%$ of *loxTB;Mc4r-Cre*.

mRNA levels produced by the *Mc4r-Cre* allele are about 52% (i.e., [76-50]/50) of the wild type allele. On an HFD, heterozygous *Mc4r-Cre* mice with wild type BRS3 had increased body weight, adiposity, food intake, and metabolic efficiency demonstrating that the *Mc4r^{Cre}* allele produces a mild obesity phenotype (Figure S1), which was less prominent at 28 weeks of age (Figure S2A-E). Previous experiments demonstrated that *Brs3^{flox/y}* mice have a wild type phenotype and *Brs3^{loxTB/y}* mice have the expected null phenotype [14]. Selective deletion of BRS3 in *Mc4r*-expressing neurons produced an increase in body weight (+20%), adiposity, and food intake on an HFD (Figure 2, *flox* vs *flox;Mc4r-Cre*, red color). Re-expression of BRS3 only in *Mc4r-Cre*-expressing cells had no detectable effect on body weight (-1%) or adiposity (Figure 2, *loxTB* vs *loxTB;Mc4r-Cre*, blue color). At 29 weeks, compared to *Brs3^{flox}* mice, the *Brs3^{flox};Mc4r-Cre* mice had increased weight (+14%) and adiposity, and liver, BAT, and iWAT masses, and decreased eWAT mass (Figure S3A-E). Deletion of BRS3 by *Mc4r-Cre* increased metabolic efficiency, while re-expression

reduced it (Figure 2E). Thus BRS3 in *Mc4r*-expressing neurons contributes to these adiposity phenotypes.

3.2. BRS3 in *Mc4r*-expressing neurons regulates insulin sensitivity and glucose metabolism

We investigated the role of BRS3 in *Mc4r*-expressing neurons in glucose tolerance and insulin sensitivity. *Mc4r-Cre* by itself did not affect insulin sensitivity or glucose tolerance (Figure S4). BRS3 deletion in *Mc4r*-expressing neurons impaired insulin tolerance and increased fed insulin levels and BRS3 re-expression reduced fed glucose (Figure 3A-D). While no difference in glucose tolerance or fasted glucose levels were seen (Figure 3F-H), BRS3 deletion increased fasted and glucose stimulated insulin levels (Figure 3I). These results suggest that BRS3 in *Mc4r*-expressing neurons regulates insulin sensitivity and glucose metabolism, possibly both due to increased body weight and independent of it (Figure 3E, J). Neither *Mc4r-Cre* by itself (Figure S2F-J) nor BRS3 manipulation in *Mc4r*-expressing neurons (Figure S3F-J) had a consistent, obvious effects on serum triglyceride, free fatty acid, cholesterol, adiponectin, or leptin levels.

3.3. BRS3 in *SIM1*-expressing neurons regulates body weight/adiposity and food intake

We next explored the contribution of BRS3 in *SIM1*-expressing neurons, which are predominantly in the paraventricular nucleus of the hypothalamus (PVH). There was a 90% reduction of *Brs3* mRNA levels in PVH of the *Brs3^{flox};Sim1-Cre* mice. In the PVH of *Brs3^{loxTB};Sim1-Cre* mice, the *Brs3* mRNA levels recovered to that of wild type mice (Figure 4). The *Sim1-Cre* allele itself does not affect body weight [18]. Mice with BRS3 deleted in *SIM1*-expressing neurons had increased body weight (+9%), fat mass, food intake, and metabolic efficiency (Figure 5, *flox* vs *flox;Sim1-Cre*, green color). Each of these changes was less than that seen in global null (*Brs3^{loxTB/y}*) mice (body weight +24%) and each was partially and significantly reversed (body weight -12%) with selective re-expression of BRS3 in *SIM1* neurons (Figure 5, *loxTB* vs

