Supporting Information

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SI Materials and Methods

RNA Isolation and Real-Time Quantitative RT-PCR. Total RNA was extracted from tissues and cells using the RNeasy Mini Kit (Qiagen) and ISOGEN (WAKO). cDNAs were transcribed using RNAs as templates with Molony murine leukemia virus reverse transcriptase (Invitrogen). cDNAs were amplified by PCR with Taq DNA polymerase (TaKaRa) using primers shown in Table S1. Quantitative RT-PCR (qRT-PCR) analyses were performed using DNA Engine Opticon-2 (MJ Research) as described previously (1). For each condition, expression was quantified in duplicate.

In Situ Hybridization. For the in situ hybridization of histological sections, mouse embryos and human sympathetic ganglia were frozen in powdered dry ice, and 16-μm sections were cut using a cryostat and stored at −80 °C to await hybridization. ^{35}S-labeled mouse and human antisense *Gpr41* RNA probes were transcribed using T7 RNA polymerase with uridine 5′-α-[^{35}S]thiotriphosphate (Perkin-Elmer). Sections were examined by in situ hybridization using a labeled probe, followed by exposure to X-ray films (Kodak) for 10 d as described previously (2). Sections were finally counterstained with hematoxylin–eosin.

Immunohistochemistry. For immunostaining, sections were fixed with 4% paraformaldehyde, washed in PBS, and treated with 5% BSA in PBS. The sections were permeabilized with 0.1% Triton X-100 (Sigma) and immunostained with a primary antibody raised against tyrosine hydroxylase (TH; Chemicon) to detect sympathetic neurons. Sections and whole hearts were stained with an antibody to TH. Following hybridization with the secondary antibody, tissue samples were incubated with 3, 3′-diaminobenzidine. Total volumes of superior cervical ganglion (SCG) and nerve area were measured by ImageJ software as described previously (2, 3). The volume of SCG was estimated according to Cavalieri's principle.

Animal and Diabetic Model. All experimental procedures involving mice were carried out according to protocols approved by the relevant animal ethics committees. *Gpr41*$^{-/-}$ and *Gpr43*$^{-/-}$ mice from a C57BL/6 background were generated. Type I diabetes was induced by an i.p. injection containing streptozotocin (STZ) (200 mg/kg; Sigma). STZ was dissolved in cold citrate buffer (50 mM; pH 4.5) immediately before injection. Glucose levels were measured from tail-vein blood 4 d postinjection with a glucometer (Lifescan). Mice with blood glucose levels >200 mg/dL were considered diabetic as described previously (4).

Generation of HEK293 Cells Expressing Mouse G Protein-Coupled Receptor 41. Flp-In T-REx HEK293 cells were transfected with a mixture containing mouse FLAG–G protein-coupled receptor 41 (GPR41) cDNA in pcDNA5/FRT/TO vector and the pOG44 vector using Lipofectamine (Invitrogen). After 48 h, the medium was changed to medium supplemented with 200 μg/mL hygromycin B to initiate selection of stably transfected cells. Following isolation of resistant cells, expression of GPR41 from the Flp-In locus was induced by treatment with 1 μg/mL doxycycline for 48 h as described previously (5). Expression of GPR41 was confirmed by RT-PCR and FACS Calibur (BD Biosciences) using FLAG-tag.

Cultures of Sympathetic Neurons, Neuro2A, and Cardiomyocytes. SCG was dissected from postnatal day 1 mice, trypsinized in 0.05% trypsin in Hanks' balanced salt solution for 20 min at 37 °C, and dissociated by trituration. Dissociated cultures of SCG neurons were plated onto poly-L-lysine (20 μg/mL; Sigma)-coated dishes in Dulbecco's modified Eagle's medium/nutrient Ham's mixture F-12 (DF) medium (Gibco) containing 1% penicillin–streptomycin solution (Gibco). The cells were cultured in conditioned medium containing NGF (10 ng/mL; Upstate Biotech) and 10% FBS (Equitech-Bio) as described (6). Neuro2A cells were cultured in DF medium (Gibco) containing 1% penicillin–streptomycin solution (Gibco) and 10% FBS (Equitech-Bio) as described (6). For coculture of SCG neurons and cardiac ventricular myocytes, freshly isolated sympathetic neurons were plated with cardiomyocytes obtained from the same animals and cultured for 2 d before analysis as described (6). Cells were cultured using the same methods as used for cultures of SCG neurons. After 1 d in culture, 1 mM cytosine arabinofuranoside (Sigma) was added to the dishes to stop cell division. For coculture of Neuro2A neurons and cardiomyocytes, Neuro2A cells were cultured in DF containing 1% N2 Supplement (Gibco) for neuronal differentiation for 24 h before plating with cardiomyocytes.

Transient Transfection and Knockdown by siRNA. For Gpr41 overexpression, Neuro2A cells were transfected with mouse FLAG-GPR41 cDNA in pcDNA3.1 vector using Lipofectamine 2000 (Invitrogen). After 24 h, for RNA interference, Neuro2A cells were transfected with 40 nM siRNA (Table S2) (Stealth Select RNAi; Invitrogen) by using the Lipofectamine RNAiMAX transfection reagent (Invitrogen) as described (7). The transfected cells were cultured in DF containing 10% FBS for 24 h and then in DF containing 1% N2 Supplement (Gibco) for neuronal differentiation.

