Supplemental Figure 1

A Vehicle — TH (green) / Insulin (red) — P6
B 6-OHDA — P6
C *TrkA^{f/f}* — TH — P6
D *TH-Cre;TrkA^{f/f}* — P6
E *TrkA^{f/f}* — P2
F *TH-Cre;TrkA^{f/f}*
G *TrkA^{f/f}* — P2
H *TH-Cre;TrkA^{f/f}*
I Vehicle — Glucagon (green) / Insulin (red) — P6
J 6-OHDA — P6
K Circularity index — Vehicle / 6-OHDA
L % Islets with core localized α-cells — Vehicle / 6-OHDA
M Number of cells / section — α-cells / β-cells — Vehicle / 6-OHDA
N *TrkA^{f/f}* — P6
O *TH-Cre;TrkA^{f/f}*
P % TH+ β-cells — *TrkA^{f/f}* / *TH-Cre;TrkA^{f/f}* — P2 / P6 / 1 Month
Q Fold expression of *TrkA* mRNA — *TrkA^{f/f}* / *TH-Cre;TrkA^{f/f}*
R Vehicle — P6
S 6-OHDA
T % TH+ β-cells — Vehicle / 6-OHDA

Supplemental Figure 2

A

α-Glut2

α-p85

TrkA^{f/f}   *TH-Cre; TrkA^{f/f}*

B

Glut2 protein level

1.5

1.0

0.5

0.0

TrkA^{f/f}   *TH-Cre;TrkA^{f/f}*

C   *TrkA^{f/f}*

Glut2   Insulin

P6

D   *TH-Cre;TrkA^{f/f}*

E   *TrkA^{f/f}*

Glucagon   Insulin

1 Month

F   *TH-Cre;TrkA^{f/f}*

G

Islet sectional area (μm^2)

12000

10000

8000

6000

4000

2000

0

TrkA^{f/f}   *TH-Cre;TrkA^{f/f}*

H   Males

Body weight (g)

25

20

15

10

5

0

TrkA^{f/f}   *TH-Cre;TrkA^{f/f}*

I   Females

Body weight (g)

25

20

15

10

5

0

TrkA^{f/f}   *TH-Cre;TrkA^{f/f}*

Supplemental Figure 3

A Matrigel

B SCG

Day 1 Day 2 Day 3 Day 4 Day 5 Day 6 Day 7

C SCG + Propranolol

D SCG + Phentolamine

E

F

G

Supplemental Figure 5

A Vehicle
TH Insulin
P6

B Propranolol

C *TrkA^{f/f}* + Iso.
TH Insulin
P6

D *TH-Cre;TrkA^{f/f}* + Iso.

Supplemental Information:

Sympathetic innervation during development is necessary for pancreatic islet architecture and functional maturation

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Supplemental Figure Titles and Legends:

Figure S1. Analyses of sympathectomized mice, related to Figure 1

(A,B) Treatment of new-born mice with 6-OHDA daily until postnatal day 6 (P6) results in depletion of sympathetic fibers (arrows) in the pancreas. Scale bars, 50μm. (C) TH immunofluorescence of tissue sections reveals intact superior cervical ganglia (SCG) at the bifurcation of the carotid arteries (*) in control $TrkA^{f/f}$ mice. (D) SCGs were absent by P6 in all the $TH\text{-}Cre;TrkA^{f/f}$ mice that were examined. Arrows point to a few remaining TH-positive cells in mutant mice. Scale bars, 200μm. n=3 mice per genotype. (E-H) Wholemount TH immunostaining of peripheral tissues reveals attenuated sympathetic innervation of the heart (E,F), and intestines (G,H) in $TH\text{-}Cre;TrkA^{f/f}$ mice at P2, compared to control $TrkA^{f/f}$ animals. While the major fiber bundles leading to the targets are intact (G,H arrows), the finer innervation into the target tissues is lost, consistent with the known role of NGF-TrkA signaling in mediating final target innervation. Scale bars, 2mm. (I,J) Mice treated with 6-OHDA show similar aberrations in islet shape and spatial distribution of islet endocrine cells, as in $TH\text{-}Cre;TrkA^{f/f}$ animals. (K) Islet shape is quantified by circularity index. (L) Percentage of islets with mis-localized α-cells. (M) Normal endocrine cell numbers in 6-OHDA-injected mice at P6. For all quantifications, n

