Brief Communication

MOLECULAR METABOLISM

Conditional deletion of melanin-concentrating hormone receptor 1 from GABAergic neurons increases locomotor activity

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ABSTRACT

Objective: Melanin-concentrating hormone (MCH) plays a key role in regulating energy balance. MCH acts via its receptor MCHR1, and MCHR1 deletion increases energy expenditure and locomotor activity, which is associated with a hyperdopaminergic state. Since MCHR1 expression is widespread, the neurons supporting the effects of MCH on energy expenditure are not clearly defined. There is a high density of MCHR1 neurons in the striatum, and these neurons are known to be GABAergic. We thus determined if MCH acts via this GABAergic neurocircuit to mediate energy balance.

Methods: We generated a *Mchr1-flox* mouse and crossed it with the *Vgat-cre* mouse to assess if MCHR1 deletion from GABAergic neurons expressing the vesicular GABA transporter (vGAT) in female *Vgat-Mchr1-KO* mice resulted in lower body weights or increased energy expenditure. Additionally, we determined if MCHR1-expressing neurons within the accumbens form part of the neural circuit underlying MCH-mediated energy balance by delivering an adeno-associated virus expressing Cre recombinase to the accumbens nucleus of *Mchr1-flox* mice. To evaluate if a dysregulated dopaminergic tone leads to their hyperactivity, we determined if the dopamine reuptake blocker GBR12909 prolonged the drug-induced locomotor activity in *Vgat-Mchr1-KO* mice. Furthermore, we also performed amperometry recordings to test whether MCHR1 deletion increases dopamine output within the accumbens and whether MCH can suppress dopamine release.

Results: *Vgat-Mchr1-KO* mice have lower body weight, increased energy expenditure, and increased locomotor activity. Similarly, restricting MCHR1 deletion to the accumbens nucleus also increased locomotor activity. *Vgat-Mchr1-KO* mice show increased and prolonged sensitivity to GBR12909-induced locomotor activity, and amperometry recordings revealed that GBR12909 elevated accumbens dopamine levels to twice that of controls, thus MCHR1 deletion may lead to a hyperdopaminergic state that mediates their observed hyperactivity. Consistent with the inhibitory effect of MCH, we found that MCH acutely suppresses dopamine release within the accumbens.

Conclusions: As with established models of systemic MCH or MCHR1 deletion, we found that MCHR1 deletion from GABAergic neurons, specifically those within the accumbens nucleus, also led to increased locomotor activity. A hyperdopaminergic state underlies this increased locomotor activity, and is consistent with our finding that MCH signaling within the accumbens nucleus suppresses dopamine release. In effect, MCHR1 deletion may disinhibit dopamine release leading to the observed hyperactivity.

Keywords MCHR1; Accumbens nucleus; Locomotor activity; GABA; Neurocircuit; Dopamine

1. INTRODUCTION

Melanin-concentrating hormone (MCH) is produced in the lateral hypothalamus [1] and has emerged as a critical player in the regulation of central energy balance [2,3]. Acute MCH administration stimulates food intake [4], and chronic MCH infusion increases body weight gain [5]; thus MCH increases orexigenic drive and promotes positive energy balance. Furthermore, MCH gene expression is upregulated in mouse models of obesity like the leptin-deficient *ob/ob* mouse [4], and MCH-overexpressing mice are more prone to diet-induced obesity [6]. By contrast, the absence of MCH increases energy expenditure, thus promoting weight loss and negative energy balance [7]. MCH knockout (*Mch-KO*) mice are lean and have increased energy expenditure and locomotor activity [8,9]. Consistent with this, deletion of MCH in *ob/ob* mice attenuates their obesity [10]. *Mch-KO* mice also display prolonged hyperactivity when treated with a dopamine reuptake blocker and are more susceptible to amphetamine sensitization [11]. Moreover, amperometry recordings from the striatum show higher dopamine output from fresh *Mch-KO* brain slices [11]. These findings indicate that a hyperdopaminergic state in the striatum mediates the hyperactivity of *Mch-KO* mice.

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In rodents, MCH acts exclusively via the MCH receptor MCHR1, which is a G_i-protein coupled receptor [12–14]. Consistent with the effects of MCH deletion, MCHR1 knockout (*Mchr1-KO*) mice are also lean, resistant to diet-induced obesity, have increased energy expenditure, and exhibit pronounced hyperactivity [15,16]. As such, the leanness observed in *Mch-KO* and *Mchr1-KO* mice is largely attributed to their increased energy expenditure and/or hyperactivity. Furthermore, both MCH and MCHR1 deletion increase amphetamine-mediated hyperactivity [17–19], which is also recapitulated following the ablation of MCH neurons in adult mice [20], thereby implicating MCH as a regulator of the dopaminergic system.

MCH nerve terminals may regulate dopamine release in the striatum [21], a key integrative region for motor control. It has been shown that MCH infusion into the ventral striatum may also stimulate food intake [22,23]. Indeed, the most prominent expression of *Mchr1* mRNA is in the striatum, which includes the caudate putamen and accumbens nucleus in the dorsal and ventral striatum, respectively [24]. These regions are almost entirely comprised of GABAergic medium spiny neurons or interneurons [25]. In aggregate, these data suggest that GABAergic neurons may mediate some actions of MCH, including dopamine-mediated locomotor activity.

To assess the actions of MCH on GABAergic neurons that effect energy expenditure and locomotor activity, we deleted MCHR1 from neurons that express the vesicular GABA transporter, vGAT, by generating the *Mchr1-flox* mouse and crossing it to the *Vgat-cre* mouse to enable cre-mediated deletion of MCHR1 from GABAergic neurons. Mice with the loss of MCHR1 in vGAT neurons were lean and display robust baseline locomotor activity and heightened sensitivity to dopamine-mediated hyperactivity. Cre-mediated deletion of MCHR1 from the accumbens nucleus using an adeno-associated virus to express Cre recombinase in *Mchr1-flox* mice also increased locomotor activity. Striatal brain slices following MCHR1 deletion have elevated dopaminergic levels when treated with a dopamine reuptake blocker during amperometry recordings. Moreover, MCH directly inhibits dopamine release to the accumbens nucleus in these striatal brain slices; an effect that is abolished by the loss of MCHR1 in vGAT neurons. Taken together, these findings indicate that MCH regulates locomotor activity via GABAergic neurons in the accumbens nucleus, and that it does so in part by inhibiting dopamine release.

