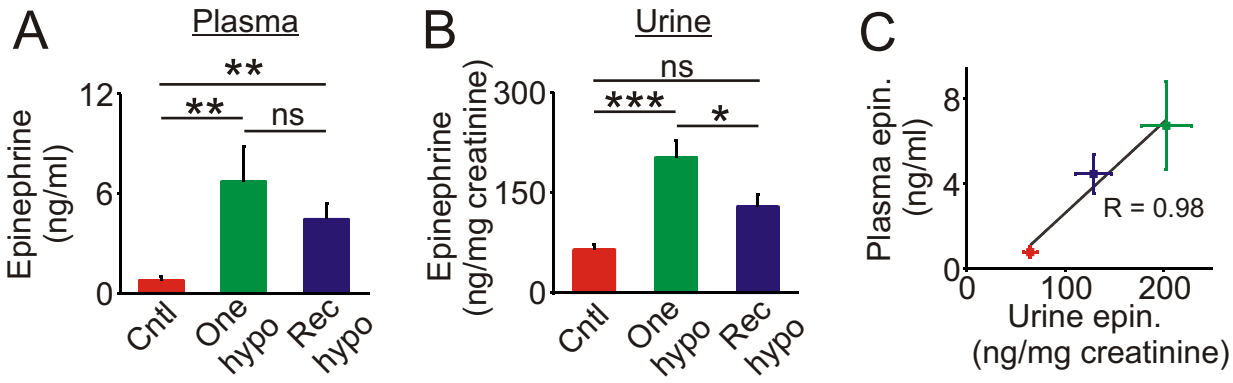
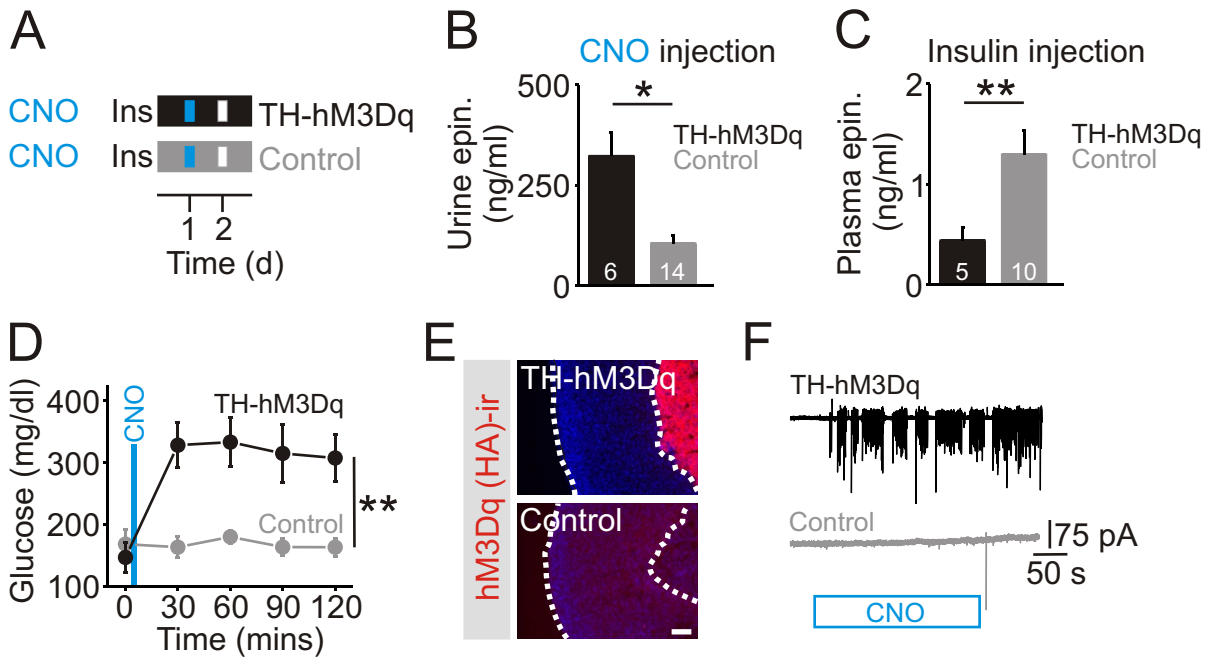