Western Blotting and cAMP Determination. Cells were first lysed in TNF buffer [10 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1 mM EDTA, 1% Nonidet P-40, 50 mM NaF, 2 mM Na_3VO_4, 10 μg/mL aprotinin, 10 μg/mL leupeption, and 2 μg/mL pepstatin]. Hearts were homogenized in 0.1 M sodium phosphate (pH 7.4) and were centrifuged at 10,000 × *g* for 20 min at 4 °C. Supernatants were analyzed by Western blotting, and proteins were detected by Western blotting using the following antibodies: rabbit antibodies against ERK1/2, phosphorylated ERK1/2, ARRB1/2, GRK2, PLCβ3 (Cell Signaling), Gq (Santa Cruz), TH (Chemicon). and a mouse antibody against β-actin (Sigma). After transfer to nitrocellulose membranes, immunoreactive bands were visualized using an enhanced chemiluminescence detection system as previously described (5, 7). For cAMP determination, the cells were lysed in a 0.1-N HCl solution. After acetylation, cAMP level was determined in duplicate using enzyme immunoassay kits (Cayman) as described (8).

Cardiographic Recording. Heart rates were measured in conscious male mice using a tail-cuff system (Softron) as described (9). For measurement of physiological parameters under resting conditions, propranolol (4 mg/kg), a β-adrenergic receptor antagonist; atropine (8 mg/kg), a muscarinic receptor antagonist; hexamethonium (20 mg/kg), a nicotinic acetylcholine receptor antagonist; and propionate (1 g/kg), octanoate (1 g/kg), β-hydroxybutyrate (500 mg/kg), acetone (0.5 g/kg); and tyramine (100 mg/kg), catecholamine releasing agents, were administrated at a volume per injection i.p. as described (10–12). Atropine was injected first, followed 10 min later by propranolol.

Biochemical Analyses. Noradrenaline (NA) concentrations in plasma and heats were measured by HPLC as described (3). Plasma glucose concentrations were measured using a glucometer (LifeScan). Plasma propionate, acetone, and octanoate concentrations were measured by GC. Plasma β-hydroxybutyrate concentrations were measured using ketone test B liquid (Sanwa). Measurements of plasma free fatty acids and triglyceride concentrations were entrusted to SRL. Plasma leptin concentrations were measured using a Leptin Mouse ELISA kit (R&D Systems).

Electrophysiological Recording. Extracellular action potentials were recording using a multichannel recording system (MED64 system; Alpha MED Sciences). SCG neurons were plated onto poly-L-lysine–coated Alpha MED Sciences probes and cultured in DF containing 10% FBS and NGF (10 ng/mL). Cells were starved in serum-free medium for 2 h before stimulation. Frequency was measured using Conductor software v.2.1e (Alpha MED Sciences) as described (13).

Indirect Calorimetry. Oxygen consumption (VO_2) was determined with an O_2/CO_2 metabolic measuring system (model MK-5000; Muromachikikai) at 24 °C as described elsewhere (14). Mice were kept unrestrained in the chamber for 1 h for three times without food and water. We determined VO_2 when the minimum plateau shape was obtained during the light cycle, which corresponded to a period of inactivity. VO_2 was expressed as the volume of O_2 consumed per kilogram weight of lean body mass per minute.

Thermometry. The body temperatures of the conscious mice were measured with life chip by using a Pocket Reader (Destron Fearing). The life chip was placed s.c. in anesthetized mice. After implantation of the life chip, mice were allowed to recover for at least 24 h before data collection.

Statistical Analyses. Values are presented as mean ± SEM. Differences between groups were examined for statistical significance using Student's *t* test (when two groups were analyzed) or ANOVA (for three or more groups). *P* values of <0.05 were considered statistically significant.

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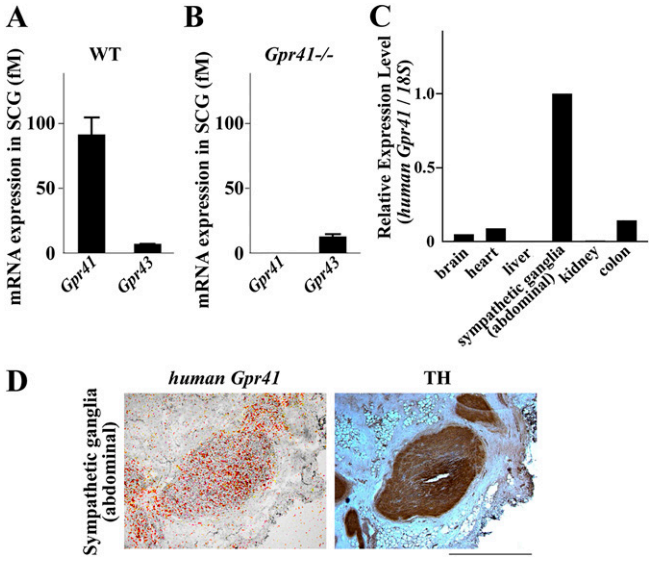


Fig. S1. *Gpr41* mRNA expression in the sympathetic ganglia. (*A* and *B*) *Gpr41* and *Gpr43* expression in SCG of wild-type and $Gpr41^{-/-}$ mice (P49). Quantification of *Gpr41* and *Gpr43* expression was performed by qRT-PCR ($n = 3$). (*C*) Expression of *Gpr41* mRNA in human tissues. Expression of *Gpr41* was measured using qRT-PCR. *18S* rRNA expression was used as an internal control. (*D*) *Gpr41* mRNA localization in human abdominal sympathetic ganglia as determined by in situ hybridization using an ^{35}S-labeled antisense human *Gpr41* RNA probe. (*Left*) Red grains superimposed on a hematoxylin–eosin-stained section show human *Gpr41* mRNA localization. (*Right*) Anti-tyrosine hydroxylase (TH) immunostaining (brown). (Scale bar: 1 mm.)

