Regulatory role of AMP-activated protein kinase in corticotropin releasing hormone secreting neurons in the paraventricular hypothalamus in social stress-induced alteration of food selection behavior

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ABSTRACT

INTRODUCTION

Psychological stress stimulates intake of sweet foods or high carbohydrate diets. The paraventricular hypothalamus (PVH) plays a key role in macronutrient selection between carbohydrate and fat in mice and rats. Our laboratory recently revealed that AMP-activated protein kinase (AMPK) in a subset of corticotropin releasing hormone (CRH) expressing neurons in the PVH (PVH-CRH neurons) increased a high-carbohydrate diet (HCD) selection over a high-fat diet (HFD) selection in mice after overnight fasting or neuropeptide Y (NPY) injection into the PVH. CRH is involved in the regulation of stress-related behavior as well as adrenocorticotropic hormone (ACTH) secretion from pituitary and subsequent glucocorticoid secretion from adrenal gland [hypothalamic-pituitary-adrenal (HPA) axis]. However, the role of AMPK in PVH-CRH neurons in stress-induced change in food selection behavior remains elusive. The objective of present study is to unravel whether AMPK in PVH-CRH neurons regulates food selection behavior between a HCD and HFD in mice in response to stress.

METHODS

C57BL/6J mice were subjected to social defeat stress, which was composed of 5 min of

direct interaction and 24 h of sensory contact to an ICR mouse (as an aggressor). CRH mRNA expression in the PVH, plasma corticosterone concentration, and locomotor activity were measured as stress markers. Phosphorylation of AMPK was measured by immunoblot and immunohistochemical analysis. NPY mRNA expression in hypothalamic nuclei was examined by quantitative polymerase chain reaction.

Lentivirus expressing short hairpin RNA (shRNA) for CRH (shCRH) was injected into the PVH of C57BL/6J mice. Lentivirus expressing shRNA for α1- and α2 subunits of AMPK (shAMPKs$^{f/f}$) or constitutively active form of AMPK (CA-AMPK$^{f/f}$) in a Cre recombinase (Cre)-dependent manner was injected into the PVH of Cre-knock-in mice expressing Cre under the control of CRH gene promoter and enhancer (CRH-Cre mice). Adeno associated virus (AAV) expressing inhibitory DREADD (Designer Receptors Exclusively Activated by Designer Drugs), hM4Di, in CRH neurons was injected into the PVH of CRH-Cre mice. Neuropeptide Y (NPY) Y1 receptor (Y1R) selective antagonist BIBP3226 was injected into the PVH of C57BL/6J mice.

Mice were subjected to measurements of stress markers and food selection before and after social defeat stress. Food selection experiments were performed with a combination of a HFD (55% of calories from lard) and a HCD (HCD1: 57% of calories

from sucrose or HCD2: 50% of calories from starch). Constituents other than carbohydrate and fat were same among HCDs and HFD.

RESULTS

Social defeat stress increased CRH mRNA expression in the PVH and plasma corticosterone concentration, and decreased locomotor activity. All mice chose HFD over HCDs when fed ad libitum. In contrast, social defeat stress increased selection of HCDs (HCD1 or HCD2) and decreased that of HFD in two-diet choice experiment. Total calorie intake did not change. Inhibition of CRH expression in the PVH by shCRH blunted the enhancement of carbohydrate selection as well as the increased CRH mRNA expression and plasma corticosterone concentrations after social defeat stress. Specific inhibition of neuronal activity of PVH-CRH neurons by DREADD system also suppressed stress-induced increase in carbohydrate selection. Immunoblot and immnohistochemical analysis revealed that phosphorylation of AMPK in a subset of PVH-CRH neurons was increased by social defeat stress. Specific inhibition of AMPK expression in PVH-CRH neurons by shAMPKs$^{f/f}$ abolished the social defeat stress-induced change in food selection. In contrast, it did not affect change in CRH mRNA expression, plasma corticosterone concentration, or locomotor activity after social defeat stress. Expression of CA-AMPK$^{f/f}$ in PVH-CRH neurons

increased selection of HCD selection and decreased that of HFD, similar to those after social defeat stress. However, it did not affect stress markers: CRH mRNA expression, plasma corticosterone concentration, and locomotor activity. Social defeat stress significantly increased mRNA expression of NPY in the dorsomedial hypothalamus (DMH) but not arcuate hypothalamus. Administration of Y1R antagonist BIBP3226 into the PVH partially suppressed stress-induced carbohydrate selection.