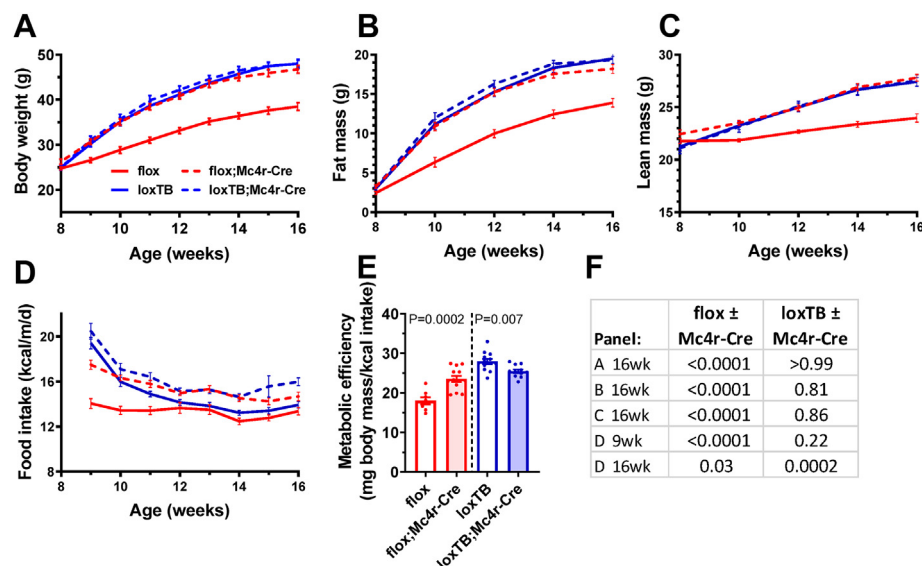


Figure 2: Role of BRS3 in *Mc4r*-expressing neurons on body weight and energy intake. Mice were singly housed and fed a high fat diet (HFD) starting at 8 weeks of age. (A) Body weight, (B) fat mass, (C) lean mass, (D) food intake, and (E) metabolic efficiency over 8–16 weeks of age. (F) P values by t-test for the littermate comparisons in the indicated panels and times. Data are mean \pm SEM, $N = 8-13$ /group.

