Supplemental Data

Chronic activation of a designer G_q-coupled receptor improves β-cell function

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Supplemental Methods

Glucose and insulin tolerance tests. For glucose tolerance tests, mice that had been fasted overnight (for 10-12 hr) were given i.p. glucose (2 mg/g). For insulin tolerance tests, fasted (overnight) mice were injected with insulin (Humulin, 0.75 mIU/g, i.p.; Eli Lilly, Indianapolis, IN). Tail blood was collected at defined time intervals for glucose measurements (Glucometer Elite; Bayer Diagnostics, Pittsburgh, PA). Plasma insulin concentrations were determined via ELISA (Crystal Chem Inc., Downers Grove, IL).

Real-time qRT-PCR analysis of islet gene expression. Total RNA was isolated from mouse pancreatic islets (80-100 islets per mouse) using the QIAzol lysis reagent (Qiagen, Valencia, CA). During this procedure, a 15 min on-column DNase I treatment step was performed (RNeasy Mini Kit; Qiagen). Total RNA was reverse transcribed into cDNA using the Superscript III first strand synthesis kit (Invitrogen, Carlsbad, CA), and gene expression levels were measured by monitoring SYBR green fluorescence intensity over time using an ABI 7900HT Fast Real-Time PCR System (Applied Biosystems). Each PCR reaction (final volume: 20 µl) consisted of cDNA (~100 ng of initial RNA sample), 10 µl of SYBR Green PCR master mix (Applied Biosystems), and 100 nM of each PCR primer. For each primer pair, qRT-PCR reactions were performed in triplicate using a 96 well plate format. Primers were selected from previously validated primer sets obtained from the Harvard Primer Bank. PCR cycling conditions were as follows: 50 ^{o}C for 2 min,

95 ^{o}C for 10 min, and 40 cycles at 95 ^{o}C for 15 s and 60 ^{o}C for 1 min respectively. The expression of 18s rRNA served as an internal control. The results were expressed as fold change in expression of a particular RNA transcript relative to 18s rRNA expression between control mice and CNO-treated littermates (for primer sequences, see Table S3).

Irs2 RNA expression in INS-1 M3 cells was determined using the same experimental strategy as described above.

Morphometric analysis of pancreatic islets. Morphometric studies were performed with pancreatic islets taken from β-Rq mice and WT littermates subjected to different experimental treatments (see text for details; 3-4 mice/experimental group). For bromodeoxyuridine (BrdU; Sigma-Aldrich, St Louis, MO) labeling studies, mice were injected with a single dose of BrdU (200 mg/kg i.p.) 4 hr prior to being sacrificed. Pancreata were rapidly isolated, fixed in 10% neutral buffered formalin overnight, and embedded in paraffin. For each pancreas, three consecutive 5 μm-thick sections from five distinct levels, 150 μm apart, were mounted on slides, blocked with normal goat serum for 1 hr, and incubated overnight (4 ^{o}C) with a guinea-pig anti-insulin antibody (Dako-A0564; dilution 1:500) or a mouse anti-BrdU antibody (Dako-M0744; dilution 1:50). The two primary antibodies were detected with biotinylated goat anti-guinea-pig IgG (BA-7000, dilution: 1:250; Vector Laboratories, Burlingame, CA) or biotinylated horse anti-mouse IgG (Vectastain; PK6102, dilution: 1:250) secondary antibodies, respectively. Subsequently, the secondary antibodies were detected with Vectastain ELITE ABC reagent (Vector Laboratories; PK6102; detection of horseradish peroxidase activity) or Vectastain ELITE ABC-AP reagent (Vector Laboratories; AK-5000; detection of alkaline phosphatase activity). 3,3'-Diaminobenzidine (DAB; Sigma-Aldrich-D3939) and 5-bromo-4-chloro-3-indolyl phosphate p-nitro blue tetrazolium chloride (BCIP/NBT; Sigma-Aldrich-B1911) served as chromogenic substrates, resulting in brown- (insulin) or purple-colored (BrdU) stains, respectively. All sections were lightly counterstained with hematoxylin to visualize nuclei. BrdU-labeled sections were photographed at 200 x using an Axiocam MRm digital camera (Zeiss) and analyzed using AxioVision software (version 4.5; Zeiss). BrdU labeling data were expressed as percent $BrdU^{+}$ cells, as compared to the total number of insulin-positive islet cells.

