Cellular/Molecular

GABA Type B Receptor Signaling in Proopiomelanocortin Neurons Protects Against Obesity, Insulin Resistance, and Hypothalamic Inflammation in Male Mice on a High-Fat Diet

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There is evidence suggesting that the GABA system in the arcuate nucleus, where orexigenic neuropeptide Y and agouti-related peptide as well as anorexigenic proopiomelanocortin (POMC) are expressed, plays an important role in energy balance. In this study, we generated POMC-specific $GABA_B$ receptor-deficient [knock-out (KO)] mice. Male KO mice on a high-fat diet (HFD) showed mild increases in body weight (BW) at the age of 9 weeks compared to wild-type (WT) mice, and the differences remained significant until 16 weeks old. However, there was no difference in BW in females between genotypes. While food intake was similar between genotypes, oxygen consumption was significantly decreased in the male KO mice. The insulin tolerance test revealed that the male KO mice were less insulin sensitive compared to WT mice at the age of 8 weeks, when there was no significant difference in BW between genotypes. Despite increased BW, POMC mRNA expression in the arcuate nucleus was significantly decreased in the KO mice compared to WT mice at the age of 16 weeks. Furthermore, the expression of TNFα as well as IL-6, proinflammatory markers in the hypothalamus, was significantly increased in the KO mice on a HFD compared to WT mice. This demonstrates that the deletion of $GABA_B$ receptors in POMC neurons in the male mice on a HFD results in obesity, insulin resistance, and hypothalamic inflammation. Furthermore, the decreased POMC expression in the obese KO mice suggests that the regulation of POMC expression through $GABA_B$ receptors is essential for proper energy balance.

Introduction

The anorexigenic proopiomelanocortin (POMC) is expressed in the hypothalamic arcuate nucleus. The POMC neurons as well as those expressing orexigenic neuropeptide Y (NPY) and agouti-related peptide (AgRP) integrate peripheral signals related to energy balance such as leptin, and send these signals to other hypothalamic neurons (Morton et al., 2006). POMC manifests its effects on energy balance via α-melanocyte-stimulating hormone (α-MSH) and β-MSH, which are cleaved from POMC precursors and bind to melanocortin type 3 and 4 receptors (Harrold et al., 2003; Coll et al., 2004). A pivotal role for POMC neurons in energy homeostasis is supported by several studies; central injection of α-MSH or β-MSH decreases food intake (Fan et al., 1997; Jonsson et al., 2001; Honda et al., 2012), whereas the injection of a melanocortin receptor antagonist increases food intake in rodents (Fan et al., 1997; Aizawa-Abe et al., 2000; Obici et al., 2001; Nogueiras et al., 2007); whole-body knock-out (KO) mice for POMC (Yaswen et al., 1999) as well as those for melanocortin type 4 receptors showed obesity (Huszar et al., 1997). Previous studies also revealed that POMC neuron-specific deletion of leptin receptors (Balthasar et al., 2004) or molecules related to leptin signaling (Kievit et al., 2006; Xu et al., 2007; Banno et al., 2010) leads to obesity in mice.

GABA, the predominant inhibitory neurotransmitter in the CNS, acts on two types of receptors: ionotropic $GABA_A$ receptors ($GABA_A$Rs), which are mainly located postsynaptically, and metabotropic $GABA_B$ receptors ($GABA_B$Rs), which are located presynaptically as well as postsynaptically (Enna and McCarson, 2006; Gassmann and Bettler, 2012). It has been demonstrated that (1) the AgRP neurons (Cowley et al., 2001) and POMC neurons (Dicken et al., 2012) are GABAergic, (2) leptin increases the frequency of action potentials in the POMC neurons by reducing GABA release from the AgRP neurons (Cowley et al., 2001), and (3) mice that lack GABA release from the AgRP neurons are lean (Tong et al., 2008). These data suggest that the GABA system in the arcuate nucleus plays an important role in energy homeostasis, although the relative contribution of $GABA_B$Rs compared to

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$GABA_A$Rs in the arcuate nucleus in the energy homeostasis has not been studied yet.

In the present study, we generated mice that specifically lack functional $GABA_B$Rs in POMC neurons. We analyzed their phenotypes with respect to energy balance and glucose tolerance to delineate the role of $GABA_BR$ signaling in the POMC neurons.

Materials and Methods

Mice. All animal procedures were approved by the Animal Care and Use Committee of Nagoya University Graduate School of Medicine and performed in accordance with the institutional guidelines, which conform with the National Institutes of Health animal care guidelines. Mice were maintained on a 12 h light/dark cycle in a temperature-controlled barrier facility, with free access to water and food. Age-matched littermates were used for all experiments.

Mice with POMC-specific deletion of $GABA_{B1}$. $GABA_{B1}R^{lox511/lox511}$ mice were generated previously (Haller et al., 2004). $GABA_B$Rs consist of principal $GABA_{B1}$ and $GABA_{B2}$ subunits that associate with auxiliary KCTD (potassium channel tetramerization domain-containing) subunits (Gassmann and Bettler, 2012). Principal subunits are essential for function while auxiliary subunits modulate the receptor response, and genetic deletion of the $GABA_{B1}$ subunit leads to complete lack of functional $GABA_B$Rs (Schuler et al., 2001; Gassmann and Bettler, 2012). *POMC-Cre* transgene mice that express functional Cre-recombinase only in POMC cells (Balthasar et al., 2004; hereafter termed *POMC-Cre* mice) and mice that express enhanced green fluorescent protein (EGFP) upon Cre-mediated excision in cells harboring the deletion event (Novak et al., 2000; hereafter termed *Z/EG* reporter mice) were purchased from the Jackson Laboratory. Primer sequences used for genotyping of $GABA_{B1}R^{lox511/lox511}$ and *POMC-Cre* mice were as follows: $GABA_{B1}R$ forward, 5′-TGGGGT GTGTCCTACATGCAGCGGACGG; reverse, 5′-GCTCTTCACCTTTCAACCCAGCCTCAGGCAGGC; *POMC-Cre* forward, 5′-TGGCTCAATGTCCTTCCTGG; reverse, 5′-CACATAAGCT GCATCGTTAAG [to detect wild-type (WT) gene] or 5′-GAGATATCTT-TAACCCTGATC (to detect transgene). *Z/EG* reporter mice were genotyped using the following *GFP* primers: forward, 5′-TCATGGCCGACAAGCAGA AGAACG; reverse, 5′-CGGCGGCGGTCACGAACT. DNA was extracted from a drop of blood from each experimental mouse at the age of 8 weeks to check for the occurrence of spurious germline deletion using the following primers: $GABA_{B1}R\,\Delta/\Delta$ forward, 5′-ATCTCTTCCTTGGCT GGGTCTTT-GCTTCGCTCG; reverse, 5′-GGGTTATTGAATATGATCGGAATTC-CTCGACT; *GAPDH* (for an internal control) forward, 5′-AACGAC CCCTTCATTGAC; reverse, 5′-TCCACGACATACTCAGCAC. All $GABA_{B1}R^{lox511/lox511}$ mice and *POMC-Cre* mice were backcrossed >10 generations onto a C57BL/6 background.