A
Plasma
Epinephrine (ng/ml)
12
6
0
**
**
ns
Cntl
One hypo
Rec hypo
B
Urine
Epinephrine (ng/mg creatinine)
300
150
0
ns
***
*
Cntl
One hypo
Rec hypo
C
Plasma epin. (ng/ml)
8
4
0
R = 0.98
0
100
200
Urine epin. (ng/mg creatinine)

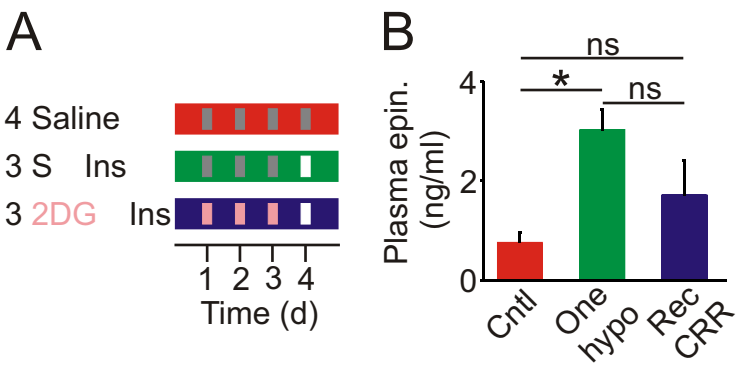
Supplementary Figure 1. **Hypoglycemia-induced epinephrine release quantified in plasma and urine.**

(A). Epinephrine levels measured in plasma 1 hr after final injection of saline or insulin using the experimental protocol shown in Figure 1A. Recurrent insulin treatment blunted epinephrine release compared to single insulin injection ((although this did not reach statistical significance); n = 10 mice per group, Kruskal-Wallis rank sum test)). (B). Epinephrine levels measured in urine collected for 24 hr after final injection of saline or insulin using the experimental protocol shown in Figure 1A (same data as shown in Figure 1B). Recurrent insulin injection significantly blunted epinephrine release compared to single insulin injection. After recurrent hypoglycemia, epinephrine levels were not significantly different from control (recurrent saline) values. (C). Comparison of epinephrine levels in plasma and urine from mice exposed to the experimental protocol shown in Figure 1A. Linear regression of this data reveals that epinephrine levels in plasma and urine are highly correlated (correlation coefficient, R = 0.98). *$p < 0.05$, **$p < 0.01$, *** $p < 0.001$, ns not significant.


A
CNO
Ins
TH-hM3Dq
CNO
Ins
Control
1 2
Time (d)
B
CNO injection
Urine epin. (ng/ml)
500
250
0
*
TH-hM3Dq
Control
6
14
C
Insulin injection
Plasma epin. (ng/ml)
2
1
0
**
TH-hM3Dq
Control
5
10
D
Glucose (mg/dl)
400
300
200
100
CNO
TH-hM3Dq
Control
**
0 30 60 90 120
Time (mins)
E
hM3Dq (HA)-ir
TH-hM3Dq
Control
F
TH-hM3Dq
Control
75 pA
50 s
CNO

