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Supplemental Information

**CRFR1 in AgRP Neurons Modulates
Sympathetic Nervous System Activity
to Adapt to Cold Stress and Fasting**

Yael Kuperman, Meira Weiss, Julien Dine, Katy Staikin, Ofra Golani, Assaf Ramot, Tali Nahum, Claudia Kühne, Yair Shemesh, Wolfgang Wurst, Alon Harmelin, Jan M. Deussing, Matthias Eder, and Alon Chen

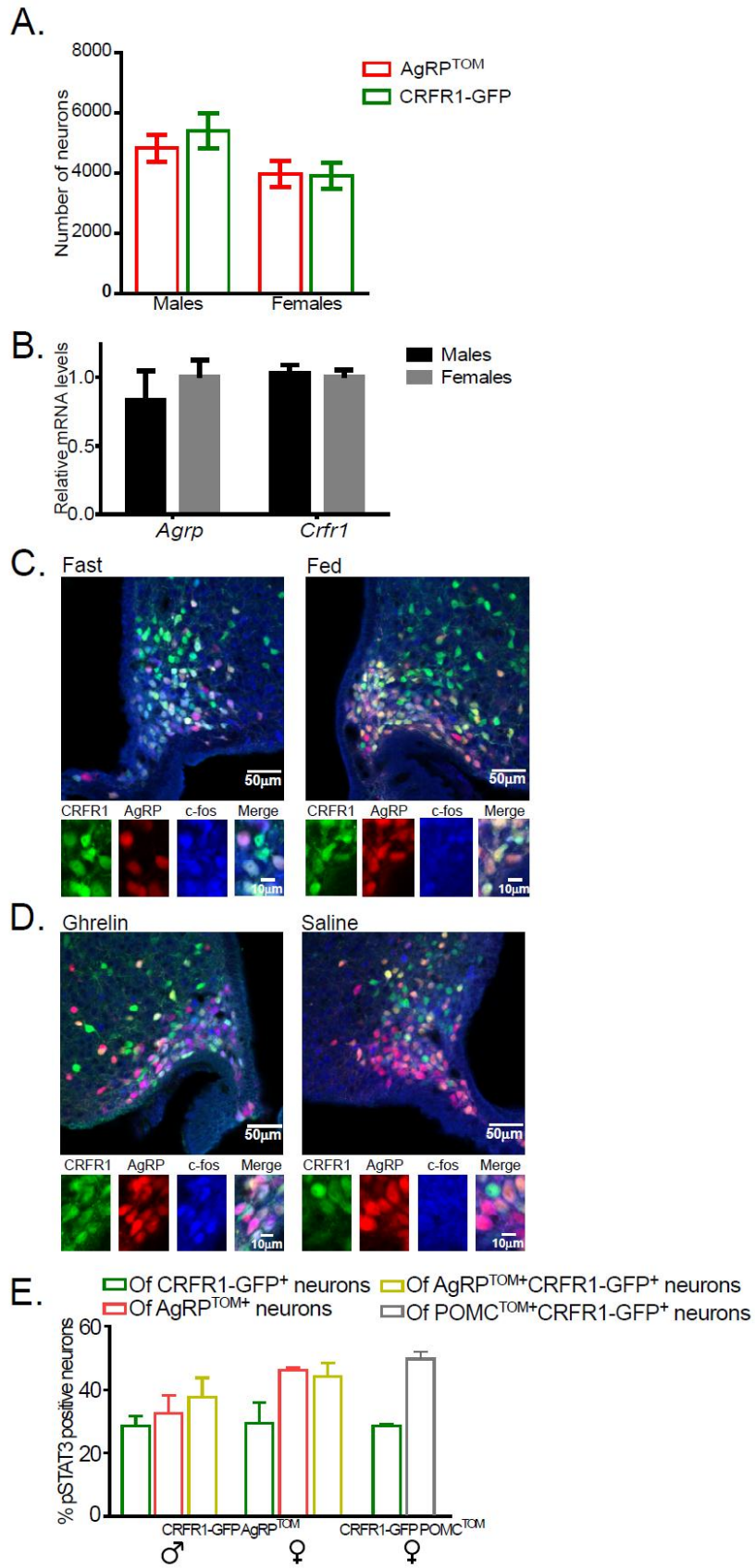


Figure S1. Characterization of AgRP⁺CRFR1⁺ neurons. Related to Figure 1.

