Supporting Information

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SI Materials and Methods

Glucose and Insulin Tolerance Tests. For glucose and insulin tolerance tests, mice that had been subjected to an overnight (10–12 hr) fast were given glucose, either orally (via oral gavage) or i.p. (2 mg/g body weight), or insulin (Humulin, 0.75 milliunits/g; Eli Lilly), respectively. Blood was collected by means of retro-orbital sinus puncture before (time 0) and at defined times after injection of glucose or insulin for the measurement of glucose (Glucometer Elite; Bayer). Serum insulin and concentrations were determined by ELISA (Crystal Chem).

Body Temperature Measurements. Body temperature measurements were taken between 1–3 p.m. by using a rectal thermometer (model TH-5; Braintree Scientific).

Quantification of M_3 mAChR Expression Levels by Using an Immunoprecipitation Strategy. To quantitate the expression of M_3 mAChR protein in mouse tissues, we used a combined radioligand binding/immunoprecipitation strategy [Yamada M, et al. (2001) Mice lacking the M3 muscarinic acetylcholine receptor are hypophagic and lean. *Nature* 410:207–212]. Membranes were prepared from mouse brain and salivary glands (submandibular gland), as described previously [Gautam D, et al. (2000) A critical role for β cell M_3 muscarinic acetylcholine receptors in regulating insulin release and blood glucose homeostasis in vivo. *Cell Metab* 3:449–461], and then incubated for 1 hr at room temperature (22 °C) with a saturating concentration (2 nM) of the non-subtype-selective muscarinic antagonist, quinuclidinyl [^{3}H]benzilate ([^{3}H]QNB; specific activity 42 Ci/mmol; PerkinElmer). Subsequently, [^{3}H]QNB-labeled M_3 receptors were immunoprecipitated by the use of an M_3 receptor-specific rabbit polyclonal antiserum, as described by Yamada et al. The amount of [^{3}H]QNB-labeled M_3 receptors in the immunoprecipitates was determined by liquid scintillography.

Histology. Whole brains or pituitary glands were harvested from control and Br-M3-KO mice. Tissues were fixed in 10% neutral buffered formalin, processed into paraffin blocks, sectioned at 5 μm, and stained with H&E according to standard protocols.

For immunohistochemical staining of pituitary somatotroph cells, 5-μm-thick pituitary sections were deparaffinized and endogenous peroxidases were quenched with H_2O_2 for 10 min. Sections were then incubated with a rabbit polyclonal anti-GH antibody (1:400 dilution; DAKO, catalog #A0570) at room temperature for 60 min, followed by incubation (at room temperature for 30 min) with the Envision anti-rabbit secondary antibody (DAKO). Binding of the secondary antibody was visualized by using DAB+ (DAKO) as a chromogen. Sections were counterstained with Meyer's hematoxylin for 2 min.

In Situ mRNA Hybridization Studies. Coronal sections (12 μm thick) of mouse hypothalamus were cut in a cryostat and mounted on positively charged microscope slides. The slides were kept at −80 °C until used. Before hybridization, the slides were fixed in 4% buffered formaldehyde and processed as described in the protocols link of http://intramural.nimh.nih.gov/lcmr/snge/. Briefly, a digoxigenin-labeled M_3 mAChR-specific riboprobe (complementary to nucleotides 1504–1958; NM_033269) was generated by using digoxigenin-tagged UTP and T7 polymerase. After hybridization, the probe was developed by using a digoxigenin antibody conjugated to horseradish peroxidase (HRP). Finally, a tyramide-Alexafluor-594 conjugate (Perkin-Elmer) was used to visualize cells expressing M_3 receptor mRNA. A GHRH-specific ^{35}S-labeled riboprobe (complementary to nucleotides 323–480; M31654) was generated by using ^{35}S-labeled α-thio-UTP and T7 polymerase. After hybridization (together with the nonradioactive M_3 receptor probe) and visualization of the M_3 receptor signal, the slides were coated with emulsion (Kodak NTB2) and developed 5 days later to visualize the autoradiographic grains over labeled cells (cells expressing GHRH mRNA). The slides were evaluated by using a Leica DMI-6000 inverted fluorescent microscope.