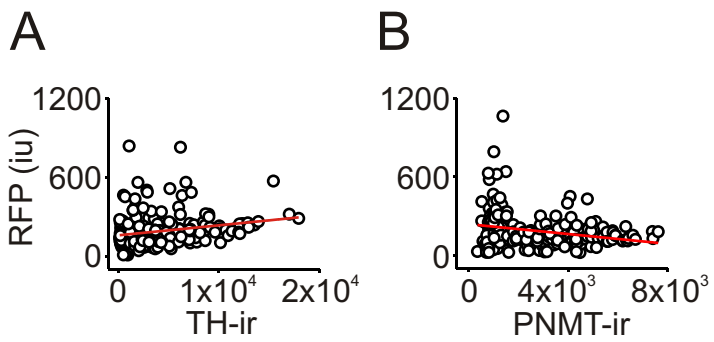
Supplementary Figure 2. **Use of DREADD technology to evoke epinephrine release *in vivo*.**

(A). Experimental protocol for recurrent adrenal activation: injection of CNO (blue bar), the DREADD agonist, into TH-hM3Dq mice (black) will activate adrenal chromaffin cells (and other TH-expressing neurons) and elicit epinephrine release. One day later, insulin (white bar) is injected to induce hypoglycemia. Control mice (TH cre or wild type littermates; gray) are exposed to the same treatment. Thus TH-hM3Dq mice (but not controls) experience recurrent activation of the counter-regulatory response. (B). Epinephrine levels are significantly higher in urine from TH-hM3Dq mice injected with CNO (0.5 mg / kg i.p.) compared to control animals, consistent with CNO-induced adrenal activation (n = 6 – 14, Student's t test). (C). In contrast, plasma epinephrine levels are significantly *lower* in the TH-hM3Dq mice after insulin-induced hypoglycemia compared to controls (n = 5 – 10, Student's t test). *Thus recurrent activation of epinephrine release (by CNO and insulin) leads to a suppression of the counter-regulatory response*. (D). CNO injection in TH-hM3Dq mice evokes hyperglycemia (consistent with epinephrine release), but does not alter blood levels in control animals (n = 3 – 5, respectively, 2-way ANOVA). (E). In TH-hM3Dq mice, the HA-tagged DREADD receptor is expressed in the adrenal medulla. (F). Representative on-cell recording of CNO-evoked action currents in a chromaffin cell in an adrenal slice from a TH-hM3Dq mouse. CNO (10 μM) did not significantly alter activity in a chromaffin cell from a representative control animal. *$p < 0.05$, **$p < 0.01$. Scale bar, 100 μm.


A
4 Saline
3 S Ins
3 2DG Ins
1 2 3 4
Time (d)
B
ns
*
ns
Plasma epin. (ng/ml)
4
2
0
Cntl
One hypo
Rec CRR

Supplementary Figure 3. **Recurrent injection of 2DG suppresses insulin-induced epinephrine release.**

(A). Experimental protocol to induce recurrent activation of the counter-regulatory response by repeated injection of the glucoprivic agent, 2DG. In this protocol, mice in which the CRR has been activated once (green) or recurrently (blue) receive the same number of insulin injections. (B). Plasma epinephrine levels measured 1 hr after the final saline or insulin injection. Plasma epinephrine was not significantly different in the saline- and recurrent 2DG-injected groups. It was also lower (but did not reach significance) in the recurrent 2DG-injected group compared to the single insulin-injected group (n = 4 mice per group). *$p < 0.05$, ns not significant, 1-way ANOVA.


A
B
1200
600
0
RFP (iu)
0
1x10^4
2x10^4
TH-ir
0
4x10^3
8x10^3
PNMT-ir

Supplementary Figure 4. **Channelrhodopsin expression in adrenal chromaffin cells in TH-ChR(tdTomato) mice.**

Quantification of channelrhodopsin expression in TH-ChR(tdTomato) mice. (A). RFP fluorescence and TH-ir and (B) RFP fluorescence and PNMT-ir in chromaffin cells *in vitro* (n = 4 experiments, ≤50 cells per experiment). Red line is a linear fit (correlation coefficient, R = 0.22; -0.25, respectively). Low R values indicate that channelrhodopsin is expressed uniformly in the medulla and is not restricted to a sub-population of chromaffin cells.