Isolating DNA from tissues for detection of recombination of floxed alleles. Tissues (pituitary, hypothalamus, hindbrain, cerebral cortex, liver, skeletal muscle, and fat) of mice at the age of 8 weeks were digested by 50 mM NaOH for 10 min at 95°C, and 1 M Tris-HCl (pH 8.0) was added to the digestion. Samples were centrifuged for 10 min at 12,000 × *g*, and supernatants were transferred to a fresh tube.

Body composition and food intake. At weaning (3 weeks old), mice were placed on either a diet of standard chow CE-2 (CHD; calories provided by 24.9% protein, 4.6% fat, and 70.5% carbohydrate; CLEA Japan) or a custom high-fat diet (HFD; 58Y1; calories provided by 18.3% protein, 60.9% fat, and 20.1% carbohydrate; Test Diet). Body weight (BW) was monitored until the age of 16 weeks, when the nose–rump length was measured.

Energy expenditure measurements. In another cohort of mice, food intake and feed efficiency, which was calculated as grams of BW gained per grams of food consumed over a 3 d period (Sutherland et al., 1974), were evaluated at the age of 8 weeks. Mice at the age of 16 weeks were acclimated to the test cage for 24 h, and energy expenditure was measured at 5 min intervals for 24 h on the second day (Model Supermex; Muromachi Kikai). Oxygen consumption (V_{O_2}) and carbon dioxide production (V_{CO_2}) were measured using electrochemical and spectrophotometric sensors. Resting is defined as <5% average max activity counts. In this condition, we calculated the resting V_{O_2}. Respiratory quotient (RQ) was calculated as the ratio of V_{CO_2} to V_{O_2}. Locomotor activity was measured simultaneously by infrared beam interruption (Model MK-5000RQ/02; Muromachi Kikai) and reported as average counts per hour. Rectal temperature was also measured at the age of 16 weeks with a thermistor (KN-91; Natsume Seisakusho).

Serum levels of insulin and leptin. Blood was collected by tail bleeding from mice at the age of 8 and 16 weeks in the beginning of the light cycle between 09:00 and 10:00 A.M. when mice were in the fed state. Serum was separated by centrifugation at 6000 × *g*. Serum levels of insulin and leptin were measured by ELISAs (Morinaga Institute of Biological Science, Kanagawa, Japan).

Serum levels of corticosterone in basal and stressed conditions. Male mice at the age of 16 weeks on the CHD were individually restrained for 30 min in ventilated 50 ml polypropylene tubes. Blood was collected by tail bleeding from mice in basal or stressed conditions, and serum corticosterone levels were measured by ELISA (AssayPro).

Insulin tolerance test and glucose tolerance test. The insulin tolerance test (ITT) and glucose tolerance test (GTT) were performed in male mice at the age of 8 weeks on a HFD as described previously (Banno et al., 2010). Blood glucose was assayed in tail blood using a glucometer (Medisafe mini; Terumo). Measurements were taken at the onset of the light cycle between 09:00 and 10:00 A.M. The insulin dose used for intraperitoneal injections was 1.0 mU/g BW. Glucose dose used for intraperitoneal injections was 2 mg/g BW.

In situ hybridization. Mice were killed at the age of 16 weeks in the light cycle between 09:00 and 10:00 A.M., when they were in the fed state, and the brains were rapidly dissected and frozen. *In situ* hybridization was performed as described previously (Hayashi et al., 2009). The RNA probes were generated from the plasmids of POMC (Sato et al., 2007). Some hybridized sections were dipped in nuclear Kodak NTB2 emulsion (Kodak) and exposed for 2 d for *POMC* mRNA to be visualized. To assist cellular localization of the hybridized signals, the emulsion-dipped sections hybridized with *POMC* mRNA probes were stained with cresyl violet. Any neuronal cross sections with grains of more than threefold the background density were considered labeled.

Immunohistochemistry. Immunohistochemistry was performed in mice at the age of 16 weeks as described previously (Suzuki et al., 2010). The antibodies of GAD67 and vGLUT2 were purchased from Millipore, and those of Synapsin 1, EGFP, and POMC precursor from Synaptic System, MBL, and Phoenix Pharmaceuticals, respectively. After washing, the sections were incubated for 1 h at room temperature with the secondary antibodies (Alexa Fluor; Life Technologies). All fluorescently stained sections were examined with a fluorescence microscope (BZ-8000; Keyence).

Cell counting. The best-matched slice at −1.4 mm caudal from the bregma, according to the brain atlas (Paxinos and Franklin, 2000), was chosen in each mouse for the analysis. Data are presented as the average number of cells per section.