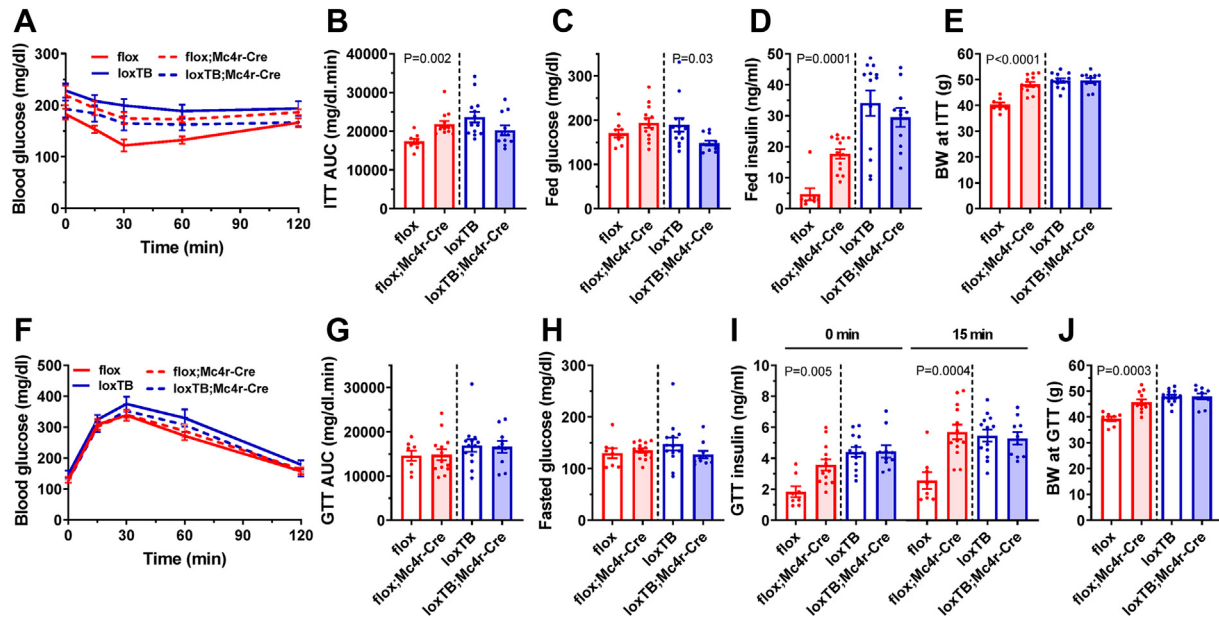


Figure 3: Effect of BRS3 manipulation in MC4R-expressing neurons on glucose homeostasis. (A) Insulin tolerance test (ITT) at 19 weeks of age (B) glucose area under the curve (AUC), (C) fed glucose, (D) fed insulin, and (E) body weight (BW) at the time of the test. (F) Glucose tolerance test (GTT) at 18 weeks of age (G) glucose area under the curve (AUC), (H) pre-test fasting glucose, (I) insulin at baseline (0 min) and 15 min after glucose dosing, and (J) body weight (BW) at the time of the test. Data are mean \pm SEM, $N = 8-13$ /group. P values were determined by t-test for the effect of Mc4r-Cre within each *Brs3* genotype.

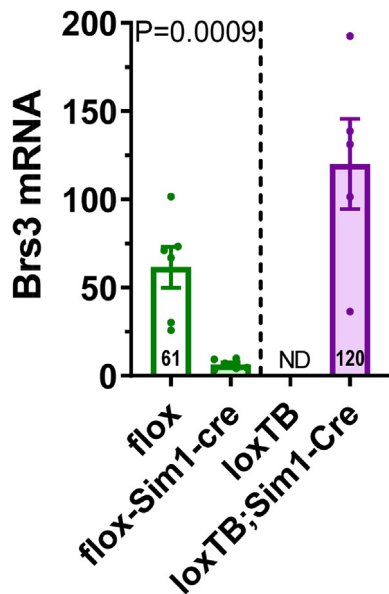


Figure 4: *Brs3* expression in Sim1-Cre cohorts of mice. *Brs3* mRNA was quantified by RT-PCR in micro-dissected paraventricular nuclei of hypothalamus (PVH). Expression is normalized to WT = 100. Data are mean \pm SEM, $N = 3-6$ /group. ND, not detected.

loxTB;Sim1-Cre, purple color). At 45 weeks of age, the effect of BRS3 deletion in SIM1 neurons was no longer statistically significant (body weight +4%), although liver and eWAT weights were changed. Re-expression of BRS3 in SIM1 neurons reduced body (−9%), liver, and iWAT weight, and increased eWAT weight at 27 weeks of age (Figure S5A–E). BRS3 manipulation in SIM1-expressing neurons had no consistent, obvious effects on serum triglyceride, free fatty acid, cholesterol, adiponectin, or leptin levels (Figure S5F–J).

3.4. BRS3 in SIM1-expressing neurons regulates insulin sensitivity

BRS3 deletion in SIM1-expressing neurons impaired insulin tolerance and increased insulin levels. BRS3 re-expression in only SIM1 neurons lowered insulin levels but did not have a statistically significant effect on insulin tolerance. Manipulation of BRS3 in SIM1 neurons had little effect on glucose levels or on glucose tolerance (Figure 6). These results suggest that BRS3 in SIM1-expressing neurons regulates insulin sensitivity, possibly with body weight effects contributing.

3.5. BRS3 in both MC4R and SIM1 neurons regulates baseline core body temperature

Brs3^{−/y} mice have a slightly lower Tb during resting periods in the light phase, but their Tb is comparable to wild type mice during active intervals in the dark phase [11], resulting in an increased Tb range. This increased Tb range is robustly quantified as Tb span, the difference between the 95th and 5th Tb percentiles during 24-h periods [14]. Selective deletion of BRS3 in either MC4R- or SIM1-expressing neurons had little or no effect on mean light and dark phase Tb, but increased the Tb span, while BRS3 re-expression reduced it (Figure 7, Table S1). This result suggests that BRS3 in both MC4R- and SIM1-expressing neurons contributes to regulating Tb.