SUPPLEMENTAL METHODS

Animals. C57BL/6J wild-type mice, LSL-hChR2-tdTomato (# 012567; Ai27D (1)), NPY(GFP) mice (# 00641; (2)), LSL-hM3DqDREADD (#026220; (3)) and NPY knock-out mice (# 004545; (4)) were purchased from The Jackson Laboratory. TH-cre mice (# 031029-UCD (5)) were from the MMRRC. All experiments used male mice (P28 - 42) that received *ad libitum* access to food and water unless specified (female mice were used in some experiments involving hM3Dq expression). Littermates were used in all experiments involving C57BL/6J wild-type and NPY ko animals. In experiments with TH-ChR mice when littermates were not available, animals with matching age and weight were used. All experiments involving animals were approved by the Animal Care and Use Committee at Louisiana State University Health Sciences Center.

Insulin-induced hypoglycemia. All experiments compared groups of mice that were euglycemic controls or exposed to single or recurrent hypoglycemic episodes. In each case a cohort of three littermate animals were separated and housed individually. Animals were food deprived each day for 3 hr then injected i.p with either saline or recombinant human insulin (Sigma #I2643). *Euglycemic controls*: i.p. injection of saline, repeated for three days. *Single hypoglycemic episode*: i.p. injection of saline repeated for two days and an insulin injection on the third day. *Recurrent hypoglycemic episodes*: i.p. injection of insulin repeated for three days. Food was replaced one hr after injection. In some experiments, a four injection protocol was used (i.e. the experiment was extended by one day). In these cases the fourth injection was used either to measure epinephrine release *in vivo* (Figure 1A-C; 3G-I) or to assess changes in adrenal proliferation/apoptosis (Figure 4A-D). A schematic indicating the four injection protocol is shown in Figure 1A. The insulin dose for wild type and NPY ko mice was 2.5 U/kg and typically 0.75 U/kg, respectively. Although fed wild type and NPY ko mice have comparable levels of blood glucose (6, 7), mice lacking NPY are more hypoglycemic than control animals in the insulin tolerance test (8). A lower insulin dose was therefore used in most experiments involving NPY ko animals to avoid severe hypoglycemia, and ensure equivalent levels of blood glucose. Blood glucose was monitored before and 1 hr after each injection *via* tail prick and commercial glucose meters (OptiumEZ, TRUEresult). Mice were sacrificed by decapitation 24 hrs after the final injection and tissue samples were collected. In experiments involving BIBP3226, the drug (1 mg / kg) was injected 15 mins before each treatment with vehicle or insulin. In some experiments (Supplementary Figure 3), mice were recurrently injected with 2-deoxyglucose (2DG; 500 mg / kg). In each figure, n corresponds to the number of animals in *each* treatment group.

Urine and plasma collection and measurement of catecholamine levels. Animals were placed in metabolic cages (Tecniplast) after the final injection on the fourth day. Epinephrine levels were quantified in the urine (this approach is minimally invasive and urine levels correlate with whole body catecholamine turnover (9)). Urine samples were collected for 24 hrs then acidified by addition of an equal volume of 0.01 M HCl and stored at -80 °C. Epinephrine levels were measured using a commercial ELISA kit (Abnova) with minimal cross-reactivity to derivatized norepinephrine and the metabolites metanephrine and normetanephrine (0.2; 0.64; 0.0009%, respectively; manufacturers' data). This suggests there was minimal contribution in the epinephrine measurements from norepinephrine released from sympathetic nerves during hypoglycemia. In most cases epinephrine levels were

normalized to the level of urine creatinine quantified using the Jaffe reaction (Cayman Chemicals). For measurement of epinephrine levels in plasma, mice were euthanized with pentobarbital (300 mg / kg i.p) 1 hr after injection with insulin, 2DG or CNO. After decapitation, trunk blood was collected in EDTA-coated tubes and stored at -80 °C.