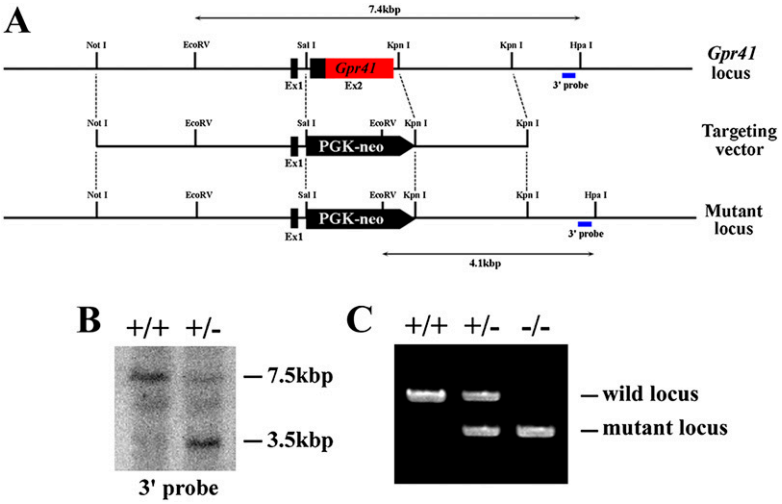


Fig. S2. Targeted disruption of *Gpr41* in mice. (*A*) A targeting vector was constructed by ligation of three fragments, the 5′ and 3′ homology recombination arms and a fragment of the LacZ-PGK-neo cassette. A 1.7-kbp fragment of mouse DNA containing the exon coding for *Gpr41* was replaced with the LacZ-PGK-neo cassette. The linearized targeting vector was then electroporated into 129/Sv ES cells. (*B*) The 3′ probe used for Southern blotting is indicated below the map of the target allele. The 7.5- and 3.5-kbp fragments, which correspond to the wild-type and mutant alleles, respectively, were detected from the genomic DNA digested with EcoRV and HpaI by Southern blotting. (*C*) Genotypes of mice were determined by PCR using the three primers. A 1.9-kbp PCR fragment was generated with P1 and P3 primers from wild *Gpr41* locus, whereas a 1.3-kbp PCR fragment with P1 and P2 primers predicted the mutated *Gpr41* locus. Primers sequences: 5′-GCAGCAGAGTGCCAGTTGTCC-3′ (P1), 5′-GGCTATTCGGCTATGACTGG-3′ (P2), 5′-GCGTGTGAGTGGTCCTTCCATCC-3′ (P3).