3.6. Tb and food intake responses to BRS3 agonist MK-5046

Treatment with a BRS3 agonist increases Tb during the light phase by $\sim 0.5^\circ\text{C}$ [32]. This effect using the BRS3 agonist MK-5046 was intact in *Brs3*^{flox} and *Brs3*^{flox};Mc4r-Cre mice, lost in *Brs3*^{loxTB}, and recovered in *Brs3*^{loxTB};Mc4r-Cre mice (Figure 8A). Similarly, MK-5046 increased Tb in *Brs3*^{flox} and probably in *Brs3*^{flox};Sim1-Cre mice, was lost in *Brs3*^{loxTB}, and recovered in *Brs3*^{loxTB};Sim1-Cre mice (Figure 8B). Thus BRS3 in neurons expressing either MC4R or SIM1 is sufficient, but neither appears to be necessary for the Tb-raising effect of MK-5046. Treatment with BRS3 agonists inhibits food intake [13]. The inhibition of food intake by MK-5046 was intact in *Brs3*^{flox} and lost in *Brs3*^{loxTB};Mc4r-Cre mice. It was lost in *Brs3*^{loxTB}, but not clearly

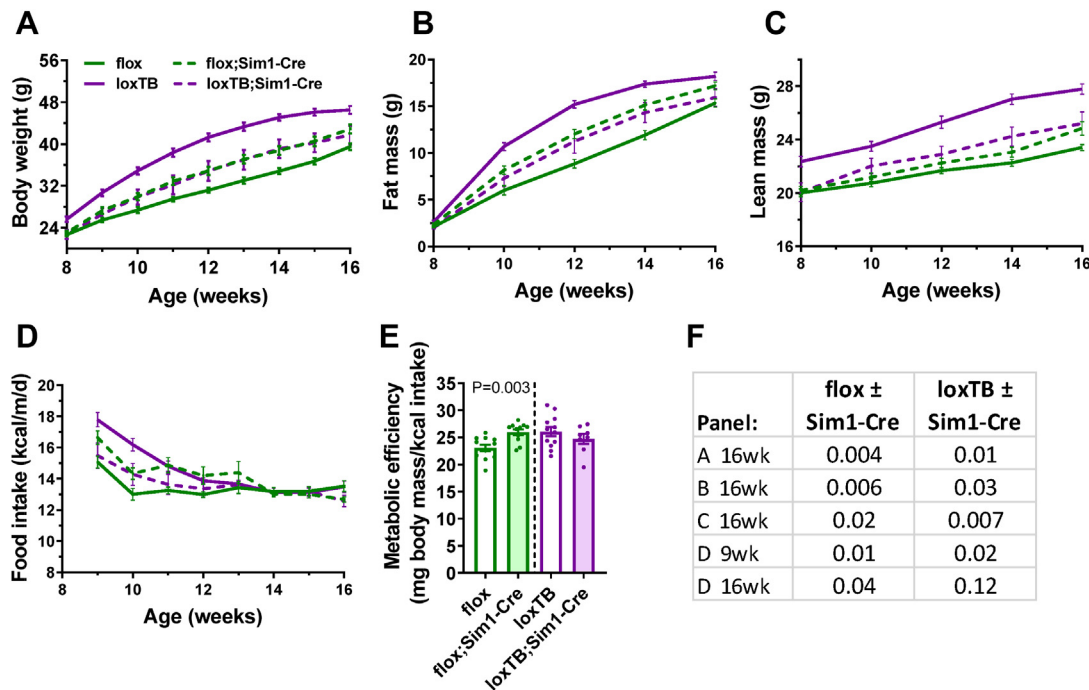


Figure 5: Role of BRS3 in SIM1-expressing neurons on body weight and energy intake. Mice were singly housed and fed a high fat diet (HFD) starting at 8 weeks of age. (A) Body weight, (B) fat mass, (C) lean mass, (D) food intake, and (E) metabolic efficiency over 8–16 weeks of age. (F) *P* values by t-test for the littermate comparisons in the indicated panels and times. Data are mean ± SEM, *N* = 8–13/group.