Determination of mRNA levels by real-time PCR. Mice were killed in the light cycle between 09:00 and 10:00 A.M., and the whole hypothalamus was rapidly dissected and frozen in liquid nitrogen. Total RNA was extracted from the hypothalamus using TRIzol (Life Technologies) and the RNeasy kit (QIAGEN). cDNA was synthesized from 1 μg total RNA using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems). The relative mRNA levels of *POMC*, *NPY*, *AgRP*, *IL-6*, *TNFa*, *Gfap*, and *CD68* in the hypothalamus were assessed by quantitative real-time PCR (qRT-PCR) using *GAPDH* as internal control. The qRT-PCR reactions were performed using Brilliant III SYBR Green QPCR Master Mix (Agilent Technologies), and samples were run using the Stratagene Mx3000p. The sequences of primers were as follows: *POMC* forward, 5′-GAGGCCACTGAACATCTTTGTC; reverse, 5′-GCAGAGGCAAA-CAAGATTGG; *AgRP* forward, 5′-CAGAAGCTTTGGCGGAGGT; reverse, 5′-AGGACTCGTGCAGCCTTACAC; *NPY* forward, 5′-TCA-GACCTCTTAATGAAGGAAAGCA; reverse, 5′-GAGAACAAGTTTCA TTTCCCATCA; *IL-6* forward, 5′-GTGGCTAAGGACCAAGACCA; reverse, 5′-GGTTTGCCGAGTAGA CCTCA; *TNFα* forward, 5′-CATCTTCTCAAAACTCGAGTGACAA; reverse, 5′-TGGGAGTAGATA-AGGTACAGCCC; *Gfap* forward, 5′-AACGACTATCGCCGCCAACTG; reverse, 5′-CTCTTCCTGTTCGCGCATTTG; *Cd68* forward, 5′-CTTC-CCACAAGCAGCACAG; reverse, 5′-AATGATGAGAGGCAGCAAGAGA;

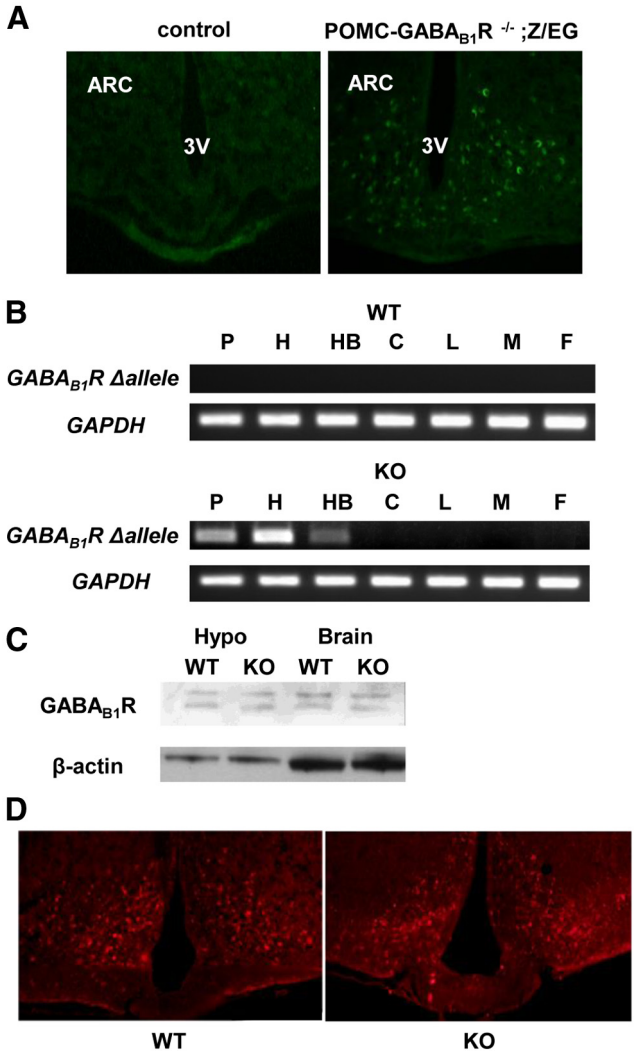

Figure 1. Generation of POMC-specific $GABA_BR$-deficient mice. ***A***, Deletion efficiency of *POMC-Cre* as assessed by immunohistochemistry for EGFP. $GABA_{B1}R^{+/+}$;*Z/EG* control hypothalamus (left) compared with *POMC-*$GABA_{B1}R^{-/(minus)}$;*Z/EG* hypothalamus (right). ARC, Arcuate nucleus; 3V, third ventricle. ***B***, Detection of deletion of $GABA_{B1}R$ alleles (Δ) in *POMC-*$GABA_{B1}R^{-/-}$ (KO) mice compared with $GABA_{B1}R^{+/+}$ (WT) mice. DNA was extracted from different tissues, and deletion of the floxed allele was detected by PCR. P, Pituitary; H, hypothalamus; HB, hindbrain; C, cerebral cortex; L, liver; M, skeletal muscle; F, fat. Recombination was detected only in pituitary, hypothalamus, and hindbrain of KO mice. A PCR reaction with *GAPDH* was used as an internal control. ***C***, $GABA_BR$ protein levels in WT mice compared with KO mice as determined by immunoblotting. β-actin protein levels are shown as a loading control. Hypo, Hypothalamus; brain, cerebral cortex. ***D***, POMC cells detected with immunohistochemistry in hypothalamus in WT mice and KO mice at the age of 16 weeks on the CHD.