- A. Number of CRFR1-GFP and AgRP^{TOM} neurons in males and females *CRFR1-GFP-AGRP^{TOM}* mice (n=7 for each sex).
- B. Relative *Crf1* and *Agrp* expression in the MBH of WT ICR mice (n=9 for each sex).
- C. Immunostaining of c-Fos in the Arc of fed and overnight-fasted *CRFR1-GFP-AGRP^{TOM}* reporter mice.
- D. Immunostaining of c-Fos in the Arc of ghrelin or saline injected *CRFR1-GFP-AGRP^{TOM}* reporter mice.
- E. Percentage of pSTAT3 positive neurons out of CRFR1-GFP⁺/AgRP^{TOM+}/CRFR1-GFP-AgRP^{TOM+} neurons in males and females *CRFR1-GFP-AGRP^{TOM}* mice (n=3 for each sex) and in female *CRFR1-GFP-POMC^{TOM}* mice (n=2).

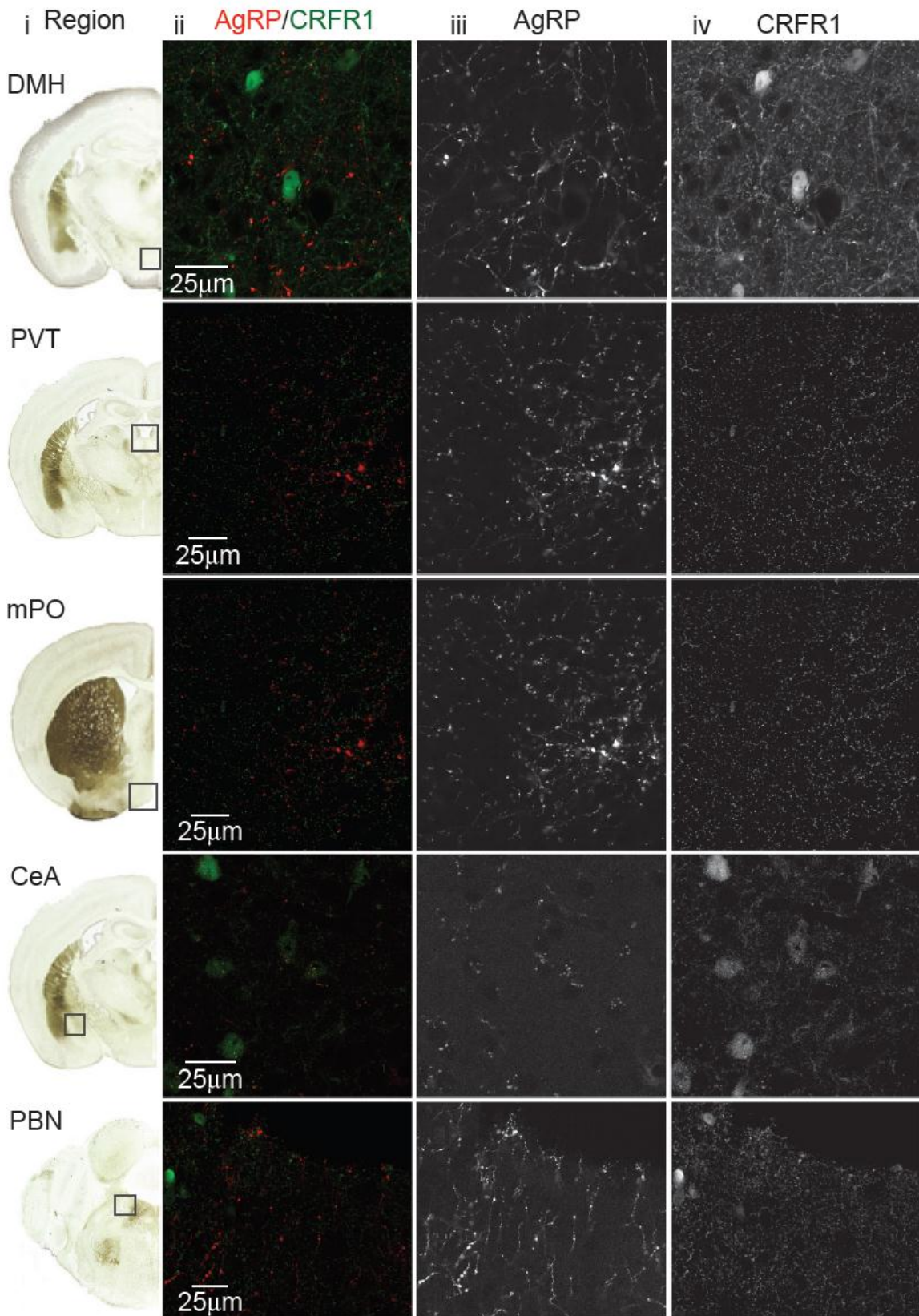


Figure S2. Projection mapping of AgRP⁺CRFR1⁺ neurons. Related to Figure 3.