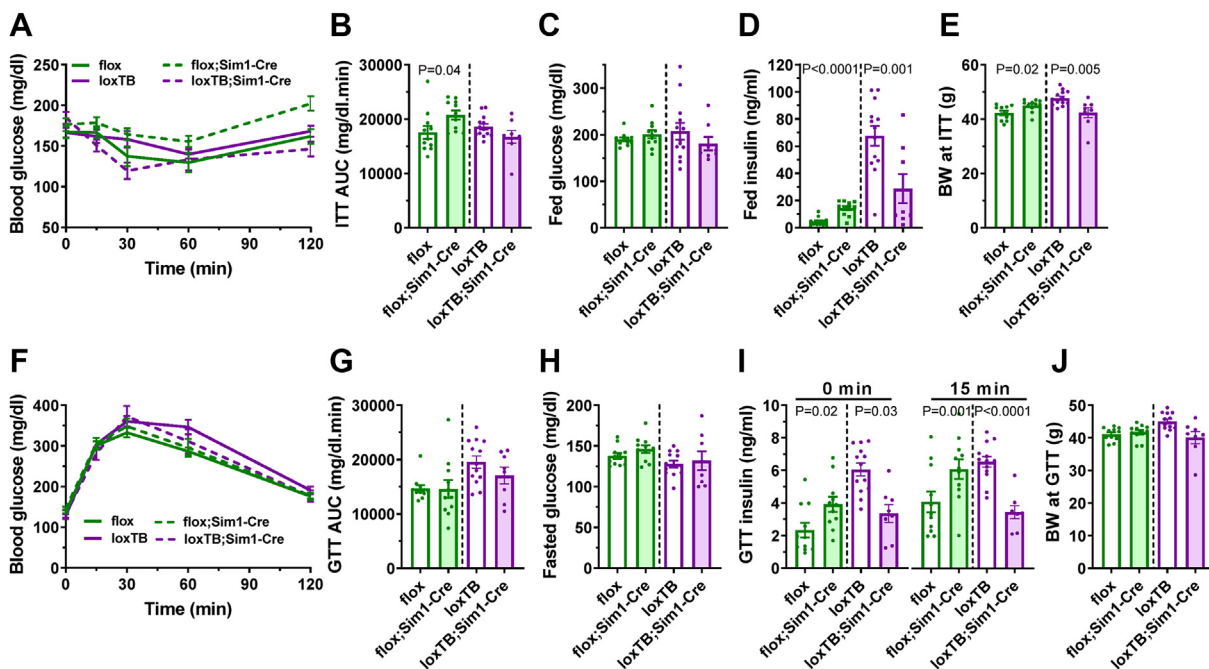


Figure 6: Effect of BRS3 manipulation in SIM1-expressing neurons on glucose homeostasis. (A) Insulin tolerance test (ITT) at 19 weeks of age (B) glucose area under the curve (AUC), (C) fed glucose, (D) fed insulin, and (E) body weight (BW) at the time of the test. (F) Glucose tolerance test (GTT) at 18 weeks of age (G) glucose area under the curve, (H) pre-test fasting glucose, (I) insulin at baseline (0 min) and 15 min after glucose dosing, and (J) body weight (BW) at the time of the test. Data are mean ± SEM, *N* = 8–13/group. *P* values were determined by t-test for the effect of *Sim1-Cre* within each *Brs3* genotype.

recovered in *Brs3^{loxTB};Mc4r-Cre* mice (Figure 8C). This suggests that BRS3 in MC4R-expressing neurons is necessary, and possibly sufficient for the food intake-suppressing effect of MK-5046. In both *Brs3^{flox}* and *Brs3^{flox};Sim1-Cre* mice, the inhibition of food intake was

intact. This effect was lost in *Brs3^{loxTB}*, and probably recovered, in *Brs3^{loxTB};Sim1-Cre* mice (Figure 8D). These results suggest that BRS3 in SIM1 neurons is not necessary but is possibly sufficient for MK-5046-induced suppression of food intake.