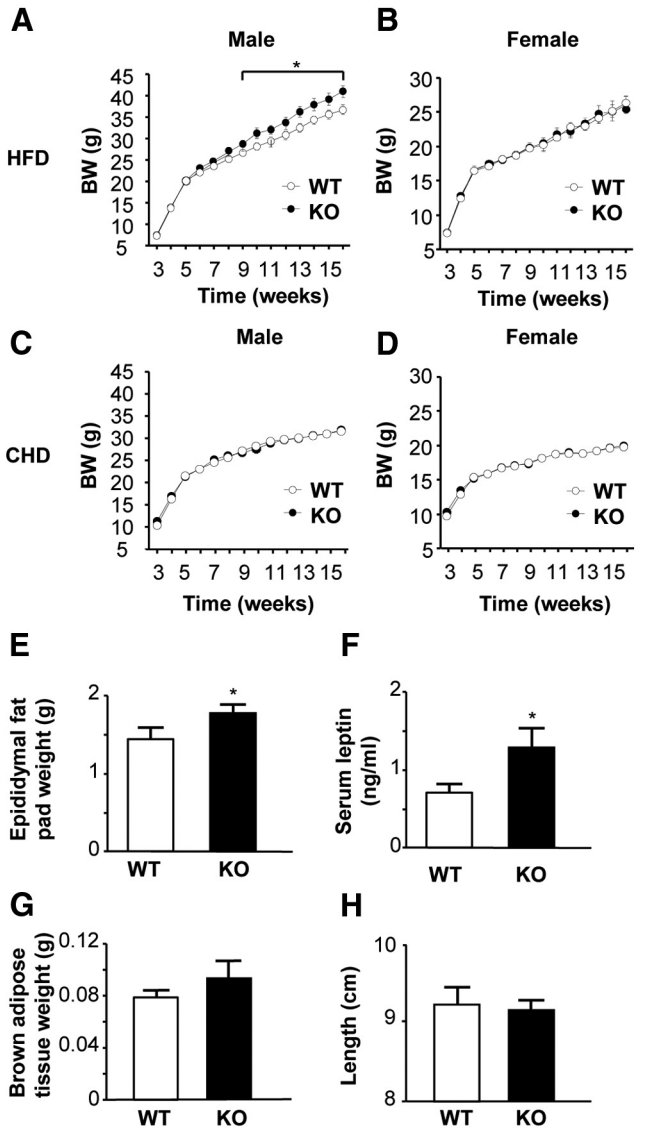

Figure 2. Body weight and composition. ***A***, ***B***, BW of male (***A***) and female (***B***) *POMC-*$GABA_{B1}R^{-/-}$ (KO) mice and $GABA_BR^{+/+}$ (WT) mice on a HFD. ***C***, ***D***, BW of male (***C***) and female (***D***) KO and WT mice on the CHD. ***E–H***, Epididymal fat pad weight (***E***), serum leptin levels (***F***), brown adipose tissue weight (***G***), and nose–rump length (***H***) of male KO and WT mice at the age of 16 weeks on a HFD. All values are mean ± SE. $n = 8$ per genotype. *$p < 0.05$ versus WT mice.

GAPDH forward, 5′-AACGACCCCTTCATTGAC; reverse, 5′-TCCACGAC ATACTCAGCAC. Relative mRNA expression was calculated using the comparative Ct method as described previously (Banno et al., 2010).

Determination of protein levels by Western blot. Tissues dissected were frozen immediately in liquid nitrogen. Whole-cell lysates were prepared in a buffer containing 10 mM Tris, pH 7.4, 50 mM NaF, 150 mM NaCl, 0.1% SDS, 2 mM Na_3VO_4, 5 mM EDTA, and 1% Triton X-100 (Sigma-Aldrich) containing fresh protease inhibitors (Rosch), and Western blot was performed as described previously (Ito et al., 2012). Membranes are incubated with $GABA_BR$ antibody (Haller et al., 2004). The levels were normalized to β actin (Abcam). Blots were quantified using NIH ImageJ software.

Statistical analysis. Statistical significance of the differences between groups was analyzed by unpaired *t* test, one-way ANOVA, or two-way ANOVA with repeated measures followed by Bonferroni's test. Results are expressed as means ± SE, and differences were considered significant at $p < 0.05$.

Results

Generation of POMC-specific $GABA_BR$-deficient mice

To generate POMC-specific $GABA_BR$-deficient mice, $GABA_{B1}R^{lox511/lox511}$ mice were crossed to *POMC-Cre* mice to generate $GABA_{B1}R^{+/lox511}$ *POMC-Cre* and $GABA_{B1}R^{+/lox511}$ mice. Subsequently, we crossed these mice to $GABA_{B1}R^{lox511/lox511}$ or $GABA_{B1}R^{+/lox511}$ mice to yield $GABA_{B1}R^{lox511/lox511}$ *POMC-Cre* mice (hereafter termed *POMC-*$GABA_{B1}R^{-/-}$ or KO mice), $GABA_{B1}R^{+/lox511}$ *POMC-Cre* mice (hereafter termed *POMC-*$GABA_{B1}R^{+/-}$ or heterozygous mice), and $GABA_{B1}R^{lox511/lox511}$ littermate controls (hereafter termed $GABA_{B1}R^{+/+}$ or WT mice). To visualize POMC cell-specific Cre-mediated recombination, we crossed $GABA_{B1}R^{-/-}$ *POMC-Cre* mice to *Z/EG* reporter mice, which express EGFP only after Cre-mediated recombination.

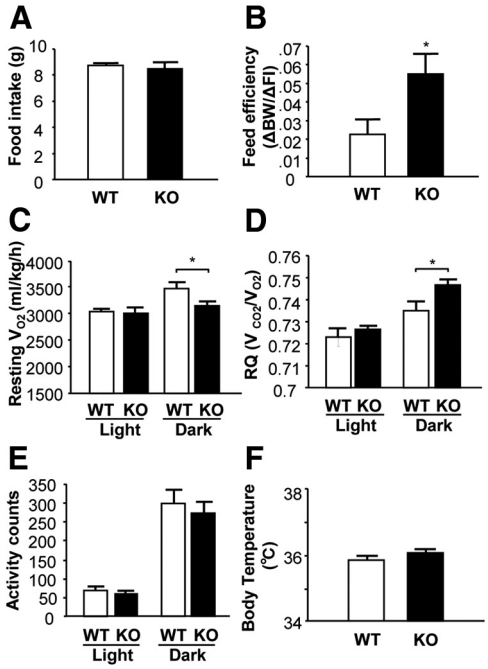

Figure 3. Analysis of energy metabolism. ***A***, Cumulative food intake for 3 d. ***B***, *POMC-*$GABA_{B1}R^{-/-}$ (KO) mice have increased feed efficiency compared with $GABA_{B1}R^{+/+}$ (WT) mice. ***C***, KO mice have decreased energy expenditure as indicated by decreased resting V_{O_2} during the dark cycle compared with WT mice. ***D***, KO mice have increased RQ values during the dark cycle compared with WT mice. ***E***, ***F***, Locomotor activity (***E***) and body temperature (***F***) were not different between genotypes. Data were collected from male mice on a HFD at the age of 8 weeks (***A***, ***B***) and 16 weeks (***C–F***). All values are mean ± SE. $n = 7$ per genotype. $^*p < 0.05$ versus WT mice.

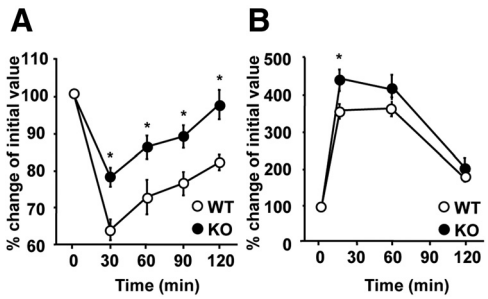

Figure 4. ITT and GTT. Insulin sensitivity and glucose homeostasis are impaired in *POMC-*$GABA_{B1}R^{-/-}$ (KO) mice. ***A***, ***B***, ITT (***A***) and GTT (***B***) in male KO mice and $GABA_{B1}R^{+/+}$ (WT) mice at the age of 8 weeks on a HFD. All values are mean ± SE. $n = 7$ per genotype. $^*p < 0.05$ versus WT mice.