To determine β-cell mass, five whole pancreatic sections per animal (150 μm apart) were stained for insulin as described above and photographed at 50 x magnification using an Axiocam MRm digital camera mounted on a Zeiss Axiovert Imager D1 imaging system (Zeiss, Thornwood, NY). Image acquisition and measurement of total pancreatic and β-cell specific area for each section were obtained using AxioVision software (version 4.5; Zeiss). To obtain a measure of β-cell mass, the ratio of islet cross-sectional area to total pancreatic area multiplied by pancreatic weight was determined.

Apoptotic β- cells were identified via TUNEL and insulin double-staining, using the DeadEnd Fluorometric TUNEL System (Promega, Madison, WI), according to manufacturer's instructions. Apoptotic cells were visualized by incubating pancreatic slices with fluorescein-12-dUTP, resulting in green fluorescence. To stain insulin-positive cells, slices were first incubated overnight with the same guinea-pig primary anti-insulin antibody as described above and then treated with a fluorescent secondary antibody (Alexafluor 568 goat anti-guinea pig IgG; Invitrogen, A11075, dilution 1:1000). All sections were counterstained with DAPI (Vectashield mounting medium with DAPI, Vector Laboratories) to visualize nuclei (purple color). Slides were imaged on a LSM 510 confocal microscope (Zeiss). TUNEL labeling data were expressed as percent $TUNEL^+$ cells, as compared to the total number of insulin-positive islet cells.

Western blotting studies. Protein extracts were prepared via lysis of isolated islets in a buffer containing 10 mM Tris (pH 7.6), 1% Triton X-100, 0.5% NP-40, 150 mM NaCl, 10 mM sodium orthovanadate, 10 mM sodium pyrophosphate, 100 mM sodium fluoride, 1 mM EDTA, 1 mM EGTA, and a cocktail of protease inhibitors (Roche), as described in detail elsewhere (1). Protein lysates were cleared via centrifugation at 8000 x g for 5 min. Protein concentrations were determined by using a BCA protein assay kit (Thermo Fisher Scientific Inc., Rockford, IL). After denaturation of proteins at 90 ^oC for 5 min, equal amounts of protein (25 μg/ well) were separated by 10% NUPAGE SDS–polyacrylamide gel (Invitrogen) electrophoresis and then transferred to polyvinylidene difluoride membranes (Invitrogen). Blots were blocked with Tris-buffered saline (TBS) containing 5% nonfat dried milk and 0.1% Tween 20 for 1 hr at room temperature, washed briefly with TBS containing 0.1% Tween 20 (TBS-T), incubated overnight at 4 ^oC with primary

antibody (Table S4) in TBS-T containing 5% BSA, washed with TBS-T, and then incubated for 1-2 hr at room temperature with a secondary antibody coupled to horseradish peroxidase. Immunoreactive proteins were visualized via chemiluminescence (Supersignal West Pico Chemiluminescent Substrate; Thermo Scientific) and exposure to an X-ray film (Kodak BioMax MS autoradiography). β-actin was used as loading control. Using a similar procedure, we also monitored siRNA-mediated knockdown of ERK1/2 protein expression in INS1-M3 cells.

Supplemental Table 1

Effect of chronic CNO treatment of β-Rq mice and WT littermates on the number of pancreatic islets and mean islet size

	number of islets per mm^2	mean islet size (μm^2)
WT + saline	1.21 ± 0.03	7,437 ± 255
WT + CNO	1.17 ± 0.02	7,542 ± 129
β-Rq + saline	1.10 ± 0.01	7,437 ± 158
β-Rq + CNO	1.29 ± 0.01	9,140 ± 126*

Mice (7-8-week old males) maintained on regular mouse chow received daily i.p. injections of either CNO (1 mg/kg i.p.) or saline for four weeks. Experiments were carried out at the end of the 4-week treatment period. Pancreata were obtained from the different experimental groups (n=3 or 4 per group). From each pancreas, 5 sections (150 μm apart) were obtained and analyzed (see Supplemental Methods for details). Data are given as means ± SEM. *$P < 0.05$, as compared to the three control groups.