2. MATERIALS AND METHODS

2.1. Animals

Experiments were performed in female mice housed under a 12 h light, 12 h dark cycle, at 22 ± 2 °C, and provided with *ad libitum* access to water and standard lab chow (3.23 kcal/g, Formulab Diet 5008, LabDiet, St. Louis, MO). All procedures adhere to the National Institute of Health *Guidelines for the Care and Use of Animals* and were approved by the Institutional Animal Care and Use Committee at the Beth Israel Deaconess Medical Center (Boston, MA).

2.1.1. Generation of *Mchr1-flox* mouse

We generated the *Mchr1-flox* mouse by designing a targeting vector that inserted loxP sites flanking exon2 of the *Mchr1* gene. A 2.7 kb genomic fragment containing exon2 was PCR-amplified from murine genomic DNA and cloned at a SmaI site 5′ of a neomycin resistance cassette flanked by Frt sites, and 3′ of a loxP site previously inserted in the pBACe3.6 cloning vector (Sanger Institute, Hinxton, United Kingdom). A 4.95 kb genomic fragment containing exon1 of the *Mchr1* gene was inserted at a NotI site immediately 5′ of the above mentioned loxP site. Similarly, a 2.9 kb genomic fragment containing downstream intron2 sequences was introduced 3′ of a second LoxP site cloned immediately 3′ of the Frt-flanked neomycin cassette. The cloned intronic sequence resided upstream of a diphtheria toxin A cassette used for selection of the ES cell clones that successfully underwent 3′ homologous recombination. All genomic fragments were fully Sanger-sequenced after completion of the transgenic construct. Mice of the F1 generation were bred with Flp-expressing mice (#003946, Jackson Laboratory, Bar Harbor, ME) in order to eliminate the neomycin cassette, and one Frt site remained 5′ of the 3′-most loxP site in intron2.

2.1.2. Generation of *Vgat-Mchr1-KO* mouse

We crossed our *Mchr1-flox* mouse with the *Vgat-cre* mouse (#016962, Jackson Laboratory) to produce the *Vgat-Mchr1-KO* mouse, in which the MCHR1 was deleted from GABAergic neurons expressing vGAT (Figure 1A). Both *Mchr1-flox* and *Vgat-cre* mice were bred onto the C57/BL6 background (#000664, Jackson Laboratory) for at least eight generations. The *Mchr1-flox* and *Vgat-cre* mouse lines were indistinguishable from their respective wild-type littermates by body weight, food intake, or locomotor activity (Supplemental Tables 1 and 2). We chose *Vgat-cre* mice as the control group for our experiments due to potential metabolic differences caused by the expression of Cre recombinase [26].

2.2. Gene expression

2.2.1. *In situ* hybridization

The *Vgat-cre* and *Vgat-Mchr1-KO* mouse brains were perfused and sliced into five series of 30 μm coronal sections as previously described [24]. A [^{35}S]-labeled antisense *Mchr1* riboprobe comprising nucleotide 30–1061 of the rat *Mchr1* mRNA was used to detect MCHR1 mRNA as previously described [24]. In brief, free-floating brain sections were pretreated with sodium citrate buffer (pH 6.0, 5 min, 90 °C), incubated with the *Mchr1* riboprobe for 18 h at 60 °C, and then washed by sequentially increasing the stringency of sodium citrate buffer exchanges. The brain slices were mounted, dehydrated, and delipidated before placing in X-ray film cassettes with BioMax MR film (Kodak, Rochester, NY) for five days.

2.2.2. Quantitative RT-PCR analysis

Microdissected brain tissue samples from the caudate putamen, accumbens nucleus, hippocampus, and cerebellum were flash-frozen in liquid nitrogen. We first isolated RNA from these tissue samples using the Direct-zol RNA MiniPrep Kit (Zymo Research, Irvine, CA) and concentrated the resulting RNA solutions via a glycogen-ethanol precipitation using 20 μg/μl UltraPure Glycogen (Invitrogen, Carlsbad, CA), as necessary. Total RNA (0.5 μg) was reverse transcribed to synthesize cDNA using the QuantiTect Reverse Transcription Kit (Qiagen, Germantown, MD). We performed quantitative RT-PCR via SYBR Green master mix (Applied Biosystems, Foster City, CA) using the 7800HT thermal cycler (Applied Biosystems). Relative mRNA expression was calculated via double delta Ct analysis and normalized to expression levels of the housekeeping gene cyclophilin. Custom primers for cyclophilin (*CypB*) and *Mchr1* were obtained from Invitrogen (Carlsbad, CA) using the following sequences: *CypB* forward, 5′-GGTGGAGAGCACCAAGACAGA-3′; *CypB* reverse, 5′-GCCGGAGTCGACAATGATG-3′; *Mchr1* forward, 5′-CAATGCCAGCAACATCTCC-3′; *Mchr1* reverse, 5′-ACCAAACACTGAAGGCATGA-3′.