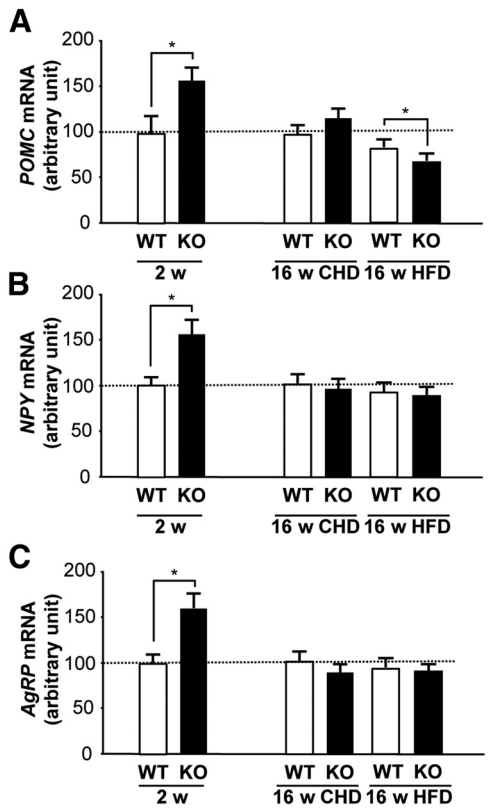

Figure 5. *POMC*, *NPY*, and *AgRP* mRNA expression in hypothalamus analyzed by RT-PCR. ***A–C***, Expression of *POMC* mRNA (***A***), *NPY* mRNA (***B***), and *AgRP* mRNA (***C***) in $GABA_{B1}R^{+/+}$ (WT) and *POMC-*$GABA_{B1}R^{-/-}$ (KO) mice at ages 2 and 16 weeks on the CHD or HFD. All values are mean ± SE. $n = 6–8$ per genotype. $^*p < 0.05$ versus WT mice.

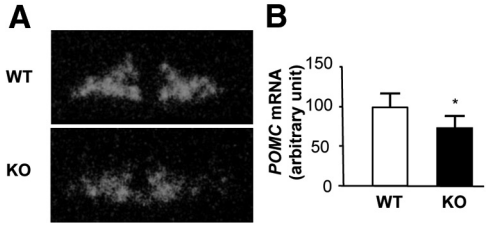

Figure 6. *POMC* mRNA expression analyzed by *in situ* hybridization. ***A***, Representative photographs showing *POMC* mRNA expression in the arcuate nucleus of male $GABA_{B1}R^{+/+}$ (WT) and *POMC-*$GABA_{B1}R^{-/-}$ (KO) mice at the age of 16 weeks on the HFD. ***B***, *POMC* mRNA expression was significantly decreased in KO mice compared to WT mice. All values are mean ± SE. $n = 8$ per genotype. $^*p < 0.05$ versus WT mice.

$GABA_{B1}R^{-/-}$; *POMC-Cre*; *Z/EG* mice expressed EGFP in the arcuate nucleus in a pattern consistent with POMC neuron localization, whereas control $GABA_{B1}R^{-/-}$; *Z/EG* mice did not express EGFP (Fig. 1*A*). Consistent with areas of endogenous POMC expression, deletion of the $GABA_{B1}R$ allele in KO mice was only detected in DNA extracts from the pituitary, hypothalamus, and hindbrain (including the nucleus of the solitary tract), whereas the recombined alleles were not detected in WT mice (Fig. 1*B*). Western blot analyses showed no difference in $GABA_BR$ protein levels in lysates obtained from whole hypothalamus or brain (Fig. 1*C*). There were no differences in the number of POMC neurons in the arcuate nucleus between WT and KO mice on the CHD (WT, 39 ± 5; KO mice, 40 ± 3) at the age of 16 weeks. Representative photographs showing POMC immunoreactivities in the arcuate nucleus are shown in Figure 1*D*.

Changes in BW, adipose tissue weight, and serum leptin levels

Analyses with two-way ANOVA with repeated measures showed that the BW of male KO mice was significantly higher than that of WT mice on a HFD at 9–16 weeks of age (Fig. 2*A*), while the BW of heterozygous mice on a HFD was between that of WT and KO mice (data not shown). In contrast, female WT and KO mice showed no significant differences in BW on a HFD (Fig. 2*B*). On the CHD, male or female mice showed no significant differences in BW between genotypes (Fig. 2*C*,*D*). Analyses with one-way ANOVA showed that adiposity was increased in male KO mice on the HFD, as evidenced by increased epididymal fat pad weight (Fig. 2*E*), while that of heterozygous mice on the HFD was between WT and KO mice (data not shown). Serum leptin levels were significantly higher in male KO mice compared with WT mice at the age of 16 weeks on the HFD (Fig. 2*F*), and the levels in