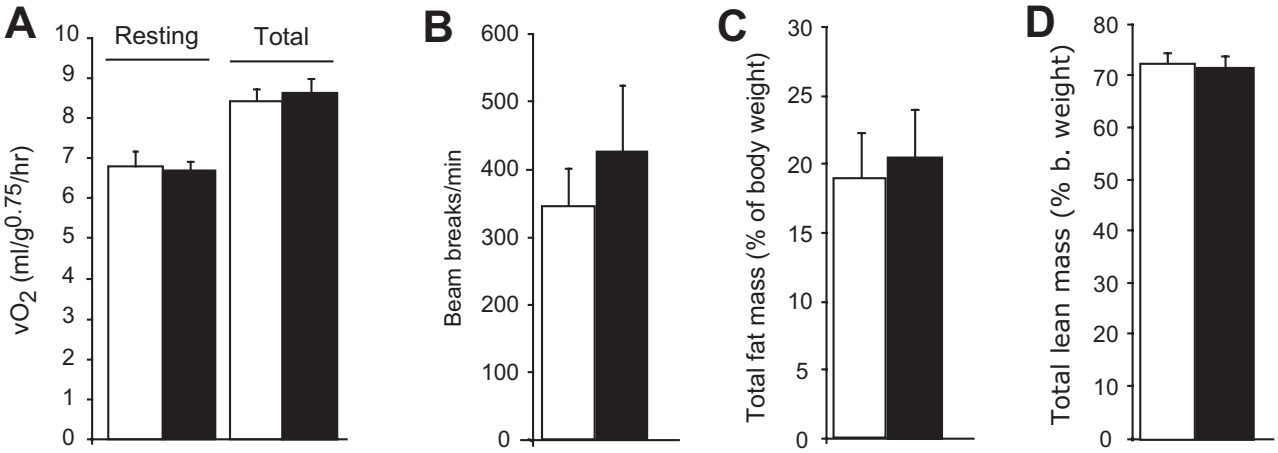

Fig. S1. Metabolic rate, locomotor activity, and body composition of Br-M3-KO mice (black bars) and control littermates (white bars). (*A*) Resting and total metabolic rate (vO_2, volume of O_2 consumed). (*B*) Locomotor activity measurements (beam breaks per min). (*C*) Total fat mass (% of body weight). (*D*) Total lean mass (% of body weight). All measurements were carried out with 20-week-old males (control, $n = 17$; Br-M3-KO, $n = 6$). The three control strains (+/+, +/+ *NesCre*, and fl/fl) gave results that did not differ significantly from each other. The data obtained with these mice were therefore pooled for the sake of clarity. Data are given as means ± SEM.