Supplemental Table 2

Effect of chronic CNO administration on the number and size of pancreatic islets in STZ-treated β-Rq mice and WT littermates

	number of islets per mm^2	mean islet size (μm^2)
WT	1.24 ± 0.06	7,797 ± 1,667
WT + STZ	0.67 ± 0.01	5,250 ± 222
WT + STZ + CNO	0.65 ± 0.02	5,389 ± 198
β-Rq + STZ	0.67 ± 0.03	5,736 ± 436
β-Rq + STZ + CNO	0.85 ± 0.02**	7,291 ± 692*

Where indicated, mice (8-week-old males) were injected with STZ for five consecutive days (50 mg/kg i.p. once per day). Mice received CNO via the drinking water (0.25 mg/ml) for 10 weeks (CNO treatment was started 1 week prior to the first STZ injection). Pancreata were obtained at the end of the CNO treatment period from the different experimental groups (n=3 per group). From each pancreas, 5 sections (150 μm apart) were obtained and analyzed (see Supplemental Methods for details). Data are given as means ± SEM. *$P < 0.05$, ** $P < 0.01$, as compared to the three STZ-treated control groups.

Supplemental Table 3

PCR primers used for qRT-PCR experiments

Mouse gene	Primer sequence	Amplicon (bp)
Glut2	Forward: 5' CATTCTTTGGTGGGTGGC Reverse: 5' CCTGAGTGTGTTTGGAGCG	221
Ins2 (Preproinsulin 2)	Forward: 5' CTGGCCCTGCTCTTCCTCTGG Reverse: 5' CTGAAGGTCACCTGCTCCCGG	204
Pcsk1 (Proprotein convertase 1)	Forward: 5' CTTTCGCCTTCTTTTGCGTTT Reverse: 5' TCCGCCGCCCATTCATTAAC	79
Pcsk2 (Proprotein convertase 2)	Forward: 5' AGAGAGACCCCAGGATAAAGATG Reverse: 5' CTTGCCCAGTGTTGAACAGGT	144
Pcx (Pyruvate carboxylase)	Forward: 5' CTGAAGTTCCAAACAGTTCGAGG Reverse: 5' CGCACGAAACACTCGGATG	162
Irs2	Forward: 5' CTGCGTCCTCTCCCAAAGTG Reverse: 5' GGGGTCATGGGCATGTAGC	124
Pdx1	Forward: 5' CCCCAGTTTACAAGCTCGCT Reverse: 5' CTCGGTTCCATTCGGGAAAGG	177
Ngn3 (Neurogenin 3)	Forward: 5' AGTGCTCAGTTCCAATTCCAC Reverse: 5' CGGCTTCTTCGCTTTTTGCTG	168
Nkx6.1	Forward: 5' CTGCACAGTATGGCCGAGATG Reverse: 5' CCGGGTTATGTGAGCCCAA	136
NeuroD1	Forward: 5' ATGACCAAATCATACAGCGAGAG Reverse: 5' TCTGCCTCGTGTTCCTCGT	110
MafA	Forward: 5' AGGAGGAGGTCATCCGACTG Reverse: 5' CTTCTCGCTCTCCAGAATGTG	113