DREADD-induced neuronal activity *in vivo*. TH-hM3Dq or control mice (TH-cre or wild type littermates) were separated and housed individually. Animals were handled daily and injected i.p. with saline for 2 -3 d before injection with clozapine-N-oxide (CNO; 0.3 mg / kg i.p.). To measure epinephrine release, mice were then placed in metabolic cages and 24 hr urine samples were collected. Mice were subsequently returned to their home cage, food deprived for 3 hrs then injected i.p. with 2.5 U / kg insulin, euthanized 1 hr later with pentobarbital and trunk blood was collected (see above). In this way, epinephrine levels could be quantified in urine and plasma in the same animals following CNO and insulin injections, respectively (see Supplementary Figure 2A-C). To quantify the effect of DREADD-induced activity on blood glucose levels, mice were injected with CNO, returned to their home cage and tail blood glucose measurements were made every 30 mins for 2 hrs after injection (Supplementary Figure 2D). In these experiments food was removed from the home cage after CNO injection.

Pyruvate tolerance test. Cohorts of four littermates were housed individually for 4 d. All animals were food deprived daily for 3 hr and two mice received a saline injection, repeated for three days (*controls*). A third animal received a daily saline injection repeated for two days and an insulin injection on the third day (*single hypoglycemic episode*). The fourth animal received a daily insulin injection (2.5 U/kg), repeated for three days (*recurrent hypoglycemic episodes*). Blood glucose was monitored before, and 1 hr after each injection *via* a tail prick. Food was returned to the cage immediately after the final daily blood glucose measurement. On the fourth day, food was removed and all animals were fasted for 16 hr. One animal then received a final saline injection (saline control). The remaining three animals were injected with pyruvate (2 g/kg). Blood glucose was monitored *via* tail prick at 0, 30, 60, 90 and 120 min after injection. For each cohort the change in blood glucose levels in response to pyruvate was calculated after subtraction of the corresponding value in the saline control mouse (to compensate for any injection-induced changes in blood glucose levels). One animal (wild type, single hypoglycemic treatment) did not respond to insulin and this data was removed (Figure 4E,G). In Figure 4, n corresponds to the number of animals in *each* treatment group.

Chromaffin cell culture. Paired experimental and control animals were sacrificed by decapitation, the adrenals were removed and chromaffin cells were isolated as described (10). In brief, the adrenal medulla was incubated in Hank's balanced salt solution (HBSS) containing 1 mg / ml collagenase type IA and 6 mg / ml BSA for 15 min at 37 °C, then transferred to HBSS containing 1 mg / ml trypsin and 6 mg / ml BSA for 30 min at 37 °C. Tissue was mechanically dispersed and dissociated cells were centrifuged at 1,000 rpm for 3.5 min. The pellet was resuspended with culture medium (DMEM containing 5.5 mM glucose and 10% FCS). Cells were plated on culture dishes or coverslips coated with poly-D-lysine and maintained at 37 °C in a humidified atmosphere of 95% air and 5% CO_2.

Measurement of liver glycogen. Tissue samples were removed, frozen on dry ice and stored at -80 °C until use. Liver glycogen was hydrolyzed using 2M HCl and glucose levels were measured using the AmplexRed Glucose Assay kit (Invitrogen). For quantification of liver glycogen levels using Periodic acid -

Schiff staining (PAS), 30 µm cryosections were prepared as described below for adrenal tissue. Sections were washed with PBS, incubated in 0.5% periodic acid for 20 mins, washed with distilled water and incubated in Schiff's reagent (Sigma) for 20 mins. Sections were subsequently mounted in a glycerol-based mounting medium.