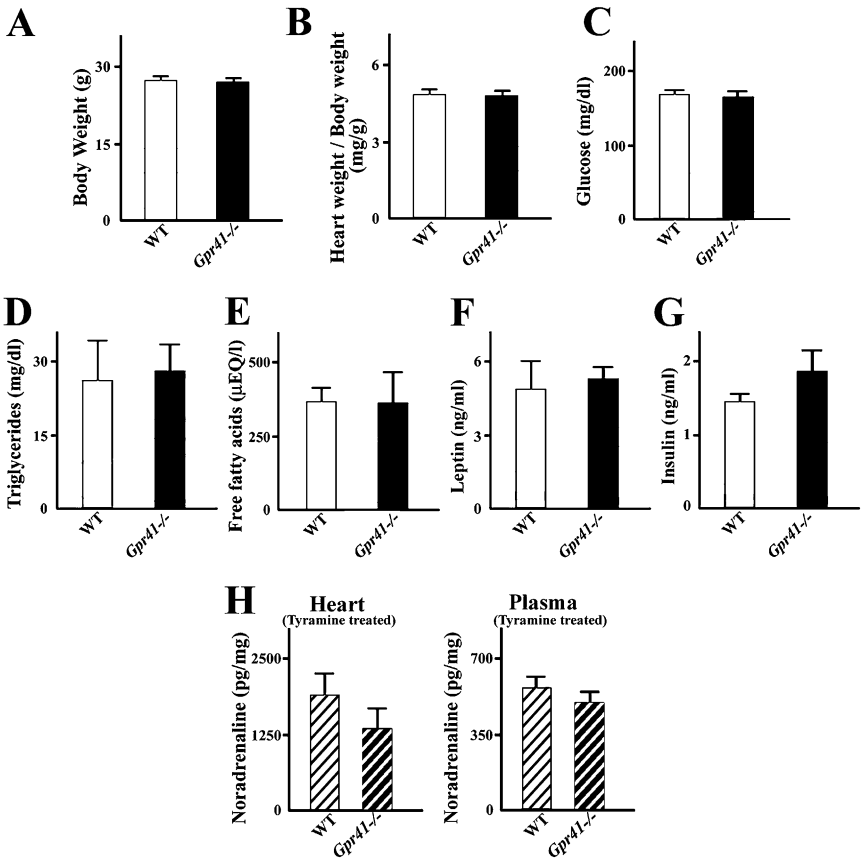


Fig. S3. Metabolic parameters of *Gpr41*$^{-/-}$ mice and NA concentration. (*A*) Body weights of wild-type mice and *Gpr41*$^{-/-}$ mice (n = 12). (*B*) Ratio of heart weights to body weights in wild-type mice and *Gpr41*$^{-/-}$ mice (n = 10). (*C*–*G*) Biochemical analysis of plasma obtained from *Gpr41*$^{-/-}$ mice. (*C*) Glucose (n = 9). (*D*) Triglycerides (n = 9). (*E*) Free fatty acids (n = 9). (*F*) Leptin (n = 5). (*G*) Insulin (n = 6). (*H*) Effects of tyramine on NA concentrations. Measurement of NA concentrations in heart (n = 12) and plasma (n = 10) at 24 h after tyramine injection (i.p.). Mice were analyzed at 12 wk of age.

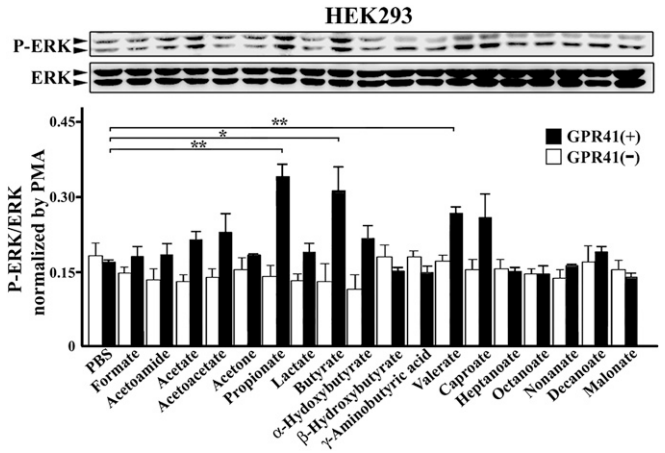


Fig. S4. Propionate as the most potent agonist for GPR41. Screening of GPR41 agonists by the phosphorylation of ERK1/2 assay in HEK293 cells expressing mouse GPR41. Monocarboxylates were added 1 mM for 5 min (n = 3). *P < 0.05; **P < 0.005.

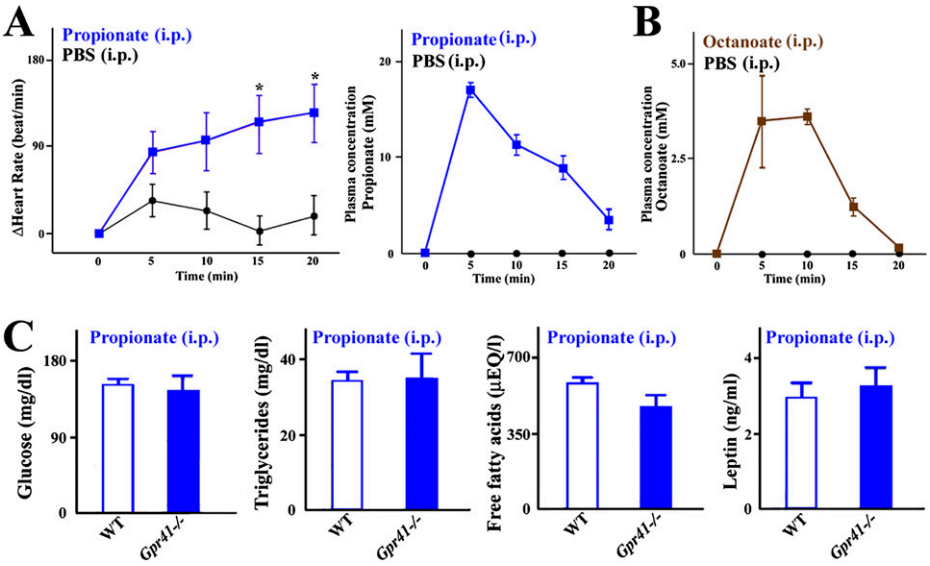


Fig. S5. Effects of propionate on heart rate. (*A*) At time 0, a bolus of propionate (1 g/kg) was administered intraperitoneally (n = 5) (*Left*). The concentration of propionate in plasma following the injection of propionate (1 g/kg) (n = 3) (*Right*). (*B*) The concentration of octanoate in plasma following the injection of octanoate (1 g/kg) (n = 3). (*C*) At 20 min after administration of propionate (1 g/kg i.p.), biochemical analysis of plasma was obtained from *Gpr41*$^{-/-}$ mice [glucose (n = 5–6), triglycerides (n = 5–6), free fatty acids (n = 5–6), and leptin (n = 5)]. Mice were analyzed at 12 wk of age. *P < 0.05.