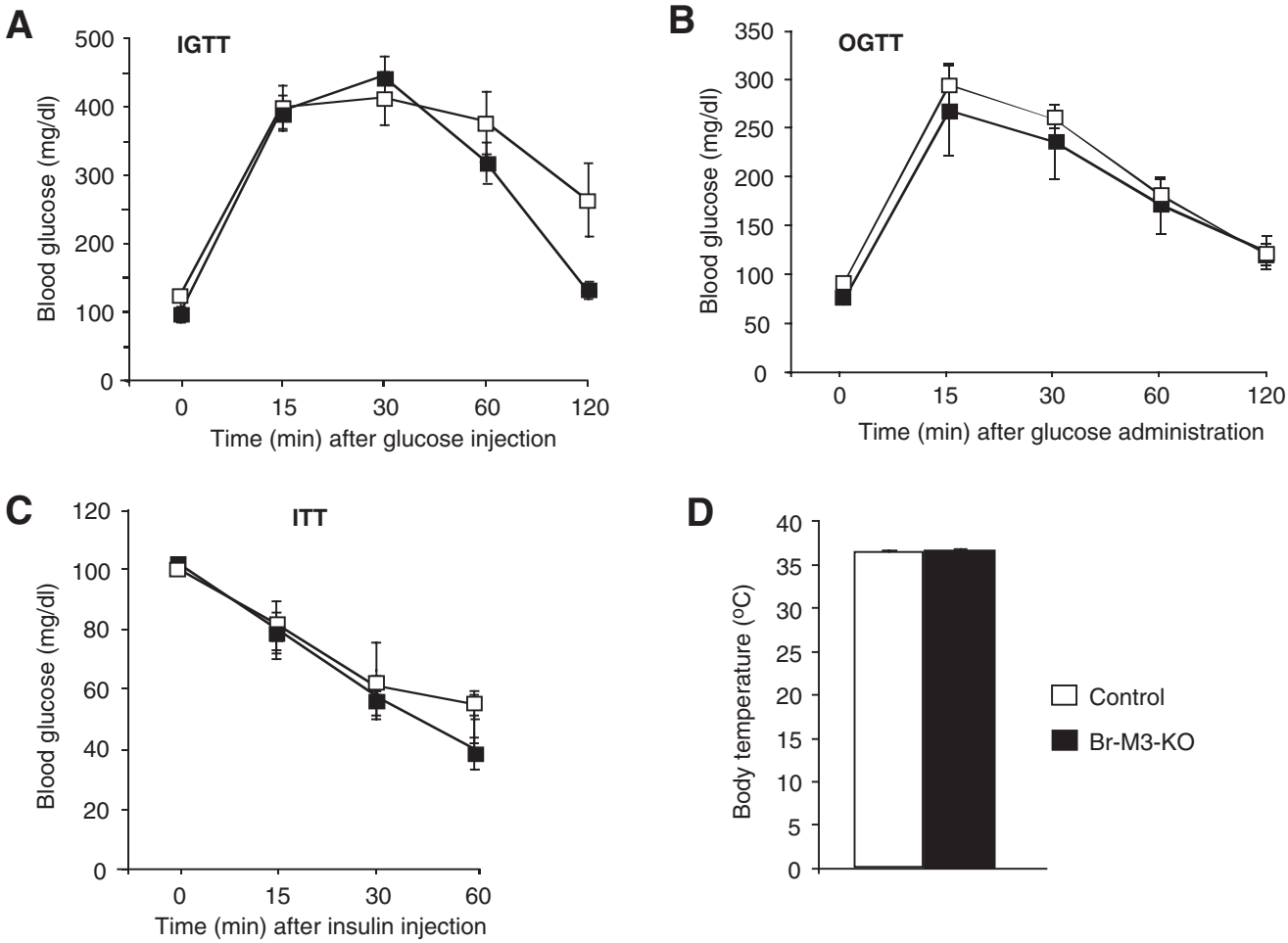

Fig. S2. Glucose tolerance, insulin sensitivity, and body temperature of Br-M3-KO mice and control littermates. (*A*) I.p. Glucose tolerance test (IGTT). Blood glucose levels were measured at the indicated times after i.p injection of 2 mg/g glucose. (*B*) Oral glucose tolerance test (OGTT). Blood glucose levels were measured at the indicated times after oral administration of 2 mg/g glucose. (*C*) Insulin tolerance test (ITT). Blood glucose levels were measured at the indicated times after i.p. injection of 0.75 milliunits/g insulin. (*D*) Body temperature. Core body temperature was measured rectally. Data are given as means ± SEM (n = 6–9 per group; 16- to 24-week-old males).

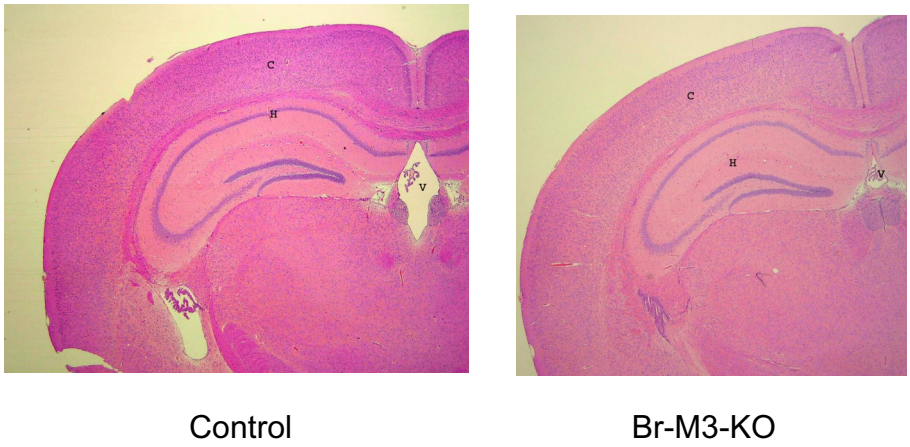

Fig. S3. Gross brain morphology of Br-M3-KO mice and control littermates. Representative frontal brain sections (H&E-stained) from a control mouse (*Left*) and a Br-M3-KO mouse (*Right*) are shown (16-week-old males). Altogether, sections from 4 control and 4 Br-M3-KO mice were examined. No obvious differences in brain morphology (besides the pituitary hypoplasia displayed by the Br-M3-KO mice) were observed.