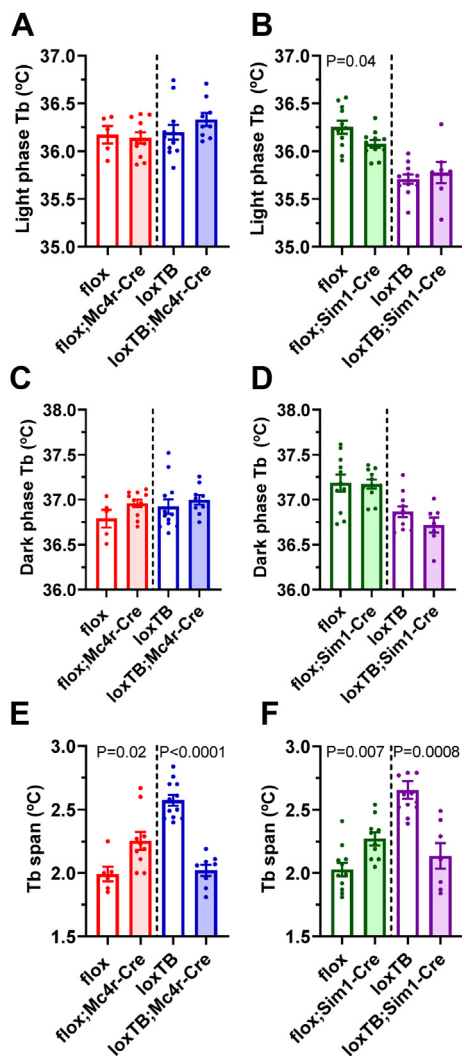


Figure 7: Effect of BRS3 in MC4R- and SIM1-expressing neurons on body temperature (Tb). Tb span, the difference between the 95th and 5th percentiles of daily Tb, was measured in 72 h of continuous telemetric Tb data from singly-housed, free-ranging mice in their home cage at 23–26 weeks of age. Ambient temperature was 22 °C and compared groups were studied simultaneously. (A, C & E) Effect of BRS3 in MC4R-expressing neurons. (B, D & F) Effect of BRS3 in SIM1-expressing neurons. Data are mean \pm SEM, $N = 8$ –13/group. P values were determined by t -test for the effect of Mc4r-Cre or Sim1-Cre within each *Brs3* genotype.

4. DISCUSSION

4.1. Manipulation of BRS3 in MC4R- or SIM1-expressing neurons

The loss of BRS3 from MC4R-expressing neurons reproduced much of the global BRS3 null phenotype for body weight/adiposity, insulin sensitivity, food intake, metabolic efficiency, and body temperature (Figure 9). However, BRS3 re-expression in MC4R neurons only modestly reversed some of these phenotypes, possibly due to confounding by the recombinase-independent obesity phenotype of the *Mc4r^{Cre}* allele. Similarly, the effect of BRS3 deletion in MC4R neurons may be exaggerated by the effects of the *Mc4r^{Cre}* allele (see 4.4). The loss of BRS3 from SIM1-expressing neurons also produced features of the BRS3 global null, including body weight/adiposity, insulin sensitivity, food intake, metabolic efficiency, and body temperature. Unlike with *Mc4r-Cre*, the phenotypes showed reversal with BRS3 re-

expression in *Sim1-Cre*-expressing neurons. Furthermore, the effects of loss or re-expression of BRS3 on body weight/adiposity and food intake are partial, suggesting that BRS3 in both *SIM1⁺* and *SIM1⁻* neurons contributes.

4.2. BRS3 neuronal populations and food intake

The BRS3 [15] and SIM1 expression patterns in adult mice overlap principally in the medial amygdala (MeA) and PVH. During embryogenesis, SIM1 is expressed in two discrete regions of the diencephalon and mesencephalon and peripherally in the developing somites, mesonephric duct, and foregut [22,23]. In adult mice, both SIM1 and *Sim1-Cre* are strongly expressed in the paraventricular hypothalamic nucleus (PVH), nucleus of the lateral olfactory tract (NLOT), supraoptic nucleus (SON), posterior hypothalamic nuclei (PH), and MeA [18].

There are historical data that various lesions in the amygdala affect food intake [33]. Focusing on the MeA, optogenetic activation of inhibitory *ARC^{AGRP}* neurons projecting to the MeA increased food intake, associated with reduced attention to territoriality [34]. The MeA may contribute to action prioritization, such as when eating competes with other behaviors [35].