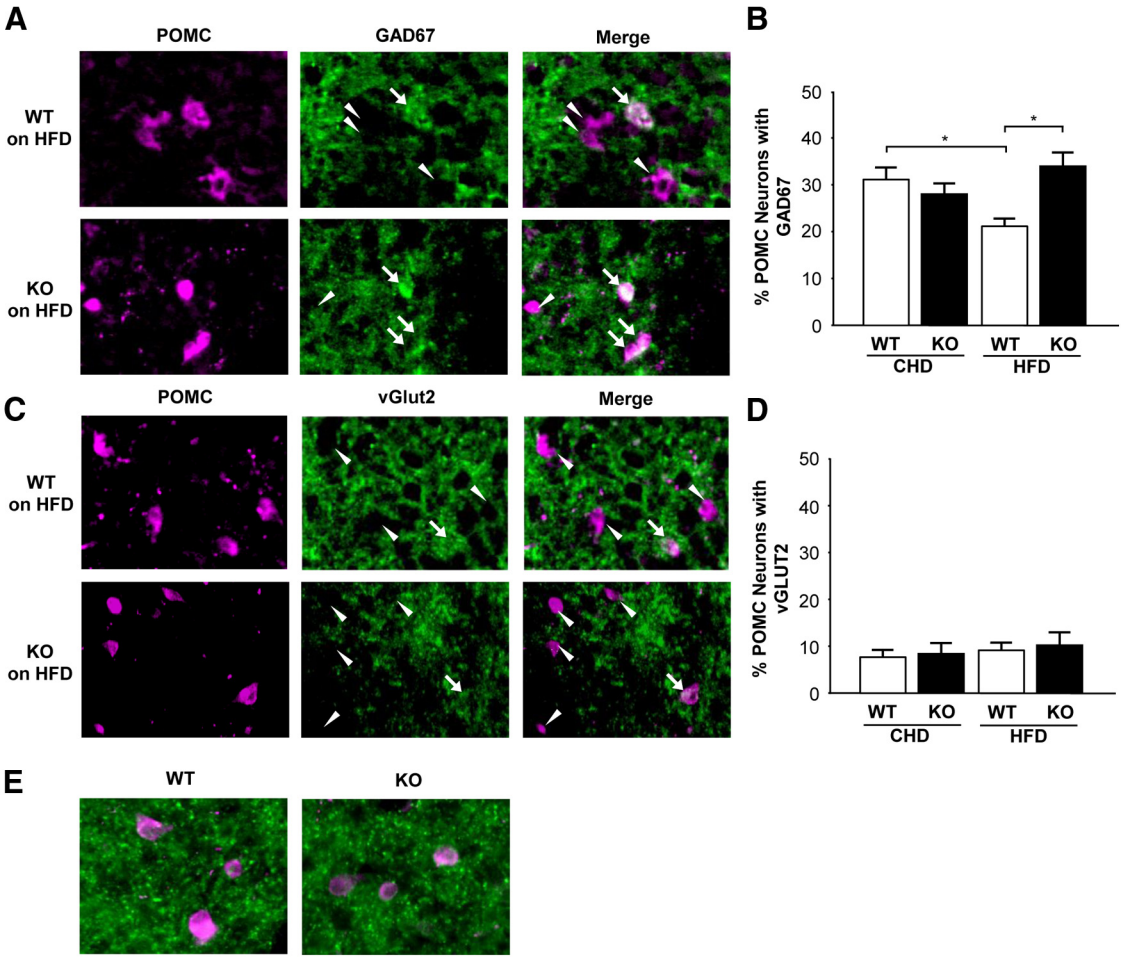

Figure 7. Changes in GABAergic and glutamatergic POMC neurons. ***A***, Representative photographs showing the staining of POMC (magenta) and GAD67 (green) in the arcuate nucleus in male WT and KO mice at the age of 16 weeks on the HFD. ***B***, GABAergic POMC neurons were significantly decreased in WT mice but not in KO mice on the HFD. ***C***, Representative photographs showing the staining of POMC (magenta) and vGLUT2 (green) in the arcuate nucleus in male WT and KO mice at the age of 16 weeks on the HFD. ***D***, There were no differences in numbers of glutamatergic POMC neurons between WT mice and KO mice on the HFD or CHD. ***E***, Expression of Synapsin 1 in the region of POMC neurons. POMC neurons (magenta) and Synapsin 1 expression (green) in the arcuate nucleus in WT and KO mice at the age of 16 weeks on the HFD are shown. POMC neurons colocalized with GAD 67 or vGLUT2 were shown by arrows, whereas those not thus colocalized were shown by arrowheads. All values are mean ± SE. $n = 6$ per genotype. $^*p < 0.05$ versus WT mice.

heterozygous mice were between WT and KO mice (data not shown). No significant differences were found in brown adipose tissue (BAT) weight or body length between male WT and KO mice on the HFD (Fig. 2*G*,*H*). There were no significant differences in adiposity, serum leptin levels, BAT weight, and body length between female WT and KO mice on the CHD or HFD (data not shown).

Food intake and energy expenditure

Food intake was similar between male WT and KO mice on a HFD (Fig. 3*A*), but feed efficiency (ΔBW/Δfood intake) was significantly higher in the KO mice compared with WT mice (Fig. 3*B*). Resting V_{O_2} was decreased and RQ was increased in male KO mice compared with WT mice during the dark cycle (Fig. 3*C*,*D*). No differences between genotypes were noted in locomotor activity or body temperature (Fig. 3*E*,*F*).

Insulin sensitivity and glucose tolerance

No differences between genotypes were noted in fasted serum glucose, serum insulin levels, or homeostasis model assessment ratio in male mice at the age of 8 weeks on a HFD (data not shown). However, male KO mice were less insulin sensitive, as measured by ITT, and showed hyperglycemia during the GTT compared to WT mice at the age of 8 weeks (Fig. 4*A*,*B*), when there were no significant differences in BW between genotypes.

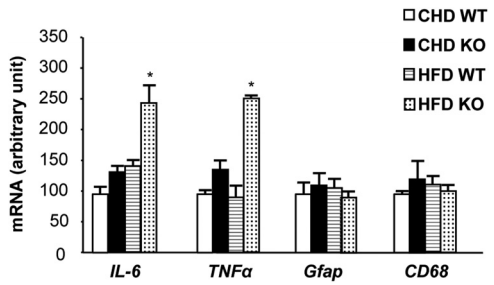

Figure 8. Hypothalamic inflammatory signaling. Hypothalamic expression of inflammation signals (*IL-6*, *TNFα*) were increased in *POMC-*$GABA_{B1}R^{-/-}$ (KO) mice on the HFD at the age of 16 weeks compared to KO mice on the CHD, or $GABA_{B1}R^{+/+}$ (WT) mice on the CHD or HFD. Expression levels of microglia-specific (*Gfap*) and astrocyte-specific (*CD68*) markers were not different between groups. All values are mean ± SE. $n = 5–8$ per genotype. $^*p < 0.05$ versus KO mice on the CHD, WT mice on the CHD, or WT mice on the HFD.

Serum levels of corticosterone in basal and stressed conditions

Serum levels of corticosterone in stressed conditions were significantly increased compared to those in basal conditions in both

male WT and KO mice, and there were no significant differences between genotypes in the levels in basal (WT, 144.3 ± 18.3 pg/ml; KO, 155.0 ± 21.3 pg/ml) or stressed conditions (WT, 271.8 ± 39.4 pg/ml; KO, 344.0 ± 57.6 pg/ml).