CONCLUSIONS

A social defeat stress induces carbohydrate selection via AMPK activation in a subset of PVH-CRH neurons in C57BL/6J mice. AMPK activation in PVH-CRH neurons is necessary and sufficient for the alteration of food selection behavior after social defeat stress. Neural activation of PVH-CRH neurons and CRH expression in the PVH are also necessary for the social defeat stress-induced carbohydrate selection. In contrast, activation of HPA axis by social defeat stress is independent of AMPK activity in PVH-CRH neurons. Thus, AMPK in PVH-CRH neurons plays a crucial role in stress-induced carbohydrate selection, via a distinct mechanism for activation of HPA axis. NPY neurons in the DMH and Y1R in the PVH are likely involved in the social defeat stress-induced food selection behavior in part.

INTRODUCTION

Stress causes abnormal eating behaviors and often leads to an abnormal energy balance. Stress affects food intake in a bidirectional manner. It has been demonstrated that 30-40% of people eat more and 40-50% eat less on average during or after stressful events[1,2]. Animal studies reveal that severe physical stress such as restraint or immobilization stress decreases food intake[3,4], whereas mild psychological stress such as a chronic mild social defeat stress increases food intake[5–7]. Stress also has an effect on food selection behavior. Human studies show that intake of sweet foods or high carbohydrate diet is increased in response to psychological stress both in men and women[8–11].

The hypothalamus is responsible for the control of feeding behavior, and the paraventricular hypothalamus (PVH) plays a key role in macronutrient selection between carbohydrate and fat. Injection of an orexigenic peptide, neuropeptide Y (NPY), into the PVH preferentially stimulates carbohydrate intake[12,13]. In contrast, opioid signals in the PVH control fat intake but not carbohydrate intake[14]. Recently, our laboratory revealed that activation of corticotropin releasing hormone (CRH) expressing neurons in the PVH (PVH-CRH neurons) enhances carbohydrate intake and decreases fat intake in a two-diet choice experiment (in submission). The alteration of food selection after fasting was caused by the

activation of a subset of PVH-CRH neurons through activation of AMP-activated protein kinase (AMPK). NPY injection into the PVH was found to stimulate carbohydrate selection through activation of AMPK in PVH-CRH neurons.

CRH, a 41-amino acids peptide, plays an important role in adrenocorticotropin (ACTH) secretion from the pituitary and glucocorticoid secretion from adrenal gland [Hypothalamic-Pituitary-Adrenal (HPA) axis][15]. The HPA axis plays an important role in the stress-induced changes in whole body metabolism and immune function[16]. Ablation of CRH gene decreases plasma corticosterone levels before and after stress in mice[17]. CRH is also involved in stress-induced alterations of behavior. CRH modulates anxiety-related behavior by acting on limbic system through CRH receptor 1[18]. Chronic upregulation of CRH expression in the PVH is linked to stress-induced depression-like behavior[19]. CRH also has an anorexic effect in animals. Intracerebroventricular (i.c.v) injection of CRH decreases food intake in food-deprived rats[20]. However, the pathophysiological role of CRH in food intake remains unknown. CRH gene-ablated mice show normal food intake during ad libitum, and decrease it after restraint stress, similar to wild type mice[21].