A larger body of evidence demonstrates the major contribution of the PVH in regulating food intake [28]. Lesions that targeted the PVH increase food intake and body weight [36] and optogenetic activation of inhibitory *ARC^{AGRP}* neurons projecting to the PVH stimulates food intake [37]. PVH neurons that inhibit food intake include those expressing SIM1 [37], MC4R [18,29,38], Nitric Oxide Synthase 1 (NOS1) [39], GLP1R [40], BRS3 [15], and prodynorphin (PDYN) [38], but not oxytocin, corticotrophin-releasing hormone, or vasopressin [39,40]. Activation of pituitary adenylate cyclase-activating polypeptide (PACAP) or thyrotropin releasing hormone (TRH) neurons in the PVH stimulates *ARC^{AGRP}* neurons, thereby increasing food intake [41]. There is incomplete information about the co-expression of these markers. Most PVH neurons express SIM1, and recent work identified that *ARC^{AGRP}* neurons project to non-overlapping subsets of PVH neurons (*PVH^{MC4R}* and *PVH^{PDYN}*) that inhibit food intake through separate pathways [38]. While the number of distinct PVH neuron types is not known, understanding this diversity will be advanced by single cell RNA profiling. Reduced food intake caused by MK-5046 in the *Brs3^{flox};Sim1-Cre* mice could also be due to BRS3 neurons in other regions, such as the parabrachial nucleus.

MC4R is expressed widely across the brain, including the cortex, thalamus, hypothalamus, brainstem, and spinal cord, specifically including the PVH and medial preoptic area [21,42,43]. Most nuclei expressing BRS3 also express MC4R but overlap at the neuronal level is not well studied. The *PVH^{MC4R}* neurons that regulate feeding are glutamatergic and express SIM1 [44]. Since the BRS3 neurons regulating food intake are glutamatergic [14] and chemogenetic activation of *PVH^{BRS3}* neurons decreases food intake [15], it seems plausible that *Mc4r-Cre*-driven deletion or re-expression of BRS3 produces its food intake and obesity effects at least in part via neurons in the PVH that express both BRS3 and MC4R.

4.3. Energy expenditure, body temperature, and BRS3 neuronal populations

For singly-housed mice under typical conditions, 1/3 or more of total energy expenditure is dedicated to cold-induced thermogenesis, devoted to maintaining the Tb [45]. Under some circumstances (for example, light phase, resting state, cool ambient temperature, and single housing), body temperature can be a surrogate for metabolic rate in mice [46]. Thus neurons controlling Tb do so in part by regulating energy expenditure. The preoptic area (POA) is an important site

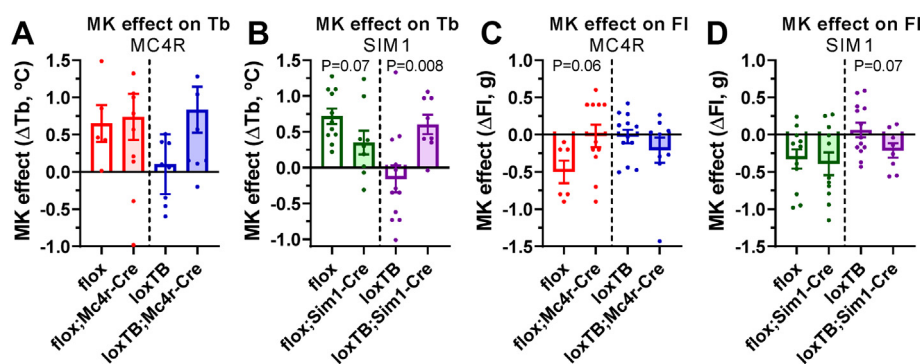


Figure 8: Response to BRS3 agonist after manipulation of BRS3 in MC4R- and SIM1-expressing neurons. (A–B) Tb response to BRS3 agonist MK-5046 (10 mg/kg, i.p.). (C–D) Food intake (FI) response to BRS3 agonist MK-5046 (10 mg/kg, i.p.). See [Materials and Methods](#) for experimental details. Data are mean \pm SEM, $N = 8$ –13/group. P values were determined by t-test for the effect of *Mc4r-Cre* or *Sim1-Cre* within each *Brs3* genotype.