Nine known AGRP neuron projection fields were analyzed for AgRP⁺GFP⁺ projections.

(i) Brain coronal section adapted from the Paxinos & Franklin mouse brain atlas (Paxinos and Franklin, 2001), black box indicates region analyzed Black box indicates region analyzed. (ii)

Overlaid images of AgRP^{TOM} fluorescence and GFP immunoreactivity. (iii) Tomato

fluorescence reveals AgRP projections. (iv) GFP immunoreactivity in each region. See also

Figure 3.

DMH- dorsomedial hypothalamus, PVT- paraventricular nucleus of thalamus, mPO- medial preoptic area, CeA- central amygdala, PBN- parabrachial nucleus. Scale bar, 25 μ m.

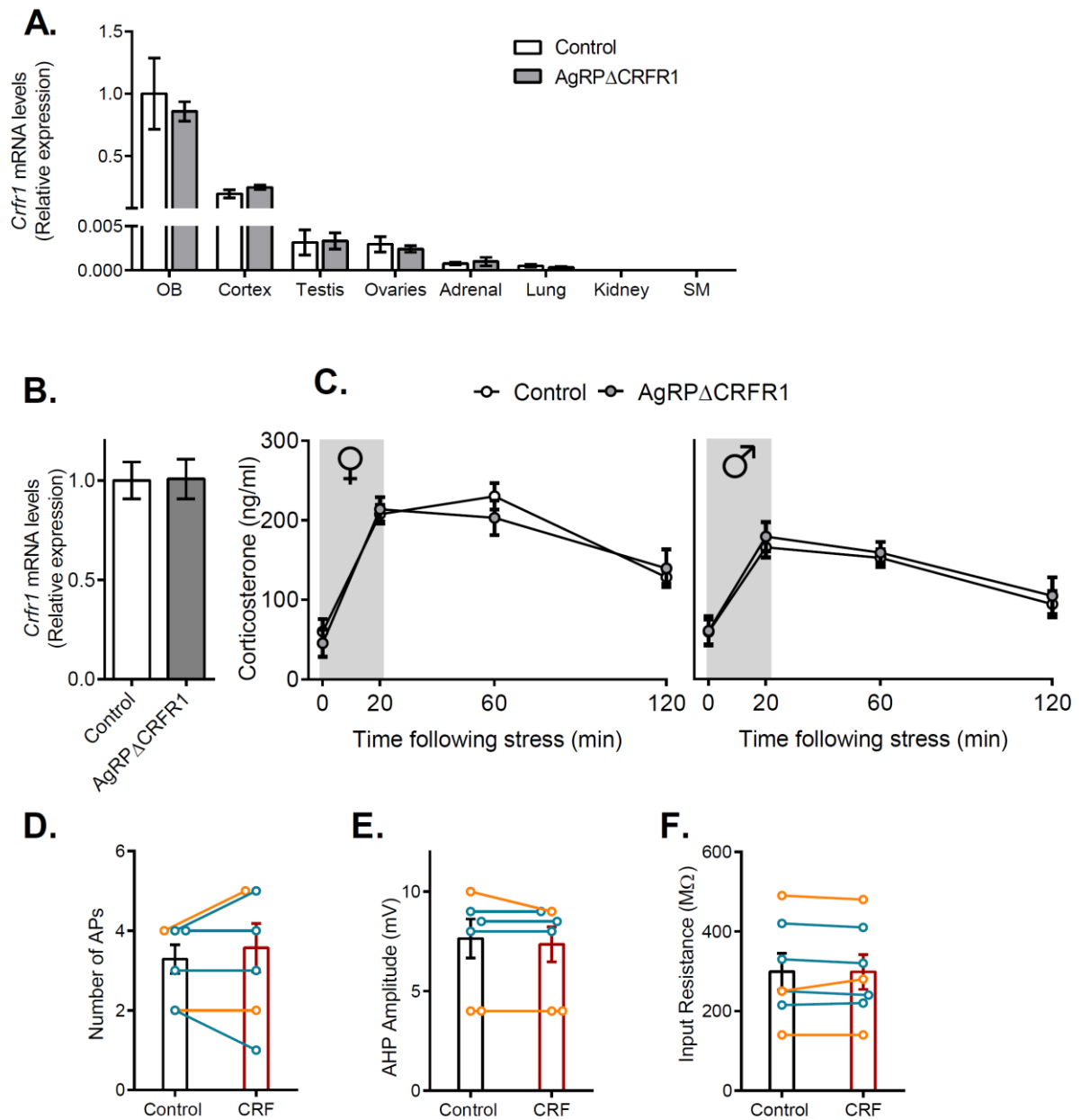


Figure S3. Unaltered HPA function in AgRP Δ CRFR1 mice. Related to Figure 4.