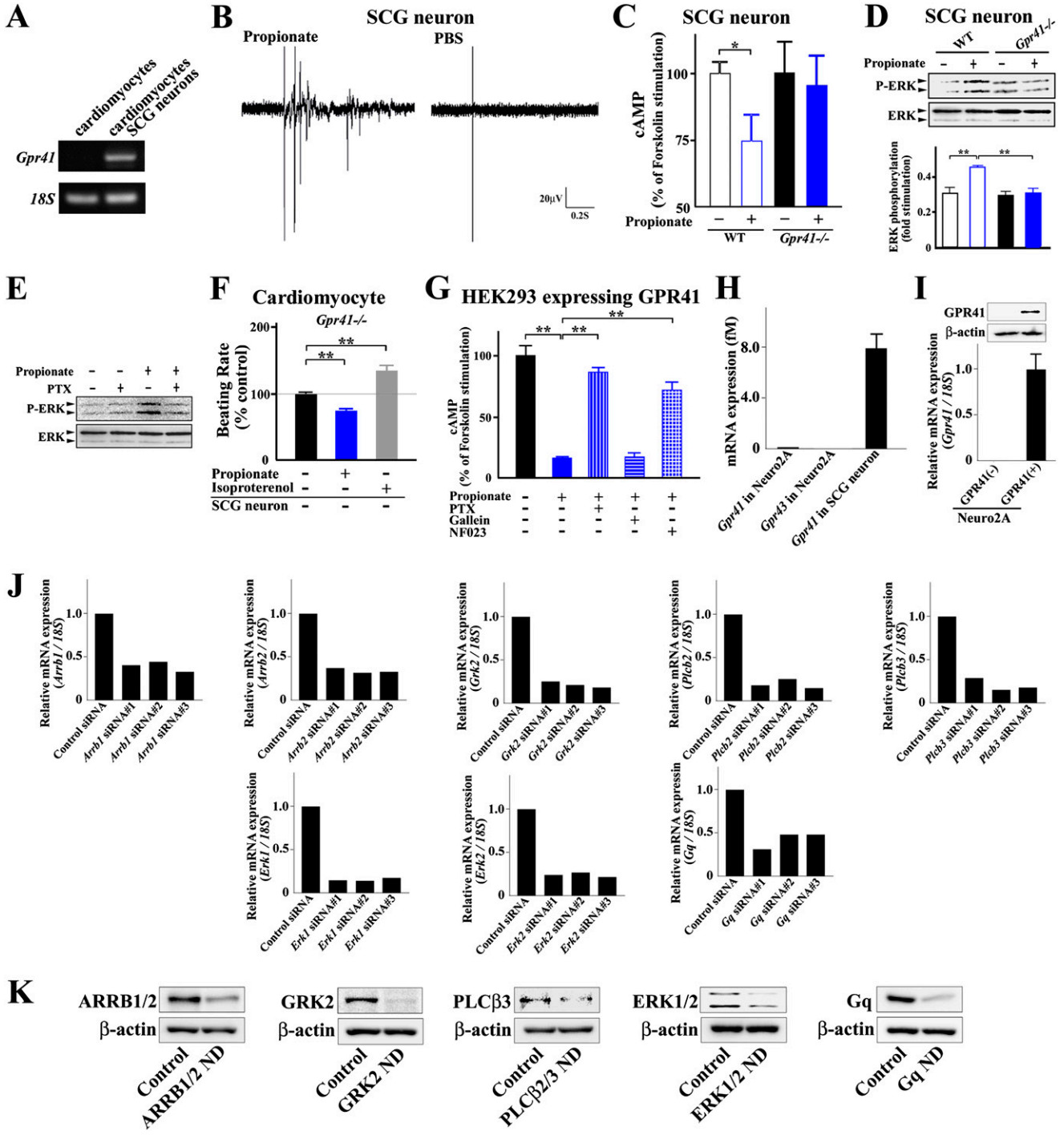


Fig. S6. Effects of propionate in primary-cultured sympathetic neurons and Neuro2A cells. (*A*) Expression of *Gpr41* mRNA in monocultured cardiomyocytes and cocultured cardiomyocytes. (*B*) Action potentials in sympathetic neurons after propionate (10 mM) or PBS stimulation. (*C*) Reduction of cAMP levels in response to propionate in sympathetic neurons. Cells were stimulated by propionate (1 mM) in the presence of IBMX (3-isobutyl-1-methylxanthine; a phosphodiesterase inhibitor) for 10 min ($n = 5$). (*D*) Effects of propionate on ERK1/2 phosphorylation. Sympathetic neurons were stimulated by propionate (1 mM) for 30 min ($n = 3$). (*E*) Effects of propionate on phosphorylation of ERK1/2. Sympathetic neurons were stimulated by propionate (1 mM) in the presence or absence of pertussis toxin (PTX) (100 ng/mL) for 30 min. (*F*) Effects of propionate (1 mM) or isoproterenol (10 μM) on change in myocyte beating rate in *Gpr41*$^{-/-}$ cardiomyocytes ($n = 6$–7). (*G*) Reduction of cAMP levels in response to propionate in HEK293 cells expressing mouse GPR41. Cells were stimulated by propionate (10 μM) in the presence of IBMX for 10 min after pretreatment with IBMX for 30 min and with or without PTX (1 μg/mL), Gallein (10 μM), or NF023 (20 μM) for 1 h ($n = 3$). (*H*) *Gpr41* and *Gpr43* expression in Neuro2A cells and sympathetic neurons. Quantification of *Gpr41* and *Gpr43* expression was performed by qRT-PCR ($n = 3$). (*I*) Efficiency of *Gpr41* transient transfection in Neuro2A cells. Expression of *Gpr41* mRNA or GPR41 protein was measured using qRT-PCR ($n = 3$) or Western blotting by anti-FLAG antibody. *18S* rRNA and β-actin expression were used as an internal control. (*J*) Knockdown efficiencies of siRNA knockdown in Neuro2A cells. Expression of *Arrb1*, *Arrb2*, *Grk2*, *Plcb2*, *Plcb3*, *Erk1*, *Erk2*, and *Gq* was measured using qRT-PCR. *18S* rRNA expression was used as an internal control. (*K*) Effects of knockdown by siRNA on protein expression in Neuro2A cells. Expression of ARRB1/2, GRK2, PLCβ3, ERK1/2, and Gq was measured using Western blotting. β-Actin expression was used as an internal control. $*P < 0.05$; $**P < 0.005$.