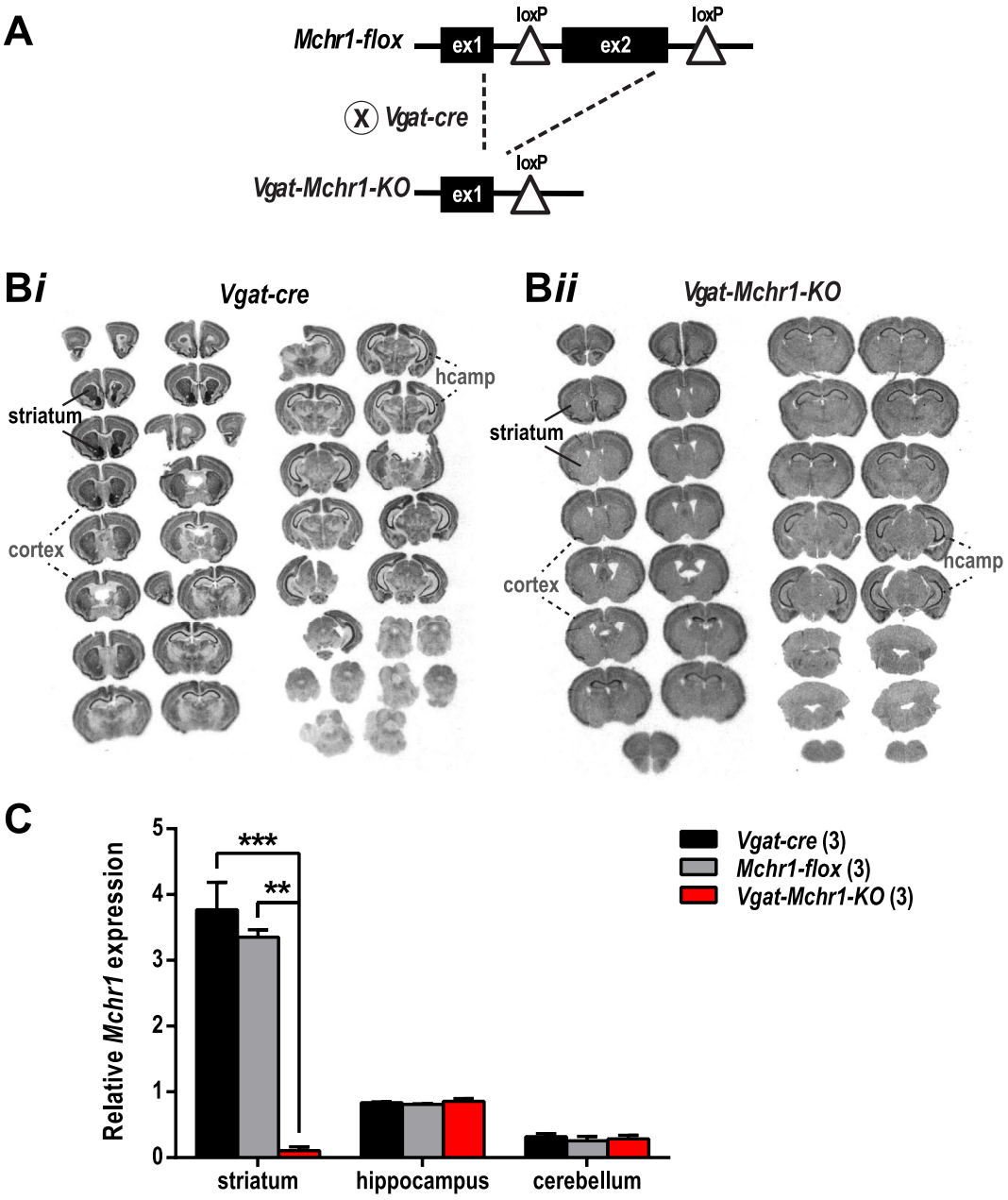

Figure 1: Cre-mediated deletion of *Mchr1* from GABAergic neurons expressing the vesicular GABA transporter. *Vgat-Mchr1-KO* mice were generated by crossing the *Vgat-cre* mouse to the *Mchr1-flox* mouse to delete exon2, flanked by *loxP* sites, of the *Mchr1* gene (***A***). Autoradiographs of ^{35}S-labeled hybridization signal for *Mchr1* mRNA in the *Vgat-cre* (***Bi***) and *Vgat-Mchr1-KO* (***Bii***) mouse brain tissue demonstrated the absence of ^{35}S-*Mchr1* hybridization in GABAergic striatal regions (black solid line), but not glutamatergic pyramidal layer of the cortex or hippocampus (gray dashed line) (***B***). qPCR analysis from the striatum, hippocampus, and cerebellum collected from the same mice shows the loss of *Mchr1* mRNA in the striatum of *Vgat-Mchr1-KO* mice only (***C***). Ordinary one-way ANOVA: **, $p < 0.01$; ***, $p < 0.001$.

2.3. Metabolic and behavioral analysis

2.3.1. Food intake

Food intake was measured by weighing food in the hoppers of individually-housed mice for either one day or over several days as indicated.

2.3.2. Body composition

Body composition was determined using EchoMRI (Echo Medical Systems, Houston, TX) at 12 weeks of age.

2.3.3. Energy expenditure

Energy expenditure was assessed in 12 week old mice by measuring oxygen consumption (VO_2) using the Comprehensive Lab Animal Monitoring System (Columbus Instruments, Columbus, OH). Mice were acclimated for two days before two days of active data collection.

2.3.4. Locomotor activity

All mice were habituated to single housing for at least 24 h before initiating locomotor recordings. We calculated locomotor activity using the Opto-M3 infrared beam break monitoring system (Columbus

Instruments, Columbus, OH), where a Multi Device Interface (v1.3) recorded the number of sequential beam breaks along the *x*-axis over a 24-hour period. Stock solutions of GBR12909 (7 mg/ml; Tocris Bioscience, Bristol, United Kingdom) were prepared using filter-sterilized double-distilled water immediately prior to use. We first tested the effects of repeated vehicle injections, which did not enhance their sensitivity to locomotor activity (data not shown). We then compared the effect of GBR12909 (20 mg/kg, i.p.) on locomotor activity to the effect of the vehicle (sterile water, i.p.), which was administered 24 h prior to the same mouse. All injections started at hour 6 of Zeitgeber time and we tracked their locomotor activity for five consecutive hours post-injection; this took place over the second half of the light cycle.

2.4. Stereotaxic injections

Mchr1-flox mice (4–6 week old) were anesthetized with a ketamine (100 μg/kg)-xylazine (10 μg/kg) mixture and placed in a mouse stereotaxic frame. We bilaterally delivered a total of 800 nl (400 nl per hemisphere) of an adeno-associated viral vector (University of North Carolina Gene Therapy Center, Chapel Hill, NC) encoding either Cre recombinase-mCherry (AAV8-hSyn-CRE-mCherry, 2.8×10^{12} genomic copies/ml) or mCherry only (AAV8-hSyn-mCherry, 8.0×10^{12} genomic copies/ml) to the rostral (anteroposterior (AP) +1.60, mediolateral (ML) ±0.60 and ± 1.40, dorsoventral (DV) −4.25 and −4.75) and caudal accumbens (AP +1.20, ML ±0.60 and ± 1.50, DV −4.30 and −4.80) using mouse atlas coordinates [27]. All mice were returned to their home cage and received buprenorphine (0.1 mg/kg, i.p.) analgesia for 24 h post-surgery. We determined their home cage locomotor activity after viral transfection and surgery recovery for seven weeks.