AMPK is an evolutionarily conserved serine/threonine protein kinase. AMPK is activated in response to increasing intracellular AMP/ATP ratio and Thr172-

phosphorylation of catalytic α-subunit (α1 and α2) of AMPK. Activation of AMPK promotes catabolic pathways producing ATP such as fatty acid oxidation, glucose utilization, and mitochondrial biogenesis, whereas it inhibits anabolic pathways consuming ATP such as protein synthesis[22]. AMPK in mediobasal hypothalamus regulates food intake by responding to hormonal and nutrient signals[23]. Fasting increases AMPK activity in the hypothalamus, and refeeding suppresses it[23]. Furthermore, orexigenic peptides, such as NPY, agouti-related peptide (AgRP) and the stomach-derived hormone ghrelin, activate AMPK in the hypothalamus[24], whereas anorexigenic signals such as glucose, insulin, leptin, and α-melanocyte stimulating hormone suppress AMPK activity in the brain region[24].

The objective of my study is to unravel whether AMPK in PVH-CRH neurons regulates stress-induced change in food selection behavior. I subjected mice to social defeat stress[5–7] and examined alteration of food selection between a high carbohydrate diet (HCD) and high fat diet (HFD) in two-diet choice experiments. Previous studies showed that social defeat stress with short period of direct interaction with an aggressive mouse induce changes in stress markers such as suppression of locomotor activity[25,26] and increased mRNA expression of CRH in the PVH[27] and plasma corticosterone level[27,28]. My findings show that a social defeat stress induces carbohydrate selection via AMPK activation in a subset of

PVH-CRH neurons in C57BL/6J mice. AMPK activation in PVH-CRH neurons is necessary and sufficient for the alteration of food selection behavior after social defeat stress. Neural activation of PVH-CRH neurons and CRH expression in the PVH are also necessary for the social defeat stress-induced carbohydrate selection. In contrast, activation of HPA axis by social defeat stress is independent of AMPK activity in PVH-CRH neurons. Thus, AMPK in PVH-CRH neurons plays a crucial role in stress-induced carbohydrate selection, via a distinct mechanism for activation of HPA axis. NPY neurons in the dorsomedial hypothalamus (DMH) and NPY Y1 receptor (Y1R) in the PVH are likely involved in the social defeat stress-induced food selection behavior in part.

METHODS

Animals

Male C57BL/6J mice were obtained from Nihon SLC (Hamamatsu, Japan), and B6(Cg)-*Crh*$^{tm1(cre)Zjh}$/J knock-in mice (CRH-Cre mice) were from Jackson Laboratories (Bar harbor, ME). Cre recombinase (Cre) was expressed under the control of endogenous *Crh* promoter/enhancer elements, due to the gene insertion of internal ribosome entry site and *Cre* cassette at the 3' UTR of CRH gene[29]. CRH-Cre mice were genotyped by polymerase chain reaction (PCR) for the knock-in cassette of genome DNA. Male C57BL/6J and CRH-Cre mice were studied at 10 to 20 weeks of age, with lentivirus or adeno associated virus (AAV) infected mice being studied at 4 weeks after infection. Mice were housed individually in plastic cages at 24˚ ± 1˚C and with lights on from 0600 to 1800 hours, and they were provided with water and laboratory chow diet (LCD) ad libitum (CE-2; CLEA, Tokyo, Japan). Male ICR mice were obtained from Nihon SLC and used as an aggressor in social defeat stress, as described below. All animal experiments were performed in accordance with institutional guidelines for the care and handling of experimental animals, and were approved by the Institutional Animal Care and Use Committee of the National Institutes of Natural Sciences.