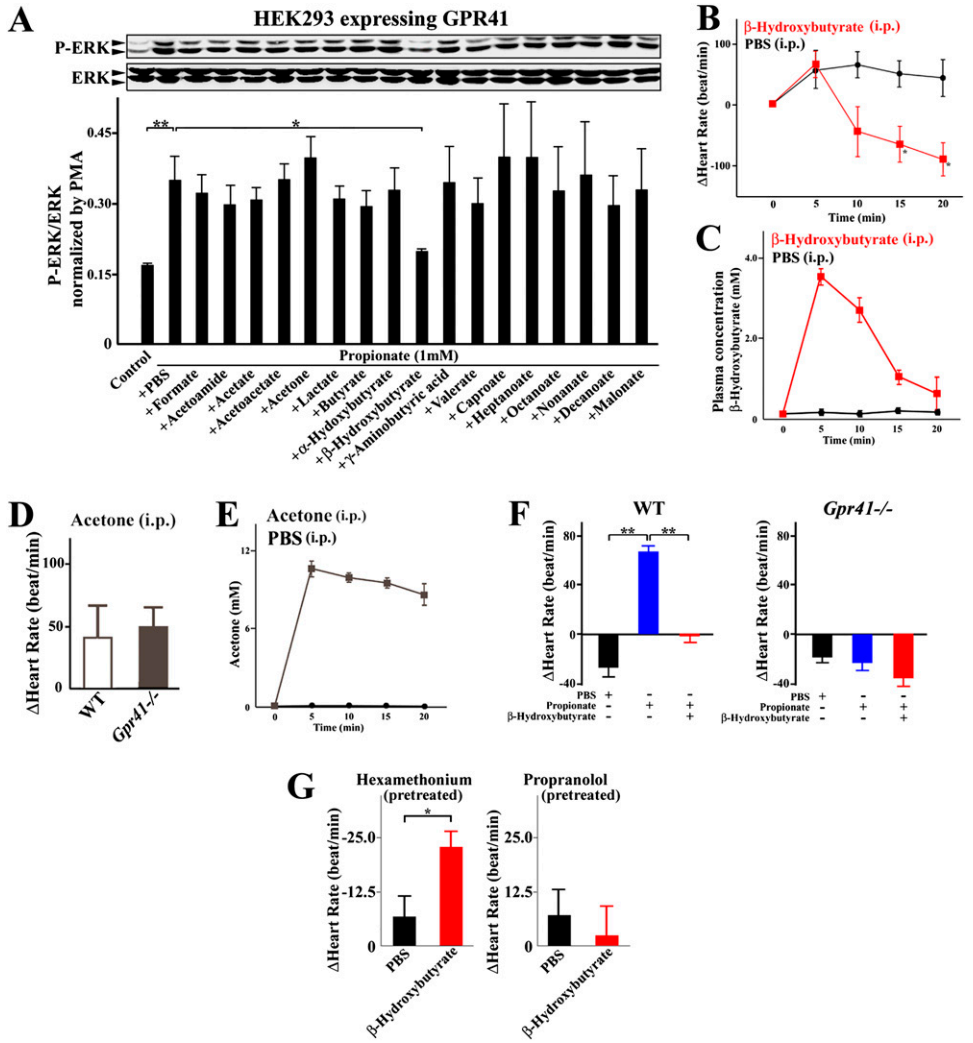
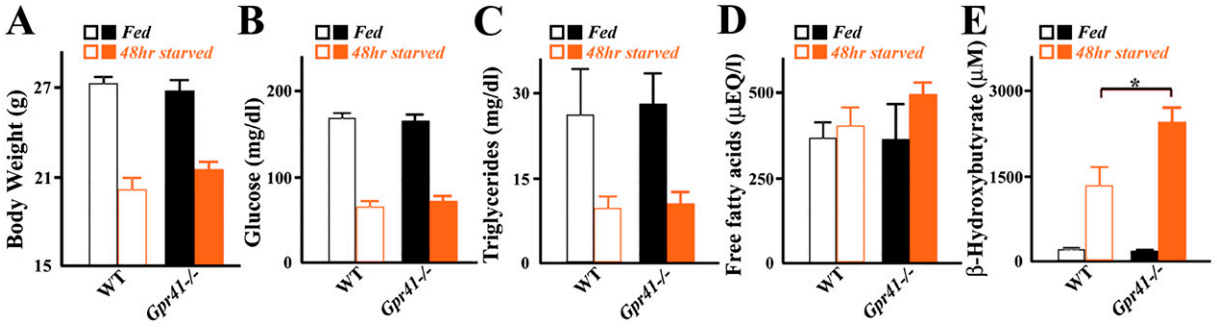