After the completion of the experiments, one mouse each from the control and experimental groups was deeply anesthetized and sacrificed by transcardiac perfusion with saline and 10% formalin for histological analysis to determine the spread of the virus throughout the striatum. Brains were sliced into 30 μm coronal sections to determine native mCherry-fluorescence at the injection site. We acquired stitched epifluorescence images with a fully motorized BX61VS microscope (Olympus, Tokyo, Japan) running VS-ASW-FL software (Olympus), and we viewed and exported the images off-line using OlyVIA software (Olympus). Brains from remaining mice were rapidly removed from the skull for quantitative RT-PCR analysis of *Mchr1* mRNA from the caudate putamen and accumbens nucleus, as described in section 2.2.2.

2.5. Amperometry

Animals were anesthetized with chloral hydrate (400 mg/kg, i.p.) and transcardially perfused with ice-cold, carbogenated (95% O_2, 5% CO_2)-ACSF containing (in mM): 110 choline chloride, 25 $NaHCO_3$, 2.5 KCl, 7 $MgCl_2$, 0.5 $CaCl_2$, 1.25 NaH_2PO_4, 25 glucose, 11.6 ascorbic acid, 3.1 pyruvic acid (pH 7.35, 305 mOsm/L). The brain was rapidly removed from the skull and coronal brain slices (350 μm) were sliced (4 °C), then incubated in the choline-based ACSF for an additional 5 min (37 °C). These acute brain slices recovered in a carbogenated bath ACSF containing (in mM): 124 NaCl, 2.5 KCl, 1.24 NaH_2PO_4, 1.3 $MgCl_2$, 10 glucose, 26 $NaHCO_3$, 2.5 $CaCl_2$ (300 mOsm/L) for 10 min (37 °C) before resting at room temperature (22 °C, >1 h) until used for recordings.

Each slice (between Bregma +1.35 mm to +0.60 mm) was transferred to the recording chamber and maintained by constant perfusion of carbogenated bath ACSF at 32 °C. A concentric bipolar stimulating microelectrode (CBBPF100, FHC, Bowdoin, ME) was placed at the brain tissue surface in the medial accumbens nucleus and a carbon fiber electrode (Carbostar-1 E1011-7, 100 μm tip length, Kation Scientific, Minneapolis, MN) was positioned within 100 μm of the stimulating electrode at approximately 50 μm beneath the tissue surface. The electrode used in each recording was calibrated with fresh 5 μM dopamine standards by sampling the current amplitude detected at one-minute intervals while holding at +600 mV in order to calculate the conversion of current amplitude to extracellular dopamine concentration. Dopamine (Sigma–Aldrich, St. Louis, MO) standard stock solutions (5 mM) were prepared in 500 mM sodium metabisulfite and stored as frozen aliquots at −20 °C.

To evoke dopamine release, we delivered a single 1 ms rectangular pulse (80 μA) to the stimulating electrode at 5-minute intervals with a constant current stimulus isolator (World Precision Instruments, Sarasota, FL) while applying a constant voltage of +600 mV to the carbon fiber tip with a Multiclamp 700B amplifier (Molecular Devices, San Jose, CA). We sampled the amperometric readings using Clampex 10 software (Molecular Devices) and maintained a stable dopamine output, where peak amplitudes were within 10% of each other, for at least 25 min before drug application. Each pharmacological compound was prepared immediately before use by dilution in carbogenated bath ACSF, then bath applied to the recording chamber for 15–20 min.

2.6. Statistical analysis

We analyzed amperometry data using Clampfit 10.7 software (Molecular Devices) and all other data sets using Excel (Microsoft Corporation, Redmond, WA). All data are reported as group mean ± SEM, with the number of mice or slices per group included in parentheses within each figure. We used Prism 6 (GraphPad, La Jolla, CA) to determine statistical significance for group means using either an ordinary one-way ANOVA with post-hoc Bonferonni test or an unpaired Student's *t*-test, where appropriate, with *, $p < 0.05$; **, $p < 0.01$; and ***, $p < 0.001$. We also used a repeated measures two-way ANOVA with post-hoc Bonferonni test to compare entire data sets over time, with †, $p < 0.05$ and ††, $p < 0.01$.

3. RESULTS

3.1. Conditional MCHR1 deletion from GABAergic neurons produced lean, hyperactive mice

We generated *Mchr1-flox* mice and mated them to *Vgat-cre* mice to delete MCHR1 from GABAergic neurons in *Vgat-Mchr1-KO* mice (Figure 1A). Following *in situ* hybridization, a comparison of autoradiographic images from *Vgat-cre* control and *Vgat-Mchr1-KO* brain tissue labeled with an isotopic [^{35}S]-*Mchr1* riboprobe show a distinct absence of *Mchr1* hybridization in GABAergic regions like the striatum but not glutamatergic pyramidal neurons throughout the hippocampus or cortex (Figure 1B). We quantified the extent of MCHR1 deletion by RT-qPCR analysis and show a complete loss of *Mchr1* mRNA from *Vgat-Mchr1-KO* striatal tissue relative to *Vgat-cre* and *Mchr1-flox* controls (Figure 1C). Meanwhile, *Mchr1* mRNA expression levels remain unchanged in the hippocampus, where MCHR1-expressing neurons are on glutamatergic pyramidal neurons [24,28], or in the cerebellum, where MCHR1 expression is minimal.