A. *Crfr1* expression in several brain regions and peripheral tissues in AgRP Δ CRFR1 mice and their control littermates (n=6).

B. Pituitary *Crfr1* expression in AgRP Δ CRFR1 mice and their control littermates (n=5-6).

Data are shown as mean \pm SEM.

C. Basal and stress induced HPA function in females (left; n=8) and males (right; n=8-9) AgRP Δ CRFR1 mice. Gray rectangle represents restraint time. Data are shown as mean \pm SEM.

D-F. CRF has no effect on AgRP neurons which do not express CRFR1. All parameters tested, firing rate (D), AHP amplitude (E), and R_{in} (F) remain constant in the presence of CRF (125nM; n=7 neurons). Each single experiment is represented with blue (female) or orange (male) circles and the histogram the mean \pm SEM.

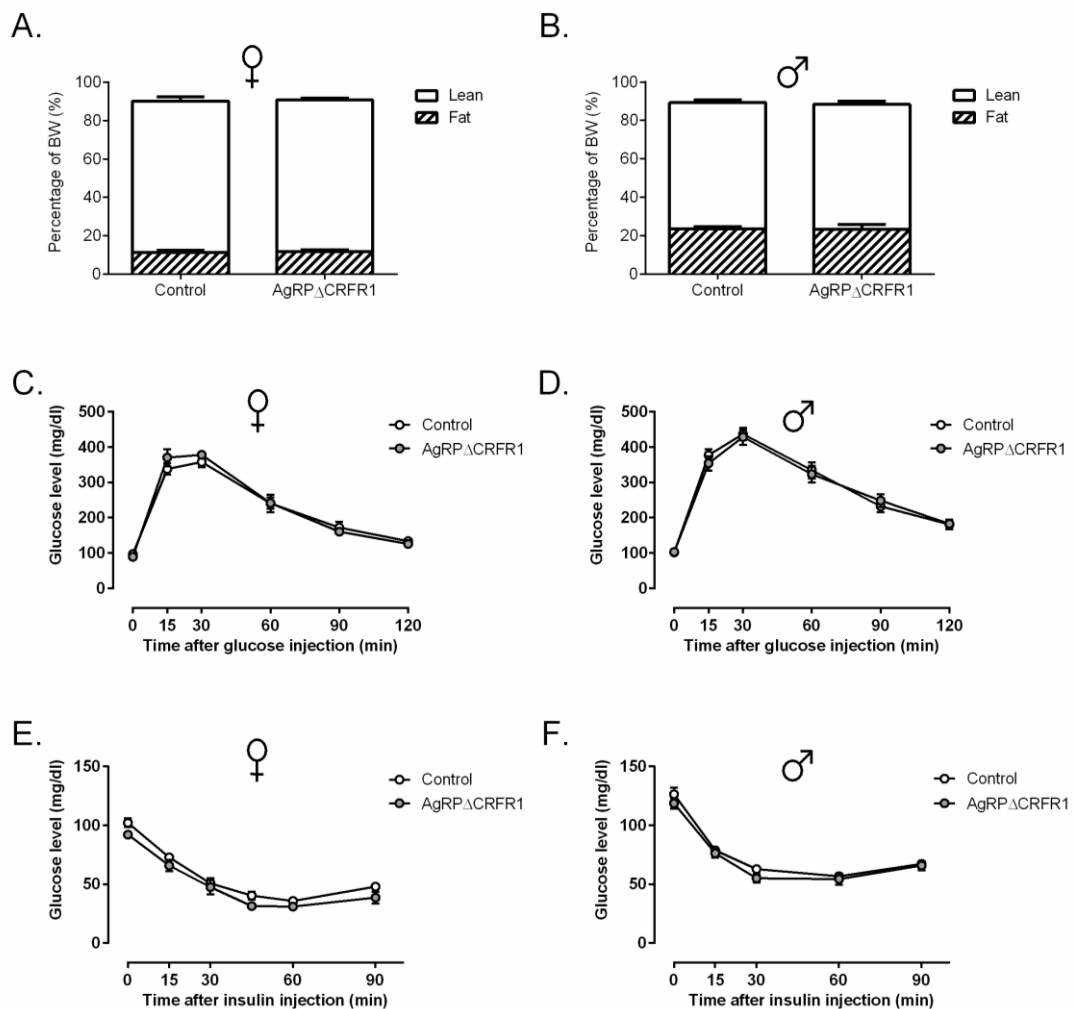


Figure S4. Unaltered body composition and glucose homeostasis in AgRP Δ CRFR1 mice.