POMC, NPY, *and* AgRP *mRNA expression*

Male KO mice had increased hypothalamic *POMC, NPY*, and *AgRP* mRNA expression compared to the WT mice at the age of 2 weeks (Fig. 5), whereas there were no differences in the expression levels between 2-week-old male WT and *POMC-Cre* mice (data not shown). While no differences in *POMC*, *NPY*, and *AgRP* mRNA expression were noted between 16-week-old male KO and WT mice on the CHD (Fig. 5), male KO mice on a HFD had significantly decreased hypothalamic *POMC* mRNA expression (Fig. 5*A*); no significant changes were observed in *NPY* and *AgRP* mRNA expression (Fig. 5*B*,*C*). *In situ* analysis hybridization also confirmed that *POMC* mRNA expression in the arcuate nucleus was significantly decreased in male KO mice on a HFD compared to WT mice (Fig. 6*A*,*B*). No differences were found in the number of cells expressing *POMC* mRNA in the arcuate nucleus between male WT (38 ± 2) and KO mice (36 ± 2) on a HFD.

GABAergic and glutamatergic POMC neurons

GABAergic POMC neurons in WT and KO mice on the CHD comprised ~30% of the total number of POMC neurons, and they were decreased in number in WT mice on the HFD, but not in KO mice (Fig. 7*A*,*B*). On the other hand, the number of glutamatergic POMC neurons was low (less than10%), and there were no changes between genotypes on the CHD or HFD (Fig. 7*C*,*D*). No differences were found in the number of POMC neurons in the arcuate nucleus between male WT (40 ± 3) and KO mice (36 ± 3) on the HFD. There were no apparent differences in the staining of Synapsin 1, a marker of total synapses (Cesca et al., 2010), in the region of POMC neurons between WT and KO mice on the CHD or HFD (Fig. 7*E*).

Hypothalamic inflammatory signaling

IL-6 and *TNFα* mRNA expression were significantly increased in male KO mice on the HFD compared with that in KO mice on the CHD or that in WT mice on the CHD or HFD (Fig. 8). Expression levels of *Gfap* and *CD68*, makers of astrocyte and microglia activation, respectively (Thaler et al., 2012), were not significantly different between groups (Fig. 8).

Discussion

In the present study, we generated POMC-specific $GABA_{B1}R$ KO mice. We demonstrated that the male KO mice on a HFD exhibited mild increases in BW accompanied by decreased energy expenditure. The male KO mice also showed insulin resistance at the age of 8 weeks, when there were no significant differences in BW between genotypes. Furthermore, the *POMC* mRNA expression in the arcuate nucleus decreased and hypothalamic inflammation increased in the male KO mice at the age of 16 weeks. On the other hand, it is reported that there is no difference in BW between *POMC-Cre* and WT mice on a HFD (Banno et al., 2010). We also demonstrated that expression levels of *POMC*, *NPY*, and *AgRP* mRNA are not different among *POMC-Cre* and WT mice, excluding the possibility that the Cre protein itself generated the phenotypes. Together, we suggest that signaling through $GABA_BRs$ in POMC neurons is essential in the regulation of both energy balance and glucose homeostasis.

While $GABA_ARs$ mediate fast GABA responses by triggering chloride channel opening, $GABA_BRs$ mediate slower GABA responses by activating G-proteins that regulate second messenger systems as well as effector K^+ and Ca^{2+} channels (Gassmann and Bettler, 2012). $GABA_BRs$ are located both presynaptically and postsynaptically. Presynaptic $GABA_BRs$ are present at GABAergic (autoreceptors) and glutamatergic terminals (heteroreceptors; Gassmann and Bettler, 2012). While most POMC neurons are reported to be GABAergic, it is also shown that some POMC neurons release glutamate, and that the released GABA and glutamate from POMC neurons regulate the activity of the neurons themselves as well as the activity of interconnected POMC neurons (Dicken et al., 2012). Thus, the effects of deletion of $GABA_BRs$ in POMC neurons would be determined by the balance between the effects of deleting postsynaptic and presynaptic $GABA_BRs$. Consistent with previous studies (Jarvie and Hentges, 2012), our data show that ~30% of POMC neurons are GABAergic, while glutamatergic POMC neurons comprise <10%. It is also demonstrated that GABAergic POMC neurons were decreased in WT mice on a HFD, but not in KO mice. While the mechanisms by which GABAergic POMC neurons were decreased in WT mice on a HFD remain to be clarified, it was reported that the density of excitatory and inhibitory synapses onto POMC neurons was changed in leptin-deficient (*ob*/*ob*) mice (Pinto et al., 2004). Thus, the synaptic alterations may be

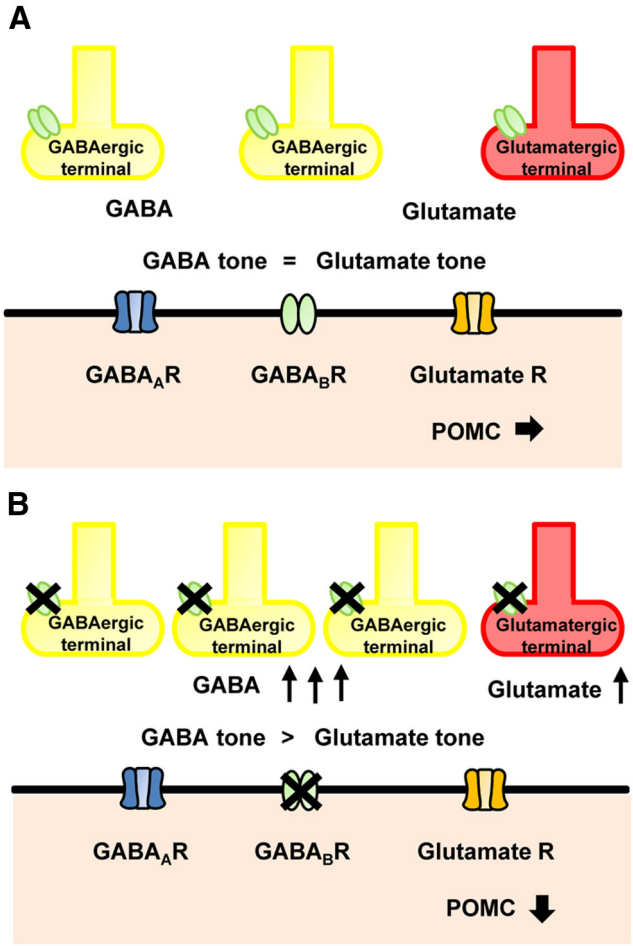

Figure 9. Possible mechanisms by which POMC expression was affected in POMC-specific $GABA_BR$ KO mice. ***A***, $GABA_BRs$ are located presynaptically and postsynaptically in POMC neurons, which release GABA and glutamate. ***B***, GABAergic POMC neurons are increased in $GABA_BR$ KO mice compared to WT mice on a HFD. Deletion of $GABA_BRs$ in GABAergic terminals leads to increases in GABA release, whereas that in glutamatergic terminals results in increases in glutamate. Increased GABA release could decrease POMC expression through action on $GABA_ARs$.