Compared to *Vgat-cre* controls, adult *Vgat-Mchr1-KO* mice weighed 10% less (Figure 2A) and had a lower body fat percentage (Figure 2B) while consuming the same number of calories (12.6 ± 0.7 kcal, n = 8; *Vgat-cre*: 14.2 ± 0.7 kcal, n = 8; $p = 0.133$). MCHR1 deletion in vGAT neurons increased VO_2 consumption (Figure 2C) and *Vgat-Mchr1-KO* mice showed a 93% increase in home cage locomotor activity (Figure 2D). These findings indicate that cre-mediated deletion of

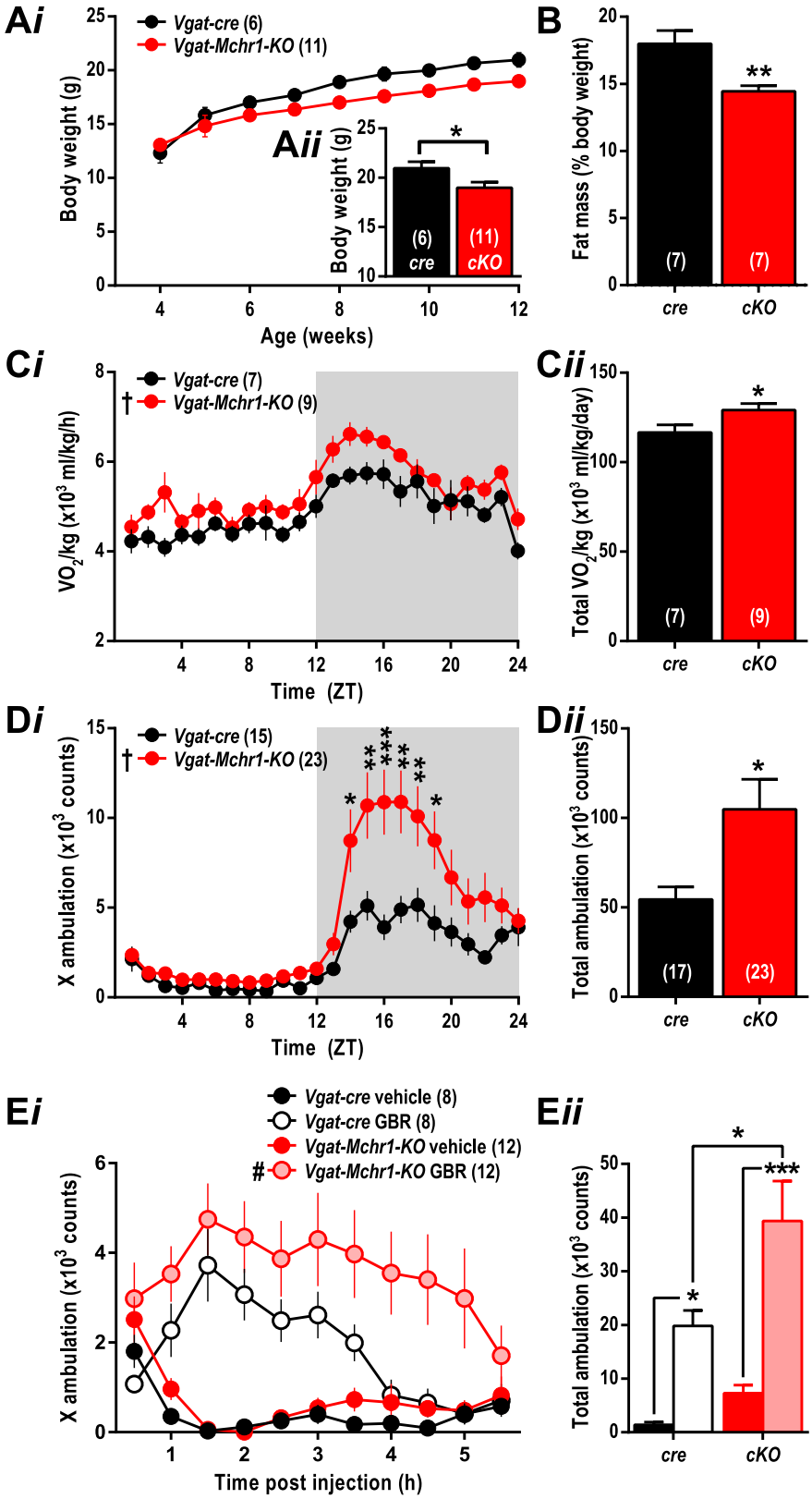

Figure 2: *Mchr1* deletion from GABAergic neurons resulted in lean and hyperactive mice with increased dopamine-mediated locomotor activity. At 12 weeks of age, *Vgat-Mchr1-KO* (*cKO*) mice weighed 10% less (***A***), consistent with lower body fat (***B***). They also have increased oxygen consumption (***C***) and home cage locomotor activity (***D***). Systemic administration of GBR12909 (GBR, 20 mg/kg, i.p.) in *cKO* mice produced a longer-lasting increase in home cage locomotor activity (***E***). Repeated measures two-way ANOVA: †, $p < 0.05$ (vs. *Vgat-cre;* ***Ci***, ***Di***); #, $p < 0.05$ (vs. *Vgat-cre* GBR; ***Ei***). Bonferroni multiple comparisons post-test: *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$ (***Di***). Ordinary two-way ANOVA (***Eii***). Student's *t* test: *, $p < 0.05$; **, $p < 0.01$ (***Aii***, ***B***, ***Cii***, ***Dii***).

Mchr1 from GABAergic neurons produces lean mice that have increased energy expenditure.

Considering the baseline hyperactivity of *Vgat-Mchr1-KO* mice, we next examined how these mice would respond to a paradigm of hyperactivity induced by the blockade of dopamine reuptake [11]. Systemic treatment of *Vgat-cre* and *Vgat-Mchr1-KO* mice with the dopamine reuptake inhibitor GBR12909 (20 mg/kg) produced a pronounced and sustained increase in the locomotor activity of all mice relative to vehicle treatment. Interestingly, conditional MCHR1 deletion heightened the sensitivity of *Vgat-Mchr1-KO* mice to GBR12909 and increased their induced hyperactivity compared to *Vgat-cre* controls. GBR12909-mediated ambulation peaked 90 minutes post-injection for all mice (Figure 2Ei), but this increase was greater in *Vgat-Mchr1-KO* mice. The effect of GBR12909 was also longer-lasting in *Vgat-Mchr1-KO* mice. *Vgat-cre* mice returned to baseline ambulation levels within four hours, while the locomotor activity of *Vgat-Mchr1-KO* mice remained sustained and elevated for at least five hours (Figure 2Ei). In effect, the cumulative ambulatory count of *Vgat-Mchr1-KO* mice treated with GBR12909 was two-fold higher than the treated *Vgat-cre* mice (Figure 2Eii). These findings suggest that a dysregulation of the dopaminergic system at least in part contributes to the hyperactivity displayed by *Vgat-Mchr1-KO* mice.