Related to Figure 5.

A,B Body composition of females (A; n=6) and males (B; n=6-9) AgRP Δ CRFR1 mice.

C-F Glucose and insulin tolerance tests in females (C,E; n=10, n=5-6) and males (D,F n=14-15; n=12-14) AgRP Δ CRFR1 mice.

Data are shown as mean \pm SEM.

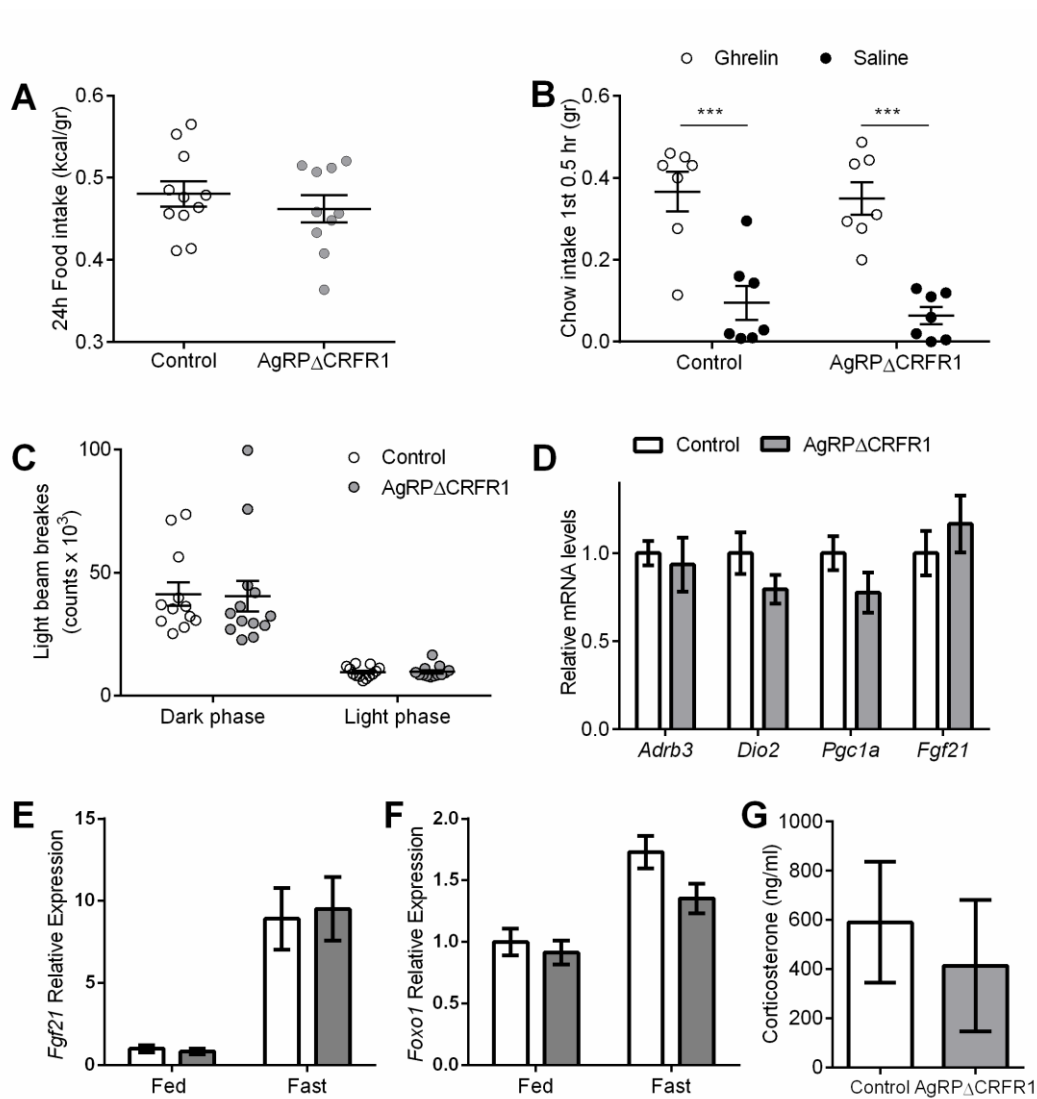


Figure S5. Phenotypes of AgRP Δ CRFR1 female mice. Related to Figures 5 and 6.