induced by changes in energy balance, and $GABA_BRs$ in POMC neurons appear to be prerequisite for the regulation of GABAergic POMC neurons on a HFD. Although our data did not reveal significant changes in glutamatergic POMC neurons or Synapsin 1 staining, a more sensitive approach may also reveal changes in the number of glutamatergic POMC neurons or in the total number of synaptic inputs onto the POMC neurons on a HFD.

The deletion of $GABA_B$ autoreceptors would increase GABA release, whereas that of the heteroreceptors would lead to increases in glutamate release (Fig. 9). In the case of POMC neurons, the effects of deletion of $GABA_B$ autoreceptors would be more dominant than those of the heteroreceptors given that there are more GABAergic than glutamatergic POMC neurons, and the increased release of GABA would inhibit POMC neuronal activity through $GABA_ARs$. This is a possible mechanism by which POMC expression is decreased in 16-week-old KO mice on a HFD, in which the number of GABAergic POMC neurons was increased compared to age-matched WT mice on a HFD (Fig. 9). To prove this hypothesis, it would be necessary in future studies to address whether central injection of GAB_AA antagonists can reverse phenotype such as obesity or insulin resistance in KO mice on a HFD. In addition to the autoregulatory mechanism, the activities of POMC neurons are regulated by other neurons including NPY neurons. Thus, further investigation is warranted to clarify the mechanisms by which POMC expression is changed in POMC-specific $GABA_{B1}R$ KO mice.

It is also important to note that effects of changes in the activity of anorexigenic POMC neurons on energy balance could be compensated by orexigenic neurons in the KO mice. In this context, the findings that there were no differences in BW between genotypes in 2-week-old KO mice, in which *POMC* expression was increased, could be explained through an upregulation of anorexigenic *POMC* expression that was compensated by increased orexigenic *NPY* and *AgRP* expression. On the other hand, decreased *POMC* expression was not accompanied by significant changes in *NPY* and *AgRP* expression in the obese 16-week-old KO mice on a HFD. As the expression of anorexigenic *POMC* is expected to increase when BW is increased, the decreased activity of POMC neurons is likely to contribute, at least in part, to late onset of obesity in POMC-specific $GABA_{B1}R$ KO mice. Our data also show that serum leptin levels were increased in POMC-specific $GABA_{B1}R$ KO mice compared to WT mice on a HFD. Leptin decreases food intake and BW by activating POMC neurons (Schwartz et al., 2000), and obesity is often associated with leptin resistance (Ahima and Flier, 2000). Therefore, it is possible that POMC-specific deletion of $GABA_{B1}R$ increased leptin resistance in the POMC neurons on a HFD, which would possibly cause the phenotypes in the KO mice.

Interestingly, while BW increases in POMC-specific $GABA_{B1}R$ KO mice are relatively mild, expression of TNFα and IL6, two proinflammatory markers in the hypothalamus, was increased more than twofold in the KO mice compared to WT mice on a HFD. These results suggest that the deletion of $GABA_BRs$ in POMC neurons leads to hypothalamic inflammation on a HFD. There is evidence to suggest that hypothalamic inflammation is related to the onset of obesity (Gregor and Hotamisligil, 2011), and previous studies showed that hypothalamic inflammation occurred even in WT mice on a HFD (Zhang et al., 2008; Kirchner et al., 2012; Thaler et al., 2012). Together with the findings in the present study, it is suggested that $GABA_BRs$ in POMC neurons are essential to protect the hypothalamus from inflammation on a HFD. It is also reported that, with a HFD, gliosis in the hypothalamus is increased and that POMC neurons died by autophagy (Thaler et al., 2012). In contrast, our data showed there is no increase in the expression of gliosis markers (*Gfap* or *CD68*) or neuronal loss of POMC. The discrepancy between studies could be explained by the difference of duration of a HFD [3 months in the present study versus 8 months in the previous study by Thaler et al. (2012)].

$GABA_BRs$ have been implicated in a wide variety of neurological and psychiatric conditions (Gassmann and Bettler, 2012). Regarding energy balance, it is reported that full $GABA_{B1}R$ KO mice are insulin resistant, with males being more affected than females (Bonaventura et al., 2008, 2012). We reported in a previous study (Sato et al., 2007) that baclofen, which crosses blood–brain barrier (van Bree et al., 1991), reduced BW in diet-induced obese mice as well as *db/db* mice, with *POMC* mRNA expression in the arcuate nucleus being increased, while the BW in lean mice was unaffected. Our clinical trial also demonstrated that baclofen reduced BW in obese subjects (Arima and Oiso, 2010). The present study further supports the role of $GABA_BR$ signaling in energy balance and points to the important role of $GABA_BR$ signaling in POMC neurons.

POMC is expressed not only in the arcuate nucleus, but also in the pituitary and hindbrain, and the recombination of the floxed $GABA_{B1}$ allele was detected at these sites in the POMC-specific $GABA_{B1}R$ KO mice. As for the pituitary, there are no differences in corticosterone levels in basal and stressed conditions between the KO and WT mice, suggesting that the hypothalamic–pituitary–adrenal axis is intact in the KO mice. However, these data do not necessarily exclude roles of $GABA_BRs$ expressed in the pituitary in the control of energy balance. Furthermore, there is evidence that POMC neurons in the hindbrain are involved in the energy balance (Ellacott et al., 2006). Thus, further studies are required to clarify the relative contributions of $GABA_BR$ ablation in the arcuate nucleus, pituitary and the hindbrain to the control of energy balance or glucose tolerance in male mice.

In conclusion, we demonstrated that regulation of POMC neurons via $GABA_BR$ signaling plays an important role in the energy homeostasis, glucose tolerance, and prevention of hypothalamic inflammation on a HFD.

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