= 3 mice per genotype; mean ± SEM; *p < 0.05, t test. (N,O) Wholemount TH staining of the pancreas reveals a reduction in sympathetic fibers (arrows in A) at P6, while the number of TH-positive β-cells increase (arrowheads). Scale bars, 200µm. (P) Mutant mice have significantly more TH$^+$ β-cells at various stages of development. n=3 mice per genotype; mean ± SEM; ***p < 0.001, t test. (Q) Quantitative RT-PCR shows *TrkA* mRNA expression levels are unchanged in mutants. *TrkA* expression levels are relative to control animals and normalized to *18s rRNA*. n=6 mice per genotype; mean ± SEM. (R-T) Treatment with 6-OHDA has no effect on the numbers of TH$^+$ β-cells. n=3 mice per genotype; mean ± SEM; t test.

Figure S2. *TH-Cre;TrkA^{f/f}* animals exhibit reduced Glut2 expression at P6, and reduced islet size and body weight at one month, related to Figure 2

(A-D) *TH-Cre;TrkA^{f/f}* animals have an early deficit in Glut2 expression and surface localization manifest by P6. n=3 mice per genotype, *t* test, mean ± SEM; *p<0.05. (E-G One month-old *TH-Cre;TrkA^{f/f}* mice have reduced islet area. n=5 mice per genotype, mean ± SEM; *p<0.05, t test. (H,I) Both male and female *TH-Cre;TrkA^{f/f}* animals exhibit significant reductions in body weight at one month as compared to control *TrkA^{f/f}* mice. Males, n=9 mice per genotype; females, n=4 mice per genotype; mean ± SEM; *p < 0.05, t test. Scale bars, 50µm.

Figure S3. Sympathetic de-innervation does not affect the levels of glucagon, several intestinal hormones, incretins, and adipose-derived leptin in *TH-Cre;TrkA^{f/f}* mice, related to Figure 3

(A) Fasting blood glucose levels are normal in one month-old *TH-Cre;TrkA^{f/f}* mice. (B-I) Plasma levels of several hormones including the pancreatic hormones, glucagon (B) and ghrelin (C), the gastro-intestinal hormones, cholecystokinin (D), gastrin (E) and secretin (F), the incretins, GLP-1 (glucagon-like peptide-1, G), GIP (glucose-dependent insulinotropic peptide, H) and leptin (I) are unaffected by the loss of sympathetic innervation in *TH-Cre;TrkA^{f/f}* mice. Plasma hormone levels were assessed in response to fasting and feeding. In both *TH-Cre;TrkA^{f/f}* and control *TrkA^{f/f}* mice, expected changes in hormone levels were seen with fasting. In the fasted state, levels of ghrelin levels are significantly increased while that of CCK, secretin, GLP-1 and leptin are all decreased. For all graphs, black bars indicate *TrkA^{f/f}* mice and red indicate *TH-Cre;TrkA^{f/f}* mice. n=3 mice per genotype, mean ± SEM; *$p < 0.05$, t test.

Figure S4. Additional *in vitro* migration assay data, related to Figure 5

(A,B) Composite images of the locations of β-cells over 7 days show that β-cells cultured alone do not migrate significantly, while those cultured with an SCG explant show directed migration. Scale bars, 200μm. (C,D) Propranolol inhibits the migration of β-cells in the presence of SCGs, while phentolamine has no effect. The averaged profiles of at least three independent experiments are plotted. (E) There is no significant difference in total β-cell number with any of the treatments, after 7 days in culture. n=3 independent experiments per condition; one-way ANOVA; mean ± SEM. (F,G) α-cell migration and numbers are unaffected by co-culture with SCG explants or treatment with propranolol. n=3 independent experiments per condition; one-way ANOVA; mean ± SEM.

Figure S5. Pharmacological manipulation of β-adrenergic receptor activity does not affect sympathetic innervation, related to Figure 6

(A,B) Propranolol injection during E18 to P6 does not impair sympathetic innervation in the pancreas. (C,D) Isoproterenol administration does not restore sympathetic innervation in *TH-Cre;TrkA^{f/f}* mice. n=3 mice were analyzed per treatment. Scale bars: 50 μm.