3.2. Deletion of MCHR1 from the accumbens nucleus increased locomotor activity

In order to identify the candidate GABAergic MCHR1 neurons that contribute to the hyperactivity of *Vgat-Mchr1-KO* mice, we bilaterally deleted *Mchr1* mRNA from the accumbens nucleus of *Mchr1-flox* mice by stereotaxic delivery of an AAV encoding Cre-mCherry. Our viral delivery confined mCherry expression to the accumbens nucleus (Figure 3A), and we evaluated *Mchr1* expression in the accumbens nucleus compared to the dorsally-located caudate putamen to verify the efficacy and specificity of this cre-mediated deletion. Additionally, we also injected *Mchr1-flox* mice with an AAV encoding mCherry only to control for decreases in *Mchr1* levels following AAV delivery.

Mchr1-flox mice transfected with Cre-mCherry showed a 50% reduction of *Mchr1* mRNA in the accumbens nucleus, while *Mchr1-flox* mice transfected with mCherry expressed similar *Mchr1* mRNA levels between the accumbens and caudate putamen (Figure 3B). Although our AAV cre-mediated strategy only produced a partial deletion of

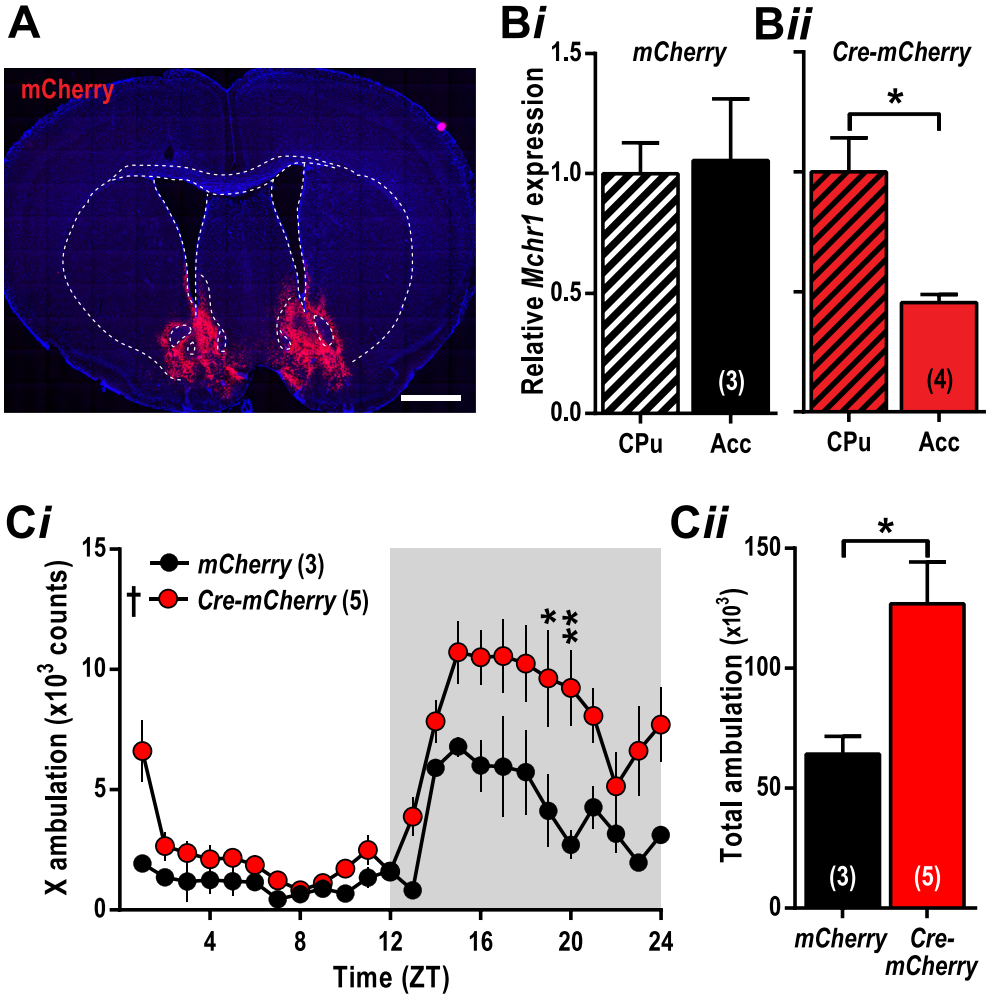

Figure 3: Deletion of *Mchr1* in the accumbens nucleus increased locomotor activity. Representative epifluorescence photomicrograph (***A***) illustrating the spread of native mCherry fluorescence in the accumbens nucleus of *Mchr1-flox* mice seven weeks following stereotaxic delivery of an adeno-associated virus encoding mCherry (AAV8-hSyn-mCherry, ***Bi***) or Cre-mCherry (AAV8-hSyn-Cre-mCherry, ***Bii***). Transfection with Cre-mCherry produced a half-fold reduction in *Mchr1* gene expression in the accumbens (Acc) relative to the caudate putamen (CPu) and increased home cage locomotor activity throughout a 24-hour period (***Ci***) and in total (***Cii***). Scale bar, 1 mm. Repeated measures two-way ANOVA: †, $p < 0.05$. Bonferroni multiple comparisons post-test: *, $p < 0.05$; **, $p < 0.01$ (***Ci***). Student's *t* test: *, $p < 0.05$ (***Bii***, ***Cii***).

accumbens *Mchr1*, cre-injected mice displayed a near two-fold increase in total ambulation that is sustained throughout their entire dark cycle (Figure 3C). There was no differences in their body weight (*mCherry*: 25.4 ± 1.3 g, n = 3; *Cre-mCherry*: 22.6 ± 0.9 g, n = 5; p = 0.116) or daily food intake (*mCherry*: 11.1 ± 2.0 kcal, n = 3; *Cre-mCherry*: 10.7 ± 2.2 kcal, n = 5; $p = 0.903$). This suggested that MCHR1 neurons within the accumbens nucleus mediate the effects of MCH on locomotor activity.