Supplemental Table 4

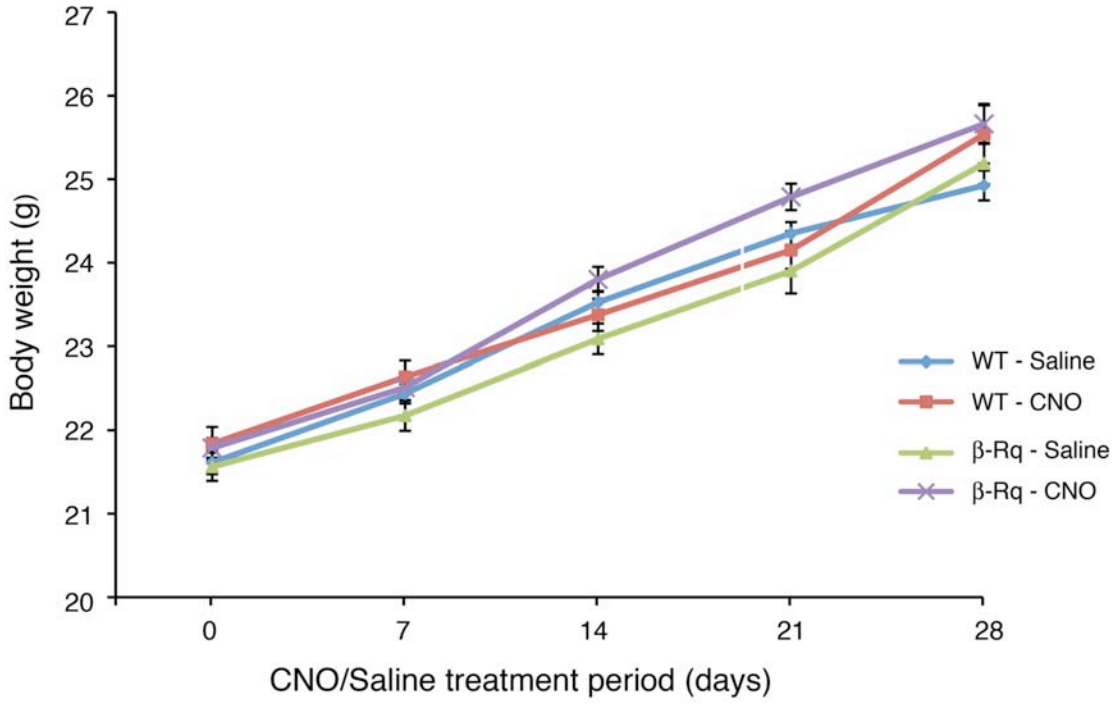
Antibodies used for Western blotting studies

Antibody target	Source of antibody	Catalog #
IRS1 (Insulin Receptor Substrate 1)	Mouse monoclonal antibody purified from hybridoma cell supernatants (ref. 2)	
IRS2 (Insulin Receptor Substrate 2)	Mouse monoclonal antibody purified from hybridoma cell supernatants (ref. 2)	
Phospho-Akt (Akt S473)	Cell Signaling Technology, Danvers, MA	9271
Akt	Cell Signaling Technology, Danvers, MA	9272
Phospho-p44/42 MAPK (Erk1/2; T202/Y204)	Cell Signaling Technology, Danvers, MA	4376
p44/42 MAPK (Erk1/2)	Cell Signaling Technology, Danvers, MA	9102
β-Actin	Cell Signaling Technology, Danvers, MA	3700
Anti-rabbit IgG, HRP-linked secondary antibody	Cell Signaling Technology, Danvers, MA	7074
Anti-mouse IgG, HRP-linked secondary antibody	Cell Signaling Technology, Danvers, MA	7076