Extended Experimental Procedures:

Antibodies, immunofluorescence and whole mount immunohistochemical analyses

Antibodies: Rabbit anti-Tyrosine Hydroxylase (TH, 1:500, Millipore); guinea pig anti-insulin (1:300, Millipore); mouse anti-glucagon (1:500, Abcam); dolichos biflorus agglutinin (DBA, 1:200, Vector Labs); rabbit anti-GFP (1:500, Invitrogen); mouse anti-β-III tubulin (1:200, Sigma); rabbit anti-GLUT2, 1:500 (Millipore); rabbit anti-carboxypeptidase A (1:250 AbD Serotec); mouse anti-PECAM (1:200, BD Biosciences), rat anti-E-Cadherin (1:500, Invitrogen); rabbit anti-NCAM (1:500, Millipore). Fluorescent secondary antibodies were all Alexafluor-488 or -546-conjugated and used at 1:200 (Invitrogen).

Immunofluorescence: For immunostaining, pancreata were dissected, fixed in 4% paraformaldehyde (PFA, Sigma), cryoprotected in 30% sucrose (Sigma), and embedded in OCT (Sakura Finetek) before being cryosectioned. Sections were frozen down at -20°C for storage.

Wholemount TH staining: Pancreata and internal organs from P2 and P6 mice were processed for wholemount TH staining as previously described (Enomoto et al., 2001) using rabbit anti-TH at 1:400 (Millipore).

In vivo blood vessel painting: Blood vessels of P6 control and mutant mice were fluorescently painted by perfusing with Alexa633 conjugated wheat germ agglutinin (WGA, Invitrogen) (Chiu et al., 2012). Mice were first perfused through the heart with ice cold PBS, followed by ice cold dye solution (6μg/mL) to perfuse 30μg of dye per 1g of animal, followed by ice cold 4% PFA. Pancreata were then removed and fixed in PFA. Thick sections (100μm) were collected and imaged on a Zeiss LSM 510 confocal microscope taking z-stacks through the entire section. Optical planes were then merged to obtain the maximal projection through the entire thickness of the tissue.

Quantitative RT-PCR and immunoblotting

Islets from 1-2 month old mice were collected, lysed in Trizol reagent (Invitrogen), and RNA purified using RNeasy mini columns (Qiagen). RNA was then reverse transcribed using a RETROscript kit (Ambion). Quantitative RT-PCR was carried out using SYBR green mix (BioRad) in a BioRad iCycler instrument, using the primers: TrkA-F: 5'-TCCAAGTCAGCGTCTCCTTCCCA-3', TrkA-R: 5'-AGCCGTTGAAC AACCAGCGCA-3', 18S rRNA-F: 5'-CGCCGCTAGAGGTGAAATTC-3', 18S rRNA-R: 5'- TTGGCAAATGCTTTCGCTC-3'. Fold change in *TrkA* transcript levels was calculated using the -2ΔΔCt method, normalizing to *18S rRNA* transcript. For western blot analyses, superior cervical ganglia (SCGs) were isolated from P2 animals, lysed in loading buffer, and subjected to SDS-PAGE. Islets were isolated from adult one month-old animals and lysed in RIPA buffer and subjected to SDS-PAGE analyses. After transferring, membranes were probed using rabbit anti-TrkA, (1:1000, Millipore), rabbit anti-Glut2 (1:1000, Millipore) and rabbit anti-p85 (1:2000, Millipore).

Light microscopy

Tissue sections were imaged using a Zeiss AxioImager M1 microscope equipped with an Axiocam HRc camera. *In vitro* migration assays were imaged using a Zeiss Axiovert 200 microscope equipped with a QImaging Retiga EXi camera. High magnification images of the neuron-islet cell co-cultures were acquired using a Zeiss LSM510 confocal microscope.

Additional References:

Chiu, Y.C., Hua, T.E., Fu, Y.Y., Pasricha, P.J., and Tang, S.C. (2012). 3-D imaging and illustration of the perfusive mouse islet sympathetic innervation and its remodelling in injury. Diabetologia *55*, 3252-3261.

Enomoto, H., Crawford, P.A., Gorodinsky, A., Heuckeroth, R.O., Johnson, E.M., Jr., and Milbrandt, J. (2001). RET signaling is essential for migration, axonal growth and axon guidance of developing sympathetic neurons. Development *128*, 3963-3974.