Fig. S7. β-Hydroxybutyrate as GPR41 antagonist. (*A*) Effect of monocarboxylates on propionate-induced phosphorylation of the ERK1/2 in HEK293 cells expressing mouse GPR41. After pretreatment of monocarboxylates (1 mM each) or PBS for 10 min, cells were stimulated with propionate (1 mM). Control: no pretreated and PBS stimulation. Effect of monocarboxylates on propionate-induced GPR41 stimulation was examined by quantifying P-ERK/ERK. (*B*) At time 0, a bolus of β-hydroxybutyrate (500 mg/kg) was administered intraperitoneally ($n = 4$). (*C*) The concentration of β-hydroxybutyrate in plasma following the injection of β-hydroxybutyrate (500 mg/kg) ($n = 3$). (*D*) Effects of acetone on heart rate in *Gpr41*$^{-/-}$ mice. Measurement of heart rate at 20 min after acetone administration intraperitoneally ($n = 6$). (*E*) The concentration of acetone in plasma following the injection of acetone (0.5 g/kg) ($n = 3$). (*F*) Inhibitory effects of β-hydroxybutyrate on heart rate of wild-type and *Gpr41*$^{-/-}$ mice. After pretreatment with β-hydroxybutyrate (500 mg/kg) for 10 min, at time 0, a bolus of propionate (1 g/kg) with or without β-hydroxybutyrate (500 mg/kg) was administered i.p. ($n = 5$). Data were measured at 20 min. (*G*) After pretreated with hexamethonium (20 mg/kg) or propranolol (4 mg/mL) for 10 min, at time 0, a bolus of β-hydroxybutyrate (500 mg/kg) was administered intraperitoneally ($n = 8$, 9). Data was measured at 20 min. Mice were analyzed at 12–14 wk of age. $^{*}P < 0.05$; $^{**}P < 0.005$.



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Parameter	Result for group				*P* value			
	WT (Control)	*Gpr41-/-* (Control)	WT (Diabetes)	*Gpr41-/-* (Diabetes)	Control (WT vs *Gpr41-/-*)	Diabetes (WT vs *Gpr41-/-*)	WT (Control vs Diabetes)	*Gpr41-/-* (Control vs Diabetes)
Heart Rate (beat/min)	708 ± 5.8	663 ± 3.1	403 ± 38	546 ± 25	$p < 0.005$ **	$p < 0.05$ *	$p < 0.005$ **	$p < 0.005$ **
Body wt (g)	28.1 ± 0.8	29.0 ± 0.7	22.7 ± 0.7	23.9 ± 0.9	$p > 0.05$	$p > 0.05$	$p < 0.005$ **	$p < 0.005$ **
Plasma Glucose (mg/dl)	162 ± 8.3	169 ± 3.5	512 ± 53	495 ± 82	$p > 0.05$	$p > 0.05$	$p < 0.005$ **	$p < 0.005$ **
Plasma β-Hydroxybutyrate (mM)	0.10 ± 0.04	0.06 ± 0.03	2.62 ± 0.7	2.40 ± 0.4	$p > 0.05$	$p > 0.05$	$p < 0.005$ **	$p < 0.005$ **

Fig. S8. Metabolic parameters of *Gpr41*$^{-/-}$ mice in starvation and STZ-induced diabetes. (*A*) Body weights of *Gpr41*$^{-/-}$ mice after 48-h starvation (n = 10–12). (*B–E*) Biochemical analysis of plasma obtained from *Gpr41*$^{-/-}$ mice after 48-h starvation. (*B*) Glucose (n = 9). (*C*) Triglycerides (n = 9). (*D*) Free fatty acids (n = 9–11). (*E*) Ketone bodies, β-hydroxybutyrates (n = 8). (*F*) Cardiographic and biochemical parameters in STZ-induced diabetic *Gpr41*$^{-/-}$ mice (n = 7). All parameters were measured at the fourth day after STZ injection. Mice were analyzed at 12 wk of age (*A–F*). *$P < 0.05$; **$P < 0.005$.

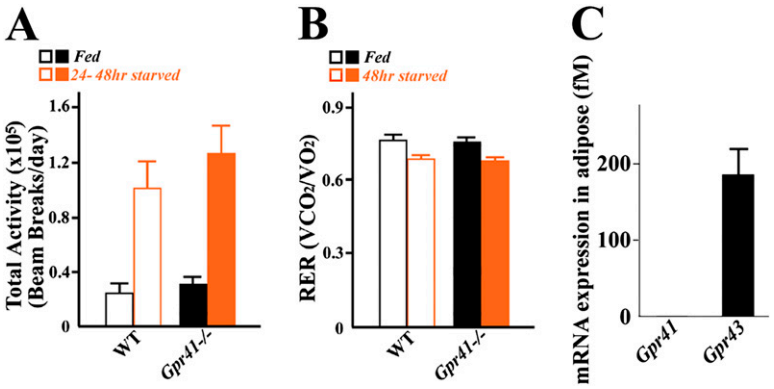

Fig. S9. Energy expenditure in *Gpr41*$^{-/-}$ mice and expression in adipose tissue. (*A*) Total activity (24 h) of *Gpr41*$^{-/-}$ mice during feeding and at 24- to 48-h starvation (n = 9). (*B*) Respiratory exchange ratio (RER) of *Gpr41*$^{-/-}$ mice during feeding and at 48-h starvation (n = 5–7). Mice were analyzed at 14–16 wk of age. (*C*) *Gpr41* and *Gpr43* expression in adipose (mesenteric) of wild-type mice (P49). Quantification of *Gpr41* and *Gpr43* expression was performed by qRT-PCR (n = 3).