Immunohistochemistry. Adrenal glands were isolated, briefly washed with PBS and fixed overnight at room temperature in 4% paraformaldehyde. In later experiments (caspase 3- and cre-ir), glands were fixed at 4 °C for 60 min which resulted in improved staining. Glands were then snap frozen in 2-methylbutane on dry ice, embedded in cryomatrix and 30 µm cryosections were prepared. In each case immunoreactivity was ultimately revealed using diaminobenzidine (ImmPACT DAB, Vector Laboratories).

PCNA immunoreactivity: cryosections were washed in PBS then subjected to citrate-based antigen retrieval (10 mM sodium citrate / 0.05% Tween 20, pH 6.0) by boiling at 100 °C for 5 min then cooled to room temperature for 20 min followed by a 5 min wash in PBS. Sections were then incubated in rabbit anti-PCNA (1:50,000; Cell Signaling Technology; #13110) overnight at 4 °C in PBS containing 0.3% Triton X-100 / 1% IgG-free BSA, then washed and incubated for 1 hr at room temperature in anti-rabbit HRP (ImmPRESS system; Vector Laboratories).

Caspase-3 and Cre immunoreactivity: adrenal glands were incubated in Tris-EDTA antigen retrieval buffer (10 mM Tris Base / 1 mM EDTA / 0.05% Tween 20, pH 9.0) for 24 hr at 4°C, then boiled for 20 minutes at 100 °C. The glands were immediately washed in PBS at room temperature and transferred to PBS / 30% sucrose for 24 hr at 4 °C. Cryosections were prepared and incubated overnight at 4 °C in primary antibody (1:2,000 rabbit anti-cleaved caspase-3; Cell Signaling Technology; #9664 or 1:500 rabbit anti-Cre; Cell Signaling Technology; #15036) in PBS / 0.3% Triton / 1% IgG-free BSA, then washed and incubated in anti-rabbit HRP.

Immunofluorescence. Adrenal glands were isolated, washed with PBS then fixed overnight at room temperature in 4% paraformaldehyde. Cryosections were prepared, permeabilized (PBS / 0.3% Triton X-100 / 5% IgG-free BSA) for 2 hr then transferred to PBS / 50 mM glycine for 30 min before incubation in blocking solution (PBS / 0.05% Triton X-100 / 5% IgG-free BSA) for 30 min. Sections were incubated overnight at 4 °C in primary antibody (1:1,000 rabbit anti-TH (Millipore; #ab152); 1:10,000 rabbit anti-NPY (Peninsula labs; T4070) or 1:5,000 rabbit anti-HA (Cell Signaling Technology; #3724) then washed with PBS and incubated in secondary antibody (1:200 donkey anti-rabbit Dylight 488; Jackson ImmunoResearch) for 90 min at room temperature and mounted in Vectashield. In some experiments staining was revealed using tyramine signal amplification (TSA) as previously described (10).

Immunocytochemistry. Chromaffin cells were fixed in PBS containing 4% paraformaldehyde for 20 min at room temperature, then stained as described (10). In brief, cells were permeabilized for 15 mins in 0.3% Triton in PBS, then blocked (PBS / 0.25% IgG-free BSA) for 30 mins and incubated overnight at 4 °C in primary antibody. After washing with PBS, the cells were incubated in secondary antibody for 90 mins at room temperature, then washed with PBS and mounted in Vectashield. Primary antibodies were rabbit anti-TH; 1:200; Cell Signaling Technology; #2792, and guinea pig anti-PNMT; 1:100; Acris;

EUD7001). Secondary antibodies were donkey anti-rabbit DyLight 488 and donkey anti-guinea pig FITC (both 1:100; Jackson ImmunoResearch).