3.3. Conditional MCHR1 deletion dysregulates dopamine transmission in the accumbens nucleus

Since both *Mch-KO* [11] and *Vgat-Mchr1-KO* (Figure 2E) mice show an enhanced sensitivity to dopamine-mediated hyperactivity, we sought to determine if MCH signaling directly interacts with the dopamine system of the accumbens nucleus. In order to measure dopamine release, we performed *in vitro* amperometry recordings in fresh brain slices containing the striatum and determined the peak current amplitude evoked by a stimulating electrode every five minutes. There were no differences in the kinetics of the evoked dopamine current from *Vgat-cre* and *Vgat-Mchr1-KO* mice, which had similar rise times (*Vgat-cre*: 64.9 ± 11.7 ms, n = 5; *Vgat-Mchr1-KO*: 105.4 ± 19.1 ms, n = 6; $p = 0.119$) and decay times (*Vgat-cre*: 273.5 ± 42.3 ms; *Vgat-Mchr1-KO*: 360.2 ± 46.0 ms; $p = 0.206$). Bath application of GBR12909 (3 μM) increased dopamine release within acute brain slices from both *Vgat-Mchr1-KO* and *Vgat-cre* mice. Notably, GBR12909 elicited a two-fold greater increase in dopamine release from *Vgat-Mchr1-KO* striatal slices (Figure 4A), thus confirming that the loss of MCHR1 signaling increased the sensitivity of *Vgat-Mchr1-KO* mice to dopaminergic dysregulation. In order to determine if MCH acutely inhibits dopamine release, we determined the effect of MCH (3 μM) on evoked dopamine release in the accumbens of striatal brain slices. Bath application of 3 μM MCH produced a 25% decrease in dopamine release that was reversible with the washout of MCH (Figure 4B). By contrast, MCH did not inhibit dopamine release in striatal slices produced from *Vgat-Mchr1-KO* brains (Figure 4B), thus suggesting that MCH may act via GABAergic MCHR1 neurons to inhibit dopamine release within the accumbens nucleus.

4. DISCUSSION

Multiple studies have implicated the MCH system in the regulation of energy homeostasis. Systemic deletion of MCH [8,9] or MCHR1 [15,16,29] in rodents results in increased energy expenditure and locomotor activity, in part by regulating dopaminergic tone [11]. This hyperactivity phenotype is also seen following the ablation of MCH neurons both in early life [20] or adult animals [30]. Consistent with the role of MCH in suppressing energy expenditure and locomotor activity, direct chemogenetic activation of these neurons also leads to decrease spontaneous locomotor activity [31]. The accumbens is a potential target for MCH action, which additionally includes its effects on food intake [22,32] and mood [33–35]. However, the identities of downstream neurons that mediate MCH actions on energy expenditure were not well described. In order to identify these neurons, we used a transgenic model to delete MCHR1 specifically from GABAergic neurons marked by the expression of vGAT, which includes the accumbens. This recapitulated the phenotype following systemic MCH or MCHR1 deletion, as we observed an increase in energy expenditure and a two-fold increase in spontaneous locomotor activity. An elevated dopaminergic tone contributes to this hyperactivity, and we localized this effect to the accumbens by site-specific MCHR1 deletion.

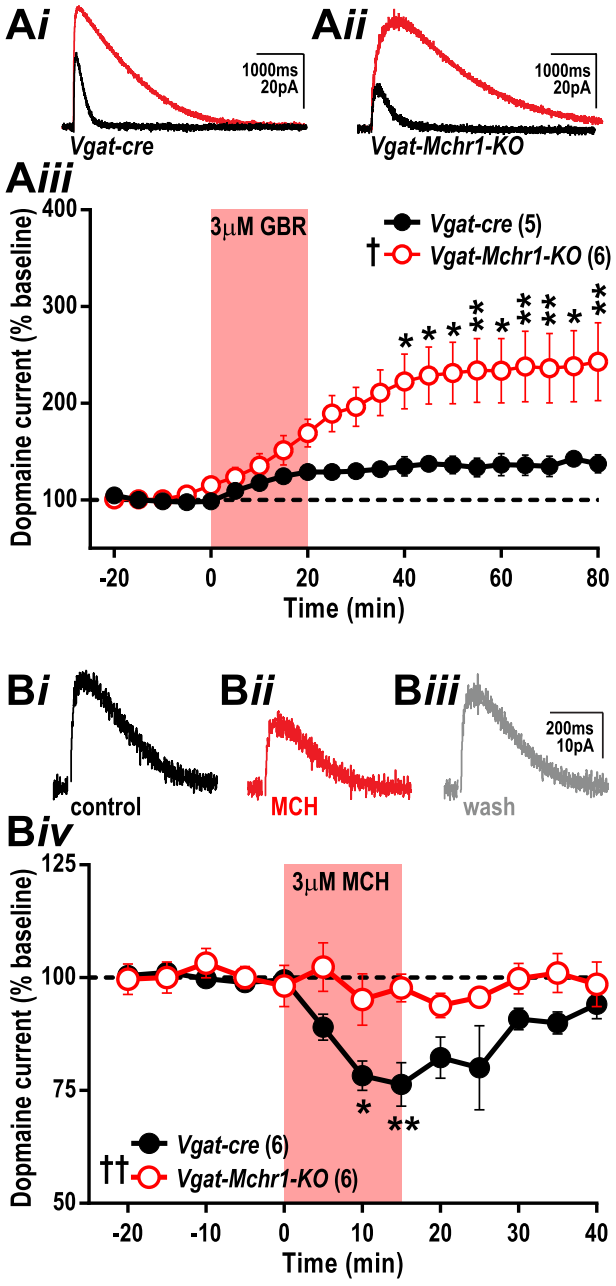

Figure 4: Reversible MCH-mediated inhibition of dopamine release in the accumbens nucleus. Representative traces from an amperometry recording of dopamine current before (black) and after a 20-minute bath application of 3 μM GBR12909 (GBR, red) to freshly prepared striatal brain slices from *Vgat-cre* (***Ai***) or *Vgat-Mchr1-KO* mice (***Aii***). GBR produced a greater increase in dopamine current amplitude from the accumbens of *Vgat-Mchr1-KO* mice (***Aiii***). Representative amperometric traces of dopamine current before (***Bi***), immediately following a 15-minute bath application of 3 μM MCH (***Bii***), and after its washout (***Biii***). MCH elicited a reversible inhibition of dopamine current in striatal slices from control but not *Vgat-Mchr1-KO* mice (***Biv***). Repeated measures two-way ANOVA: †, $p < 0.05$; ††, $p < 0.01$. Bonferroni multiple comparisons post-test: *, $p < 0.05$; **, $p < 0.01$.