A. Locomotor activity (n=11-13)

B. Food intake (n=10-11)

C. Food intake in 30min following ghrelin or saline injection (n=7)

D. Relative expression of selected genes in the BAT following exposure to 5°C (n=6-8).

E. Hepatic *Fgf21* relative expression at fast and fed state (n=7).

F. Hepatic *Foxo1* relative expression at fast and fed state (n=7).

G. Fasting corticosterone levels (n=4-5).

Data are shown as mean \pm SEM. *p < 0.05.

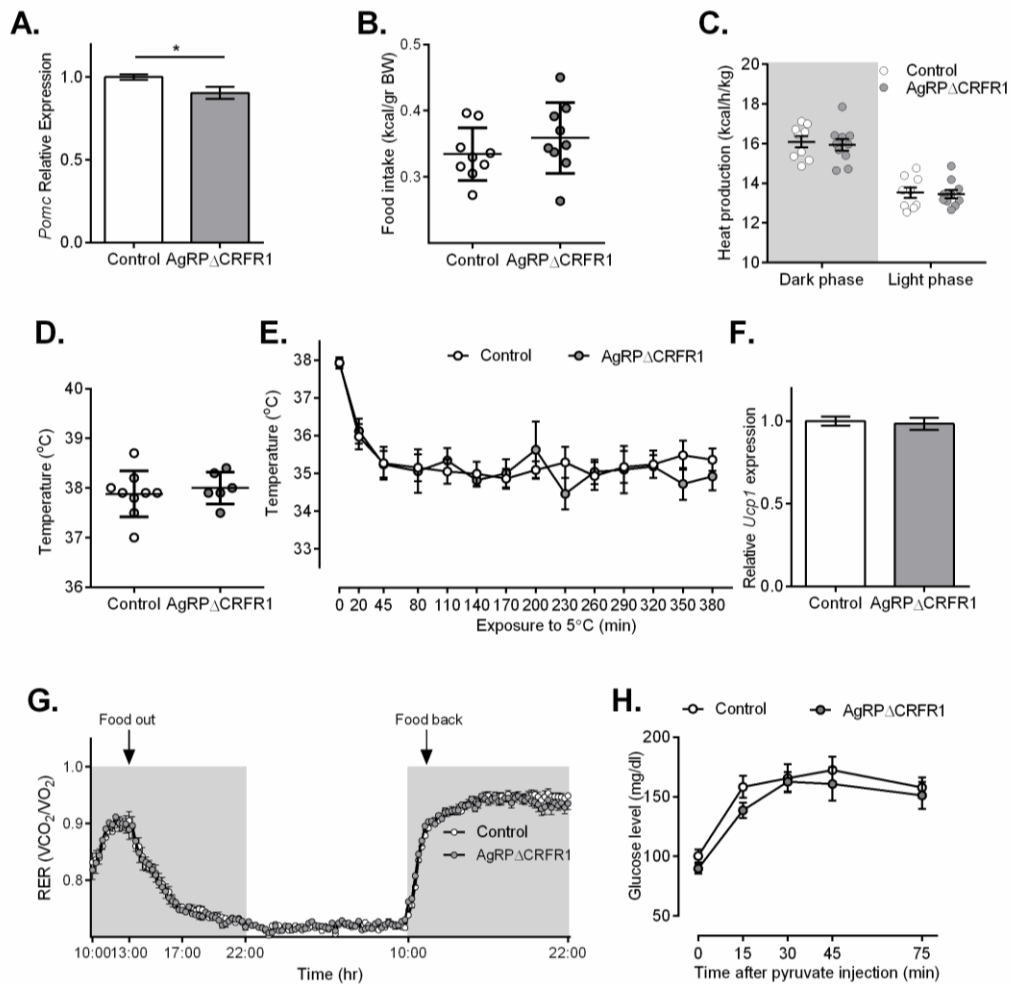


Figure S6. Phenotypes of AgRP Δ CRFR1 male mice. Related to Figures 5 and 6

A. Relative *Pomc* expression in the mediobasal hypothalamus (n=5-6)

B. Food intake (n=9)

C. Heat production (n=9-10)

D. Body temperature (n=6-9).

E. Body temperature during cold challenge test (n=5-10).

F. Relative BAT *Ucp1* expression following exposure to 7h in 5°C (n=6-7).

G. RER kinetics during food deprivation and refeeding (n=11-13).

H. Glucose levels following pyruvate injection (2gr/kg; n=6-9)

Data are shown as mean \pm SEM. *p < 0.05.

Supplemental Experimental procedures

Mice

CRFR1-GFP mice were kindly provided by the late Prof. Wylie Vale, Salk Institute, CA, USA. RIP-Cre mice (Postic et al., 1999) were kindly provided by Dr. Eran Hornstein, Weizmann Institute of Science, Israel.