Table S1. Primers used for qRT-PCR assays

Primer	Forward	Reverse
Gpr41 (mouse)	5′-GTGACCATGGGGACAAGCTTC-3′	5′-CCCTGGCTGTAGGTTGCATT-3′
18S (mouse)	5′-CTCAACACGGGAAACCTCAC-3′	5′-AGACAAATCGCTCCACCAAC-3′
Gpr43 (mouse)	5′-GGCTTCTACAGCAGCATCTA-3′	5′-AAGCACACCAGGAAATTAAG-3′
Gpr41 (human)	5′-CACTATGGATTGGGCTCTGG-3′	5′-TCTGCCACCCTCAAGAAAAC-3′
18S (human)	5′-AAACGGCTACCACATCCAAG-3′	5′-CGCTCCCAAGATCCAACTAC-3′
Ucp1 (mouse)	5′-GGCATTCAGAGGCAAATCAG-3′	5′-AGCATTGTAGGTCCCCGTG-3′
Arrb1 (mouse)	5′-CACGTCACCAACAACACCAAC-3′	5′-CGATGATGCCCAGGATTTCAC-3′
Arrb2 (mouse)	5′-GGAACTCTGTGCGGCTTATC-3′	5′-GAAGTGGCGTGTGGTTTCAG-3′
Grk2 (mouse)	5′-TCTTCCAGCCATACATTGAGG-3′	5′-GCAGAACCGTGTGAACTTGTC-3′
Plcb2 (mouse)	5′-ATGCTGGATGTCAGATGGTTG-3′	5′-TCGTAATGGAAAGGGTGGTGG-3′
Plcb3 (mouse)	5′-TGCCTGCCCTGCTTATCTAC-3′	5′-AGCCTCACTCTCCCCAATGA-3′
Erk1 (mouse)	5′-TATCAACACCACCTGCGACC-3′	5′-CATACTCCGTCAGAAAGCCAG-3′
Erk2 (mouse)	5′-CTCTCCCGCACAAAAATAAGG-3′	5′-TGGGCTCATCACTTGGGTC-3′
Gq (mouse)	5′-TGGAGAAGGTGTCTGCTTTTG-3′	5′-ATTCCCGTCGTCTGTCGTAG-3′

Table S2. siRNA used for RNA interference

siRNA	Sequence
Arrb1 #1	5′-UUCCCAGGUAGACAGUGAGCUUUCC-3′
Arrb1 #2	5′-UUUGGCGGGAUCUCAAAGGUGAAGG-3′
Arrb1 #3	5′-AUACAAUGUCGUCAUCAUUGGUGUC-3′
Arrb2 #1	5′-CAAACACGAUGUCAUCGUCUGUGGC-3′
Arrb2 #2	5′-UCAUGUUUGAGCUGCCCAUCCAAGG-3′
Arrb2 #3	5′-AGAACGUGGAACUAGGAGACACCUG-3′
Grk2 #1	5′-AACAAGUAGAAGUAUCGCCGCUGCC-3′
Grk2 #2	5′-AUUCGAUGCACACUGAAGUCAUUCA-3′
Grk2 #3	5′-ACUAUCACACUGCAGGACAAACUGC-3′
Plcb2 #1	5′-UUCAGUUCCCGCAACUCUUCCAAGC-3′
Plcb2 #2	5′-UGAAGUUUCAUCGUCCCACUUGAUG-3′
Plcb2 #3	5′-UCAAGAUCUUCUCAAAGAUAAAGGG-3′
Plcb3 #1	5′-AUUUGAUGAACUUACUCCCGCGCCG-3′
Plcb3 #2	5′-UUCGCAGGCAGACAUAGUGGUAUCC-3′
Plcb3 #3	5′-UAAUCAAGGCCUCCGCAUAGUCCUG-3′
Erk1 #1	5′-UUGAUAAGCAGAUUGGAAGGCUUCA-3′
Erk1 #2	5′-UUUGGAGUCAGAUUUAGGAAAGAGC-3′
Erk1 #3	5′-AAUGUAAACAUCUCUCAUGGCUUCC-3′
Erk2 #1	5′-UCAUGAUCUGGAUCUGCAACACGGG-3′
Erk2 #2	5′-AUAAUACUGCUCCAGGUAUGGGUGG-3′
Erk2 #3	5′-UUAGCUGAAUGGAUAUACUUUAGCC-3′
Gq #1	5′-UUGUUGUGUAGGCAGAUAGGAAGGG-3′
Gq #2	5′-AAAUGACACUUUGUAAGUCAAAGGG-3′
Gq #3	5′-AGAACUUGAUCAUAUUCGCUAAGCG-3′