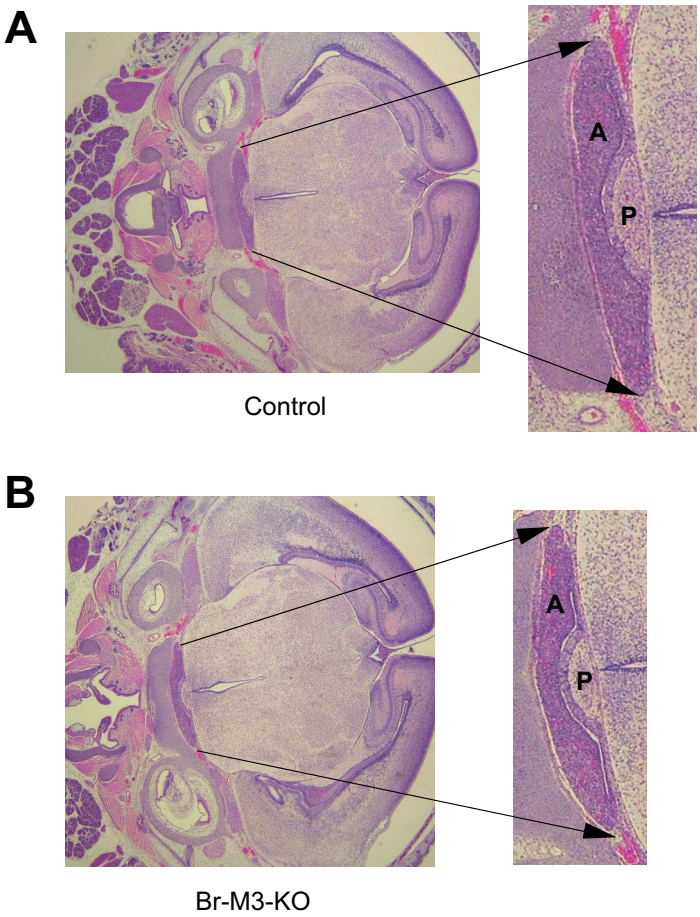

Fig. S4. Gross pituitary morphology of neonatal Br-M3-KO mice and control littermates. (*A* and *B*) Representative brain sections (H&E-stained) from a neonatal control mouse (postnatal day 1) (*A*) and a neonatal Br-M3-KO mouse (postnatal day 1) (*B*). Altogether, sections from 4 control and 4 Br-M3-KO mice were examined. No obvious differences in pituitary size and gross morphology were observed. A, anterior pituitary; P, posterior pituitary.