Supplementary References

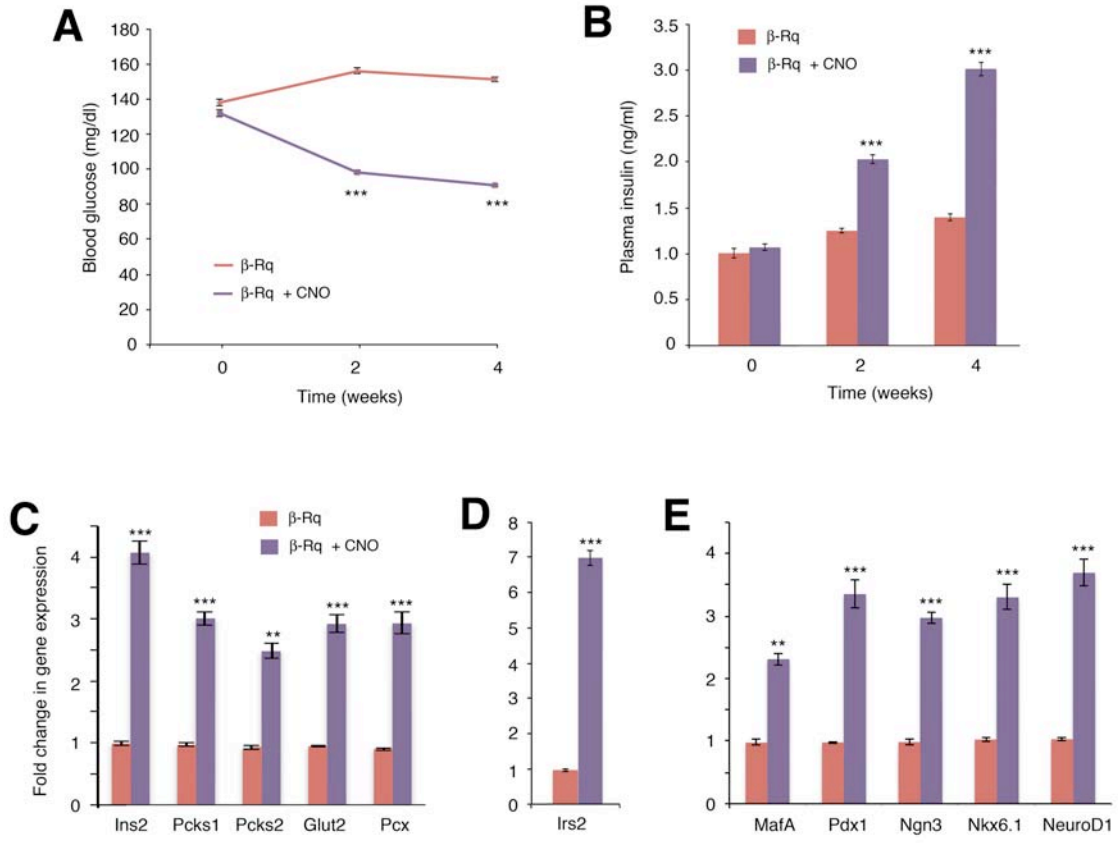
1. Lin HM, Lee JH, Yadav H, Kamaraju AK, Liu E, Zhigang D, Vieira A, Kim SJ, Collins H, Matschinsky F., et al. Transforming growth factor-beta/Smad3 signaling regulates insulin gene transcription and pancreatic islet beta-cell function. *J Biol Chem.* 2009;284:12246-12257.

2. Withers DJ, Gutierrez JS, Towery H, Burks DJ, Ren JM, Previs S, Zhang Y, Bernal D, Pons S, Shulman GI, et al. Disruption of IRS-2 causes type 2 diabetes in mice. *Nature*. 1998; 391:900-904.



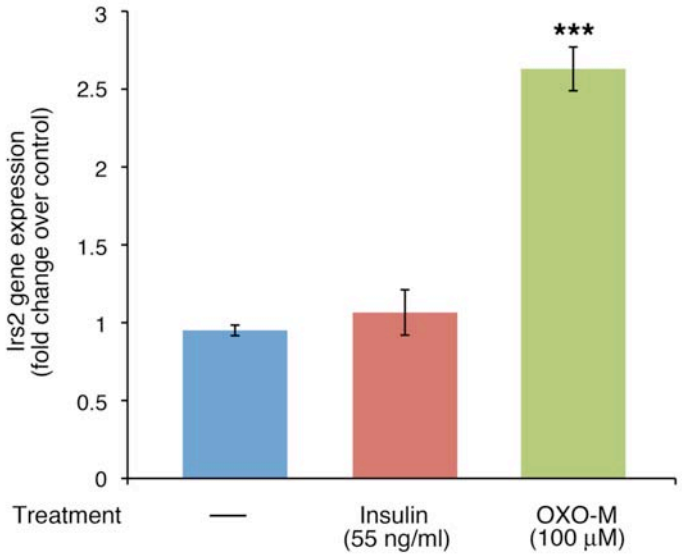
Supplemental Figure 1

Chronic CNO treatment has no significant effect on the body weight of β-Rq transgenic mice and WT littermates. Mice (7-8-week old males) maintained on regular mouse chow received daily i.p. injections of either CNO (1 mg/kg i.p.) or saline for four weeks. Data are expressed as means ± SEM (n=6 per group).