Image analysis. Images were obtained using a Nikon TE2000U microscope, 4x, 10x and 60x oil-immersion (1.4 numerical aperture) objectives, a Retiga 1300 monochrome camera and Image-Pro Plus 5.1 (Media Cybernetics) or NIS-Elements (Nikon) software. ImageJ (Rasband WS, ImageJ, US NIH, Bethesda, Maryland, USA, http://imagej.nih.gov/ij/, 1997-2016) was used for most analysis. To quantify staining in slices an ROI was drawn around the medulla (minus any cell-free areas) and the mean pixel intensity was calculated. In most experiments > 8 sections from each animal were used to calculate the mean. All slides were blinded until the analysis was complete. In one experiment (Figure 2A-C) both indirect immunofluorescence (n = 2) and TSA methods (n = 3) were used. Because this resulted in different basal levels of immunoreactivity, the data was normalized by the total immunoreactive signal in each independent replicate (n = 5).

Carbon fiber amperometry. Recording of amperometric events from chromaffin cells *in vitro* was made as previously described (10, 11) with the exception that catecholamine release was triggered optogenetically. This approach was chosen as a non-invasive way to evoke release and because we found the response to puffed application of AChR agonists varied with time in culture. In brief, TH-ChR(tdTomato) male mice were exposed to the three protocols described above (*control*: three saline injections, *insulin-induced hypoglycemia*: two saline injections and one insulin injection; *recurrent hypoglycemia*: three insulin injections), then euthanized 24 hrs after the final injection. Adrenal glands were isolated and chromaffin cells were plated on poly-d-lysine coated dishes. Amperometric recordings were made from single chromaffin cells within 12 hrs after isolation *in vitro*. Recordings were made with carbon fiber electrodes that were coated with Sylgard and mounted on the headstage of a Multiclamp 700A amplifier (Molecular Devices). Electrodes were held at 700 mV and current was sampled at 5 kHz and filtered at 2 kHz. During an experiment the cells were superfused with extracellular solution (in mM: 135 NaCl, 3 KCl, 2 $CaCl_2$, 1 $MgCl_2$, 10 HEPES, and 5.5 glucose, pH 7.3, with NaOH) at room temperature. Catecholamine secretion was evoked by brief exposure to 470 nm light from a fiber-coupled led (Thorlabs) that was placed in the bath solution close to the recorded cell. Light exposure was triggered by TTL pulses from a Digidata 1322A. Secretion was triggered by two 20 ms flashes separated by 50 ms repeated 200 times with a 1 s interval. All recorded cells were silent before optogenetic stimulation. To reduce electrode fouling, after each recording the carbon fiber was held at 700 mV for 500 ms, then stepped to -700 mV for 500 ms at a frequency of 1 Hz for 2 minutes. To maintain consistency between experiments, ≥20 cells were recorded from each animal. The amplitude of amperometric events was analyzed manually using Clampfit 10.4 (Molecular Devices). Only non-overlapping events with a rapid rising phase were selected and only cells with at least 10 amperometric events during the recording period were analyzed. All electrophysiological studies were performed blind with respect to the experimental treatment until the analysis was complete.

***In situ* slice electrophysiology.** Slices (200 µm) of adrenal gland were prepared as previously described (7). Slices were superfused with ACSF (130 mM NaCl, 2.5 mM KCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$, 1.25 mM NaH_2PO_4, 26 mM $NaHCO_3$, and 5 mM glucose, pH 7.4, saturated with 95% O_2/5% CO_2). On-cell recording of chromaffin cell activity was made at a holding voltage of 0 mV with patch pipettes (filled with ACSF) in

the loose seal configuration. Seal resistance was typically < 30 MΩ. CNO (10 μM) was applied to the slice *via* a gravity fed superfusion system.

Statistical tests. Comparisons between groups that contained normally distributed data (assessed using the Shapiro-Wilk test) were made using ANOVA (Tukey post hoc) or Student's t test. The Wilcoxon rank sum test or the Kruskal-Wallis rank sum test was used for non-normal data and the Kolmogorov-Smirnov test was used to analyze cumulative distributions. R (3.3.1; GUI Rcmdr 2.2-5), Clampfit and OriginPro7 were used for analysis. All values are mean ± SEM and $P < 0.05$ was considered significant.

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