The following lines were purchased from Jackson Laboratory (Bar Harbor, ME, USA):

AgRP-IRES-Cre (Stock# 012899), POMC-Cre (Stock# 005965), CRF-IRES-Cre (Stock# 012704) and tdTomato reporter (Stock# 007909). To generate AgRP-conditional CRFR1 knockouts, AgRP-IRES-Cre mice had been backcrossed for four generations on a C57BL/6 background and then intercrossed with CRFR1^{loxP/loxP} mice in which two loxP sites flank the second exon (Kuhne et al., 2012). Both experimental and control groups carried 2 floxed CRFR1 alleles, while only the experimental group carried the Cre allele. The genotyping reaction included the multiplex primer (Kuhne et al., 2012) which allows the detection of

AgRP-CRFR1^{-/-} mice that show somatic recombination owing to stochastic embryonic expression of AgRP-IRES-Cre. These mice were excluded. Primers sequence will be sent upon request.

Stereotaxic injections

For immunostaining of secreted neuropeptides located in the axons terminals, mice were injected with colchicine which inhibits microtubule polymerization resulting in concentration of neuropeptides in the cell body. Colchicine (2µl of 1µg/µl) was injected to the lateral ventricles (injection coordinates: AP-0.22mm; ML+0.95mm; DV-2.2mm). Mice were sacrificed by perfusion when locomotor symptoms were observed.

For observation of synapses originated from PVN-CRF neurons, 300nl AAV9-CMV-Flex-synaptophysin-mCherry viral vector (McGovern Institute for Brain Research at MIT) were injected bilaterally into the PVN of *CRF-Cre* mice crossed with *CRFR1-GFP* mice (injection coordinates: AP-0.9mm; ML±0.3mm; DV-4.5mm). Mice were perfused 1 month following the injection.

Electrophysiology

Adult (6-to10 week-old) male and female mice were anaesthetized with isoflurane (Abbott, Abbott Park, IL, USA) and decapitated. The brain was gently removed from the skull and chilled in ice-cold choline chloride-based cutting solution containing (in mM): 120 choline chloride, 3 KCl, 27 NaHCO₃, 2 MgCl₂, 17 D-glucose, pH 7.4, saturated with carbogen (95% O₂/5% CO₂). The brain was trimmed in a large block containing the hypothalamus and afterwards sliced using a vibratome (HM650V, ThermoFisher Scientific, Waltham, MA). 300-µm-thick coronal slices were cut through the full extent of the arcuate nucleus/lateral hypothalamus. Slices were incubated for 45 min at 34°C in artificial cerebrospinal fluid

(ACSF), containing in mM: 125 NaCl, 2.5 KCl, 1.25 NaH₂PO₄, 25 NaHCO₃, 1 MgCl₂, 2 CaCl₂, and 25 Glucose, pH 7.3, saturated with carbogen. Subsequently slices were maintained in ACSF at room temperature (23–25 °C) for at least 60 min prior to patch-clamp recordings. ACSF contained 50 μM APV and 5 μM NBQX to block ionotropic glutamate receptors.

All experiments were carried out at room temperature and slices were continuously superfused with ACSF containing 50 μM APV and 5 μM NBQX (4–5 ml/min). Neurons of the arcuate nucleus co-expressing AgRP-tdTomato and CRFR1-GFP, or AgRP-tdTomato alone, were visually identified by epifluorescence microscopy. After identification, the cell bodies of these neurons were visualized by infrared videomicroscopy and the gradient contrast system. Somatic whole-cell patch-clamp recordings (seal resistance >1 GΩ) were performed in bridge mode using a discontinuous single-electrode voltage-clamp amplifier (SEC-10L, npi electronics, Tamm, Germany). Only measurements of the access resistance (R_a) were done in voltage-clamp mode (holding potential -70 mV). The current/potential was low-pass filtered at 3 kHz, digitized at 9 kHz via an ITC-16 interface and stored with the standard software Pulse 8.31 (HEKA Elektronik, Lambrecht/Pfalz, Germany). The patch-clamp electrodes (open-tip resistance 4–5 MΩ) were pulled from borosilicate glass capillaries (Harvard Apparatus, Kent, UK) on a DMZ-Universal puller (Zeitz-Instruments, Munich, Germany) and filled with a solution consisting of (in mM): 130 K-gluconate, 5 NaCl, 2 MgCl₂, 5 Glucose, 10 HEPES, 0.5 EGTA, 2 Mg-ATP, 0.3 Na-GTP, 20 phosphocreatine, pH 7.3 with KOH, osmolarity 305 mOsm (all substances were from Sigma-Aldrich, St. Louis, MO).