Interestingly, although MCH has been reported to stimulate food intake [4,22,23], MCHR1 deletion from vGAT neurons in our studies did not alter food intake. However, it should be noted that the effects of MCH on food intake in mice are potentially problematic. While intracerebroventricular (ICV) administration of MCH to rats leads to rapid

and substantive increases in food intake [4,23,36], the effects of ICV administration in mice is minimal, thus either repetitive or chronic MCH administration is required to increase food intake [5]. In transgenic models, global MCH deletion initially decreased food consumption [8,37], but this effect did not persist over time [9]. Furthermore, in contrast to MCH deletion, global MCHR1 deletion produces hyperphagia [15,16], which was significantly more robust in male than female mice [15]. The absence of a food intake effect in our model could relate to our use of females [36,38] or to a less robust effect of MCH on food intake in mice.

Increased locomotor activity is associated with increased dopaminergic tone. For example, deletion of the dopamine transporter that produces a hyperdopaminergic state is associated with spontaneous locomotor activity [39,40]. Previous reports suggest that MCH is a regulator of dopaminergic tone. A comparison of accumbens dopamine release measured using amperometry recordings in *ex vivo* brain slices from mice that lack MCH [11] revealed elevated dopamine release compared to wildtype mice. Similar results were also noted in rats that lack the MCH peptide precursor [41]. Results from whole animals are also consistent with increased dopaminergic tone. MCH- and MCHR1-deficient mice display enhanced sensitization to psychostimulants like amphetamine [11,17,19] and cocaine [20,42], as well as increased locomotor responses to the dopamine reuptake blocker GBR12909 [11,20]. We found that in addition to recapitulating the hyperactivity in global MCH- or MCHR-deficient mice, MCHR1 deletion from vGAT neurons in our mice also increased dopaminergic tone, and they were more sensitive to the hyperlocomotor effects of GBR12909.

Since the absence of MCH signaling results in enhanced dopaminergic tone, MCH itself would have an inhibitory effect on the dopamine system. Consistent with this, we found that MCH acutely suppressed dopamine release in accumbens brain slices, an effect that was abolished following MCHR1 deletion in vGAT neurons. The pathways downstream of the incumbent GABAergic MCHR1 neurons in the accumbens that mediate this response have not yet been defined. In the intact animal, MCH may directly inhibit striatal inputs from dopaminergic efferents that originate in the ventral tegmental area or compact part of the substantia nigra [24,43]. Furthermore, as activation of vGAT neurons in the ventral tegmental area can suppress dopamine release in the striatum [44], the loss of the G_i-protein coupled MCHR1 receptor may disinhibit these neurons and contribute to the hyperdopaminergic state in our mouse model. Our findings confirm that the inhibitory effect of MCH on dopamine release requires MCHR1 expression on vGAT neurons, thereby identifying for the first time that GABAergic vGAT neurons are key targets of MCH.

Targeting MCHR1 deletion specifically to the accumbens also increased spontaneous locomotor activity, thus the accumbens comprises a critical GABAergic region underlying the hyperactivity of MCHR1-deficient mice. The accumbens also mediates the orexigenic effects of MCH in rats [22,32]. These aggregate data lead us to posit that the accumbens is a critical component of the neurocircuit that integrates MCH actions. However, MCHR1 is widely expressed within the brain, thus site-specific MCHR1 deletion at other discrete neuroanatomical sites would provide additional insights to elucidate the full range of MCH effects.

5. CONCLUSION

Transgenic models of MCH or MCHR1 deletion produce robust and consistent effects of increase energy expenditure and locomotor activity. While the role of MCH in the regulation of energy homeostasis is well known, the neuronal population supporting the actions of MCH are not well defined. We found that MCH acts via a GABAergic neurocircuit to regulate energy homeostasis. Furthermore, we also show that MCH regulates dopamine release, and the interaction between dopaminergic and GABAergic systems in the accumbens is a critical pathway contributing to the effects of MCH on energy expenditure.

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CONFLICTS OF INTEREST

None.

APPENDIX

Supplemental Table 1 — Baseline characterization of *Mchr1-flox* mice

	Wildtype (5)	*Mchr1-flox* (5)	*p* value[4]
Body weight (g)[1]	19.0 ± 1.0	18.4 ± 1.4	0.749
Food intake (kcal)[2]	64.0 ± 3.5	66.0 ± 5.7	0.770
Ambulation (counts)[3]	97,027 ± 9,740	83,083 ± 9,616	0.338

Body weights[1], total 6-day *ad libitum* intake of standard lab chow (3.23 kcal/g)[2], and number of sequential *x*-axis ambulatory beam breaks over a 24-hour period averaged over three days[3] were determined from eight week old wildtype and homozygous *Mchr1-flox* littermates. Numbers and text in parenthesis indicate the number of mice used and unit of measurement, respectively. All values are reported as group mean ± SEM and compared using an unpaired Student's *t* test[4].

Supplemental Table 2 — Baseline characterization of *Vgat-cre* mice

	Wildtype (6)	*Vgat-cre* (5)	*p* value[4]
Body weight (g)[1]	19.7 ± 0.6	20.0 ± 0.7	0.799
Food intake (kcal)[2]	62.5 ± 2.8	63.2 ± 3.2	0.855
Ambulation (counts)[3]	79,915 ± 18,344	80,934 ± 18,585	0.970

Body weights[1], total 6-day *ad libitum* intake of standard lab chow (3.23 kcal/g)[2], and number of sequential *x*-axis ambulatory beam breaks over a 24-hour period averaged over three days[3] were determined from eight week old wildtype and heterozygous *Vgat-cre* littermates. Numbers and text in parenthesis indicate the number of mice used and unit of measurement, respectively. All values are reported as group mean ± SEM and compared using an unpaired Student's *t* test[4].

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