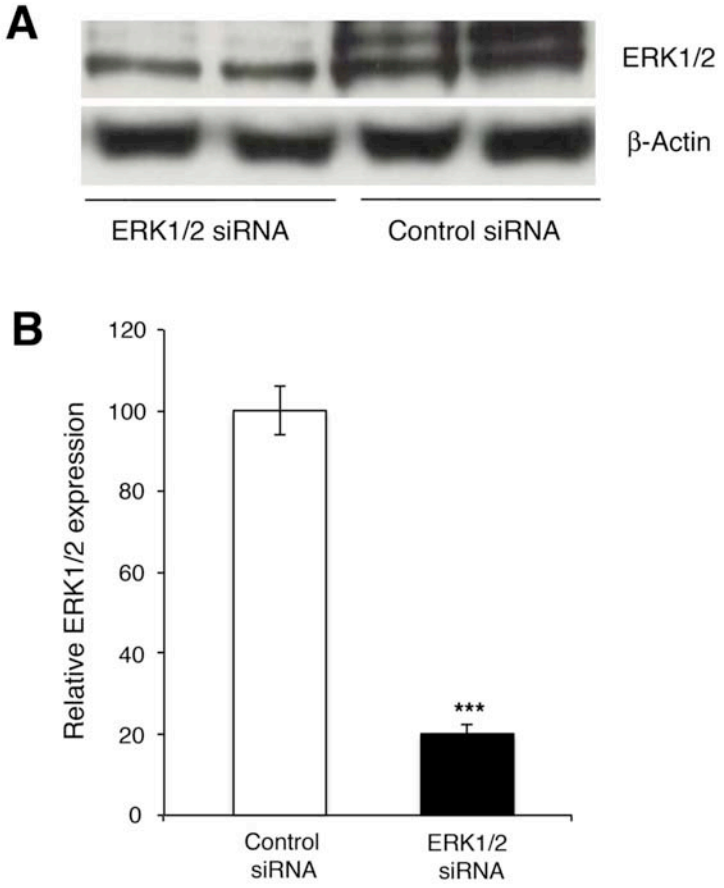
Supplemental Figure 2

Chronic treatment of β-Rq mice with CNO drinking water. β-Rq transgenic mice (7-8-week old males) were chronically treated with CNO for four weeks. CNO was administered via the drinking water (0.25 g/ml). Control mice consumed CNO-free drinking water. Gene expression was studied at the end of the 4-week treatment period. (**A**) Progressive reduction of blood glucose levels and (**B**) elevated plasma insulin levels in CNO-treated β-Rq mice (freely fed mice). (**C-E)** Enhanced expression of key β-cell genes in islets prepared from CNO-treated β-Rq mice, including genes critical for insulin synthesis (**C**), *Irs2* (**D**), and several important β-cell transcription factors (**E**). Islet gene expression was studied by real-time qRT-PCR using total islet RNA. Data were normalized relative to the expression of 18S rRNA and are presented as fold change in gene expression compared to non-CNO-treated mice. Data are expressed as means ± SEM (A, B, n=9 per group; D-E, n=2 or 3 per group). $^{**}P < 0.01$, $^{***}P < 0.001$, vs. non-CNO-treated β-Rq mice.