Mouse:	Brs3 KO	Effect of BRS3 deletion in MC4R neurons	Effect of BRS3 re-expression in MC4R neurons	Effect of BRS3 deletion in SIM1 neurons	Effect of BRS3 re-expression in SIM1 neurons
Reference for comparison:	WT	WT	KO	WT	KO
Adiposity/BW	↑	↑	no clear reversal	partial ↑	partial reversal
Metabolic efficiency	↑	↑	partial reversal	↑	no clear reversal
Food intake	↑	↑	no clear reversal	partial ↑	partial reversal
Insulin	↑	↑	no clear reversal	↑	reversed
Tb span	↑	↑	reversed	↑	reversed
Tb increase by MK-5046	lost	intact	confers response	unclear	confers response
Food intake reduction by MK-5046	lost	lost	unclear	appears intact	possibly yes

Figure 9: Effects of manipulating BRS3 in MC4R- and SIM1-expressing neurons. Summary of results. Food intake refers to the effect upon initiating a high fat diet.

for regulating body temperature and energy expenditure [47–52], and BRS3 is expressed in many and MC4R is expressed in some POA subregions. Ablation of SIM1-expressing neurons reduced energy expenditure [53], but inactivation of the SIM1 gene itself did not change energy expenditure [54]. During development, *Sim1-Cre* is expressed in other hypothalamic nuclei, including the DMH/DHA, areas where BRS3 is also expressed.

BRS3 agonists regulate energy expenditure and Tb by sympathetic activation of brown adipose tissue [11,13,16,55]. Activation of DMH^{BRS3} (but not PVH^{BRS3}) neurons increases energy expenditure and Tb [15]. However, the effects of BRS3 on energy expenditure are likely to be multifaceted, since preliminary observations in our laboratory suggest that BRS3-expressing neurons in other sites also contribute to Tb regulation.

The Tb span is increased in *Brs3*^{−/y} mice due to a lower Tb during resting light phase periods combined with a normal Tb during dark phase active intervals [11]. The loss of BRS3 from either *Mc4r-Cre*- or *Sim1-Cre*-expressing neurons caused an increased Tb span, which was normalized by re-expression in either population. It is not known which brain nuclei contribute to this phenotype. Speculatively, BRS3 loss in MC4R and SIM1 neurons in the DMH/DHA could contribute to this phenotype. *Brs3*^{−/y} mice fail to increase their Tb upon treatment with a BRS3 agonist. This Tb increase was recovered when BRS3 was re-expressed

in either *Mc4r-Cre*- or *Sim1-Cre*-expressing neurons. It is not clear how many populations of neurons account for these observations.

4.4. The *Mc4r*^{Cre} allele causes an obesity phenotype

The *Mc4r*^{Cre} allele produces lower *Mc4r* mRNA levels than a wild type *Mc4r* allele. Additionally, MC4R produced from *Mc4r*^{Cre} has a 17-amino acid C-terminal extension, which may reduce receptor function [15]. These effects presumably explain the mild obesity seen in *Mc4r*^{Cre/+} mice fed an HFD. Thus, phenotypes in mice carrying a *Mc4r*^{Cre} allele may be due to reduced MC4R expression/function in addition to the desired gene expression changes caused by Cre-mediated recombination.

4.5. Study considerations

Potential limitations or confounders of this study include the following: 1) Since both *Mc4r-Cre* and *Sim1-Cre* express the recombinase constitutively, recombinase activity in progenitor cells could cause BRS3 deletion/re-expression in differentiated neurons that no longer express the driver Cre (for example, [56]; for a SIM1 example see [57] vs [58]). 2) Obesity causes insulin resistance, obscuring primary, adiposity-independent effects on insulin sensitivity. 3) While acute effects on Tb and energy expenditure can correlate [46], indirect calorimetry experiments were not performed. 4) When parsing

attribution of phenotypes to multiple neuronal subpopulations, effect sizes become smaller, reducing statistical power.

4.6. Conclusions

BRS3 in both MC4R and SIM1 neurons regulates energy homeostasis. Prior experiments demonstrated that the relevant BRS3 neurons are glutamatergic (Vglut²⁺) and not GABAergic (Vgat⁺). While the hypomorph status of the *Mc4r^{Cre}* allele can confound phenotypes, we demonstrate that BRS3 in both MC4R- and SIM1-expressing neurons contributes to the regulation of body weight/adiposity, insulin sensitivity, food intake, and body temperature.

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CONFLICT OF INTEREST

None declared.

APPENDIX A. SUPPLEMENTARY DATA

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.molmet.2020.02.012>.

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