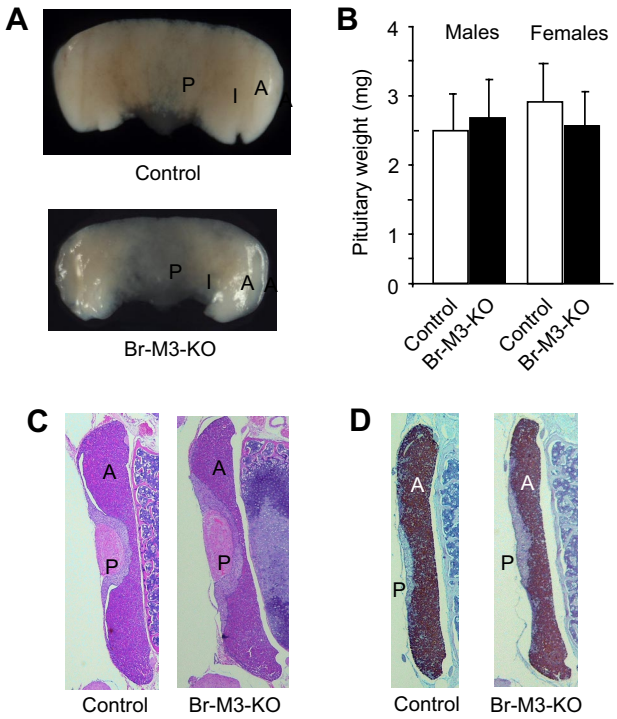

Fig. S5. CJC-1295 administration restores normal pituitary size and morphology in Br-M3-KO mice. (*A*) Gross morphology of pituitary glands taken from representative Br-M3-KO and control mice (males) after CJC-1295 treatment for 8 weeks (2 μg/mouse/day; s.c.). (*B*) Pituitary weight of Br-M3-KO and control mice (9-week-old males and females) after CJC-1295 treatment for 8 weeks. Data represent means ± SEM ($n = 4$ per group). (*C* and *D*) H&E staining and immunostaining with an anti-GH antibody of pituitary sections, respectively. Following CJC-1295 treatment for 8 weeks, pituitary glands from Br-M3-KO and control littermates (9-week-old males) were sectioned and stained with H&E (*C*) or a mouse GH antibody (*D*).

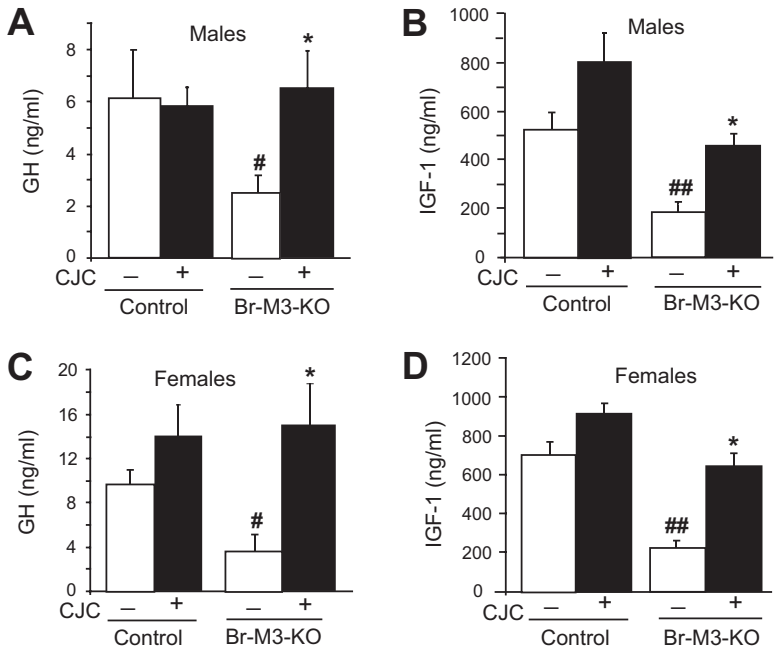

Fig. S6. CJC-1295 administration restores normal serum IGF-1 and GH in Br-M3-KO mice. (*A* and *C*) Serum GH levels. (*B* and *D*) Serum IGF-1 levels. Hormone levels were measured in male (*A* and *B*) and female (*C* and *D*) Br-M3-KO mice and control littermates (mouse age: 9 weeks). Mice were either treated with CJC-1295 for 8 weeks [(+); 2 μg per mouse per day; s.c.] or left untreated (−). Data are presented as means ± SEM ($n = 6–8$ per group). *, $P < 0.05$; **, $P < 0.01$ compared with nontreated mice of the same genotype. #, $P < 0.05$; ##, $P < 0.01$ compared with the nontreated control littermates.

Table S1. Fed and fasting blood glucose and serum insulin levels of Br-M3-KO mice and control littermates

Measurement	Control	Br-M3-KO
Blood glucose, mg/dL		
Fed	138 ± 6	135 ± 7
Fasted	87 ± 5	94 ± 7
Serum insulin, pg/mL		
Fed	1,029 ± 69	1,007 ± 37
Fasted	403 ± 76	436 ± 55

All data were obtained with 14- to 16-week-old male mice (littermates). Data represent means ± SEM ($n > 6$ per group).