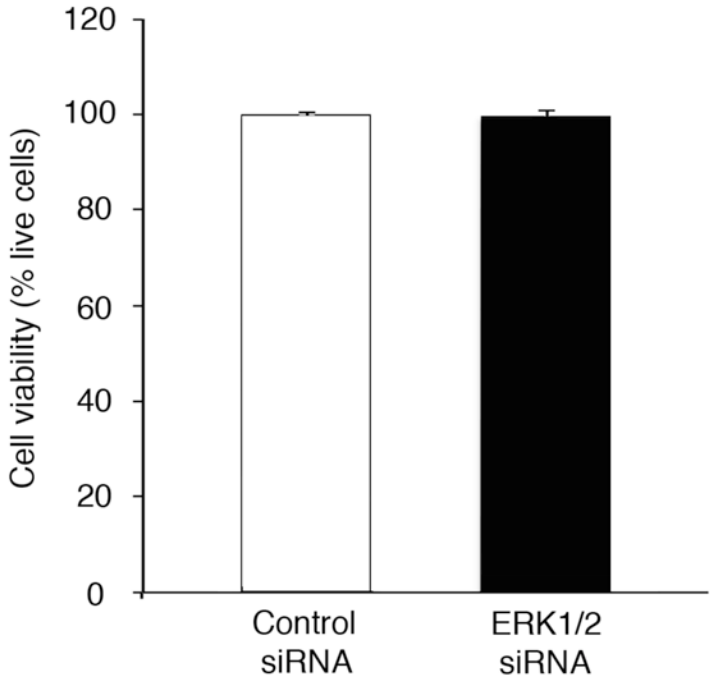
Supplemental Figure 3

Insulin treatment does not promote *Irs2* gene expression in INS1-M3 insulinoma cells. INS1-M3 cells were incubated (for 5 hr) with either insulin (55 ng/ml) or the muscarinic agonist, OXO-M (100 μM; positive control). Changes in *Irs2* gene expression levels were determined via qRT-PCR (for details, see Supplemental Methods). Data represent means ± SEM of three independent experiments. ***$P < 0.001$ (vs. untreated cells).