5 min after reaching the whole-cell configuration, injection of 400-ms-long depolarizing current pulses was used to induce mild firing (1–6 action potentials) of the neurons. CRF (125 nM, Bachem, Bubendorf, Switzerland) was bath applied and the same current pulses were

applied again 15 min after starting CRF application. Due to CRF-independent fluctuations in the resting membrane potential (RMP), analysis of CRF effects on this parameter could not be performed. If such fluctuations occurred, we used continuous current injection to shift the RMP for the experiments in the presence of CRF to the control value.

Offline analysis was performed using the Pulse Software and statistical analysis with SigmaStat 3.5. Statistical comparisons were carried out using two-tailed paired t-tests with significance declared at $p < 0.05$.

RNA preparation and quantitative-PCR

RNA was extracted from hypothalamic, mediobasal hypothalami (MBH), livers, adrenal glands, BAT or iWAT as indicated using miRNAasy mini kit (Qiagen Inc., Valencia, CA) according to the manufacturer's recommendations. RNA was extracted from olfactory bulb, cortex, testis, ovaries, lungs and skeletal muscle using TRIzol reagent (Life Technologies Corp., Carlsbad, CA) according to the manufacturer's recommendations. RNA was reverse transcribed to generate cDNA using high capacity cDNA reverse transcription kit (Applied Biosystems Inc, Foster City, California). cDNA product corresponding to 10 ng RNA was used as templates for real-time PCR analysis.

Quantitative (q) PCR analysis was carried out using the StepOnePlus system using either SYBR Green or Taqman assays (Applied Biosystems, Life Technologies Corp., Carlsbad, CA). Primers were designed to span introns using Primer Express (Applied Biosystems, Carlsbad, CA). For the following TaqMan assays were used: mCRFR1—assay #Mm01240376_m1; mHPRT—assay #Mm01545399_m1. Primers sequence will be sent upon request.

In-situ hybridization

For detection of co-localization on single cell level, double ISH (DISH) was performed as previously described (Refojo et al., 2011). The following riboprobes were used: *Crfr1*, nucleotides 1728-2428 of GenBank accession no. NM_007762; *Gfp*, nucleotides 679-1396 of GenBank accession no. U55762. Specific DNA fragments coding for the riboprobes were generated by PCR applying T7 and T3 or SP6 primers using plasmids containing the above-mentioned cDNAs as templates. Antisense and sense cRNA probes were synthesized and labeled with ^{35}S UTP or dioxygenin (DIG) by in vitro transcription from 200 ng of respective PCR product used as templates. For DIG detection anti-DIG-POD (Fab) antibody was used 1:400. Tiramide-biotin signal amplification (TSA) was performed using the NEL700A Kit.

Glucose, insulin and pyruvate tolerance tests

GTT and ITT were performed as previously described (Chen et al., 2006). Briefly, following 5.5 h of fasting, glucose (2 g/kg body weight) or insulin (0.75 units/kg bodyweight, Sigma-Aldrich Co, St. Louis, MO, USA) were injected i.p. and glucose levels were measured at the indicated time points using an automatic glucometer (Roche Diagnostics). For pyruvate tolerance test, mice were fasted for 16 h before pyruvate injection (2 g/kg, Sigma-Aldrich Co).

Body composition

Mice body composition was assessed using EchoMRI-100TM (Echo Medical Systems, Houston, TX, USA).

Ghrelin and leptin injections

For assessing leptin-induced STAT3 phosphorylation, food deprived mice were perfused 1 hour following i.p. leptin injection (3 μ g/gr; PLR Ltd, Rehovot, Israel). For assessing ghrelin-induced neuronal activation, mice were perfused 1 hour following i.p. ghrelin injection (3 μ g/gr; kindly provided by the late Prof. Wylie Vale, Salk Institute, CA, USA).

Corticosterone measurement

For the evaluation of the endocrine response to stress, tail blood samples were collected before (basal), immediately after 20 minutes of restraint stress, and 80 and 120 minutes after stress initiation. Restraint stress was induced using a 50-mL ventilated conical tube. Plasma samples were immediately centrifuged and stored at -80°C until assays were conducted.

Corticosterone concentrations were quantified using a corticosterone enzyme immunoassay kit (Cayman Chemical Co, Ann Harbor, Michigan, USA).

Ghrelin sensitivity test

Ghrelin (3 μ g/gr) was injected i.p. into fed mice during the light phase and food intake was measured during 30 minutes. As control, similar volume of saline was injected on the subsequent day.

Supplemental References

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