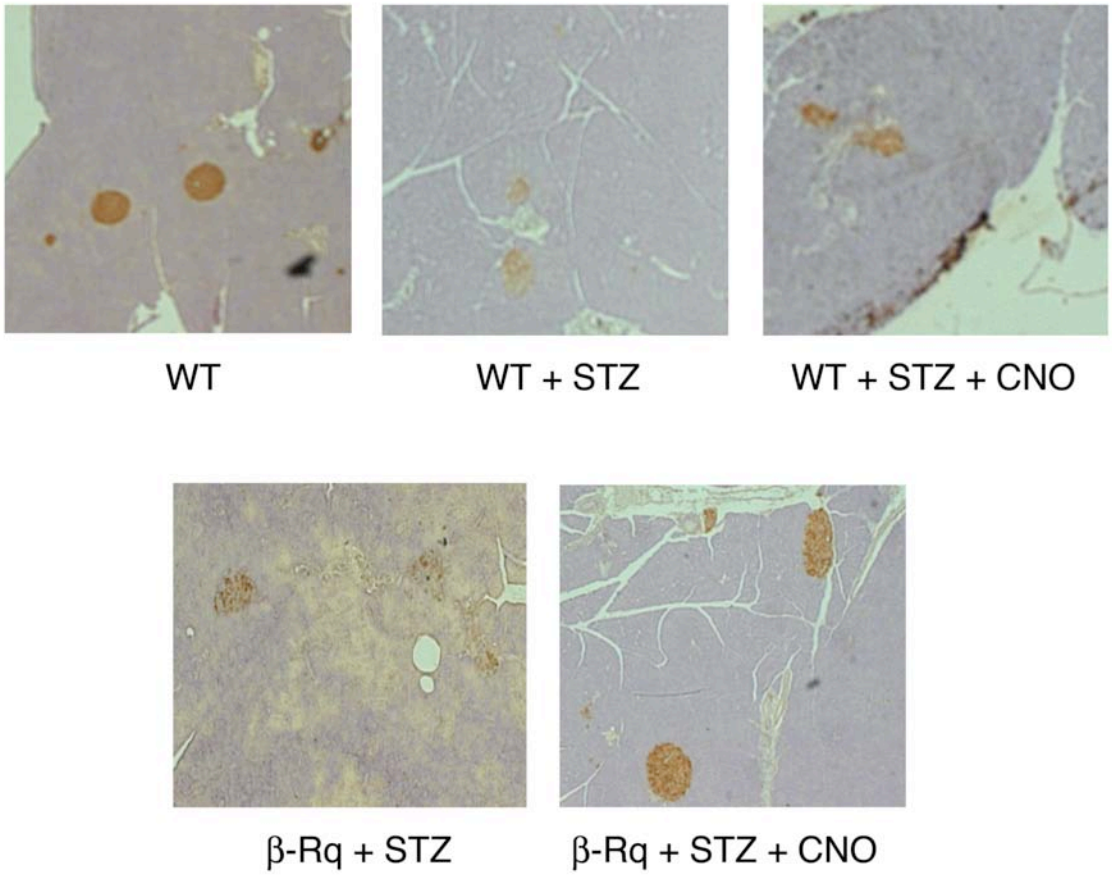
Supplemental Figure 4

Effective knockdown of ERK1/2 expression in INS1-M3 cells by the use of siRNA technology. (**A**) Representative western blot indicating ERK1/2 expression after treatment of INS1-M3 cells with ERK1/2 siRNA or scrambled control siRNA (for details, see Experimental Procedures). 25 μg of total protein were loaded per well. (**B**) Quantification of immunoblotting results. In each individual experiment, ERK1/2 expression observed with control siRNA was set equal to 100%. Data are given as means ± SEM of three independent experiments. ***$P < 0.001$.



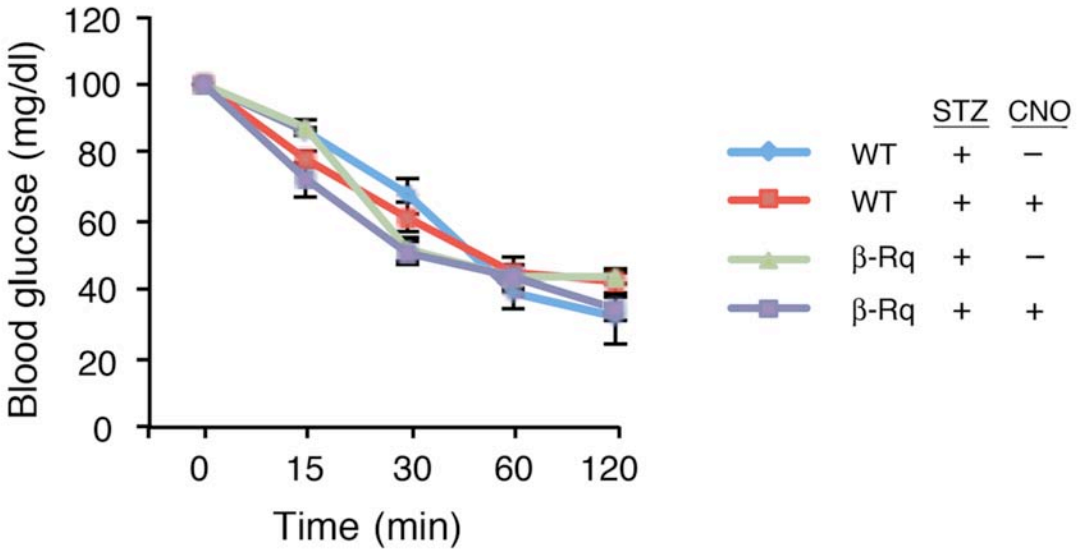
Supplemental Figure 5

siRNA-mediated knockdown of ERK1/2 expression in INS1-M3 insulinoma cells has no significant effect on cell viability. Cell viability assays were carried out by using an MTT kit (Sigma; # 7H258), according to the manufacturer's protocol. Data represent means ± SEM of two independent experiments carried out in duplicate.



Supplemental Figure 6

Images of pancreatic sections from STZ-injected β-Rq transgenic mice and WT littermates following chronic CNO treatment. Where indicated, mice (8-week-old males) were injected with STZ for five consecutive days (50 mg/kg i.p. once per day). Mice received CNO via the drinking water (0.25 mg/ml) for 10 weeks (CNO treatment was started 1 week prior to the first STZ injection). Control animals consumed regular drinking water. Representative pancreatic sections stained with an anti-insulin antibody are shown.



Supplemental Figure 7

Insulin sensitivity is unaltered in STZ-treated WT and β-Rq mice. β-Rq transgenic mice and WT littermates mice (8-week-old males) were injected with STZ for five consecutive days (50 mg/kg i.p. once per day). Mice received CNO via the drinking water (0.25 mg/ml) for 10 weeks (CNO treatment was started 1 week prior to the first STZ injection). This figure shows the results of an insulin tolerance test (insulin dose: 0.75 mIU/g, i.p.), indicating that insulin produced similar glucose-lowering effects in all four groups of mice. Data are expressed as means ± SEM (n=6 per group).