Cannula implantation

Mice were sedated by subcutaneous injection of buprenorphine (0.1 mg/kg, Lepetan; Otsuka Pharmaceutical, Tokyo, Japan), and anesthetized with intraperitoneal (i.p.) injection of ketamine (100 mg/kg, Ketalar; Daiichi Sankyo Propharma Co., Ltd., Tokyo, Japan) and xylazine (10 mg/kg, Bachem AG, Bubendorf, Switzerland). A chronic double-walled stainless steel cannula (Unique Medical Co., Osaka, Japan) was implanted stereotaxically and bilaterally into the PVH with the use of a Kopf stereotaxic frame (David Kopf Instruments, Tujunga, CA) and according to the atlas of Paxinos and Franklin[30]. The stereotaxic coordinates for the PVH were 0.7 mm posterior, 0.5 mm lateral, and 4.7 mm below to the bregma. Cannulas were anchored firmly to the skull with acrylic dental cement. Buprenorphine (0.1mg/kg) was injected subcutaneously on the next day of surgery. Correct placement of the cannula tips was verified microscopically in brain sections.

Virus production

For expression of short hairpin RNA (shRNA) coding a sequence for mouse CRH under the control of the mouse U6 gene promoter (shCRH) were inserted into pFUGW (kindly provided by P. Osten, Cold Spring Harbor Laboratory). shRNA coding a sequence for mouse

α1- and α2AMPK (shAMPKs$^{f/f}$) was introduced into pSico (Addgene, Cambridge, MA) to be expressed in Cre-dependent manner. Those shRNA coding sequences were as follows: CRH, 5'-GATCTTTgcatttagcacacaagtaaTTCAAGAGAttacttgtgtgctaaatgcTTTTTTACGCGTC-3'; α1- and α2AMPK, 5′-TgatgtcagatggtgaatttTTCAAGAGAaaattcaccatctgacatcTTTTTTC-3′.

Constitutively active form of AMPK (CA-AMPK) was described previously[31]. The mutant protein is a truncated form (residues 1 to 312) of the α1 subunit of rat AMPK that contains FLAG epitope inserted immediately after the initiating methionine and replacement of Thr172 with Asp (the phosphorylation site for AMPK kinases including calcium/calmodulin-dependent protein kinase kinase 2 and liver kinase B1). To ensure CRH neuron-specific expression of CA-AMPK, the coding sequence was placed in the Cre-inducible lentivirus vector with a synapsin 1 gene promoter based on pFSy(1.1)GW (CA-AMPK$^{f/f}$) (kindly provided by P. Osten, Cold Spring Harbor Laboratory).

Lentiviruses were produced by co-transfection of 293T fibroblasts with the transfer vectors encoding the protein or shRNA sequence of interest, the puromycin resistance gene, the HIV-1 packaging vector Δ8.9, and the gene for the envelope glycoprotein of vesicular stomatitis virus, according to the calcium phosphate protocol. Virus particles in culture

supernatants were collected by centrifugation at 6,000 × *g* for 16 to 18 h and then resuspended in phosphate buffered saline (PBS). All virus particles were applied to a Sepharose Q FF ion-exchange column (GE Healthcare Life Sciences, Pittsburgh, PA) in PBS and eluted with a linear gradient of 0 to 1.5 M NaCl. Elution was monitored by measurement of absorbance at 260 or 280 nm. The peak fractions containing the particles were collected and concentrated by centrifugation with the use of a ViraTrap Virus Purification Kit (Biomiga, San Diego, CA).

To examine the Cre-dependent expression of shRNA for α1- and α2 AMPK by pSico-based lentivirus, lentivirus expressing shAMPKs$^{f/f}$ was infected to mouse myoblastoma C2C12 cells. Infected cells were sorted with fluorescence of green fluorescent protein (GFP) by a cell sorter SH800 (Sony Corporation, Tokyo, Japan). Sorted GFP-positive cells were transfected with 3 μg of Cre expression plasmid based on pcDNA3.1 by Lipofectamine 2000 reagent (Life Technologies, Carlsbad, CA), and were subjected to real time quantitative PCR (RT-qPCR) analysis 48 h after the transfection.

Specific expression of inhibitory DREADD (Designer Receptors Exclusively Activated by Designer Drugs), hM4Di, in PVH-CRH neuron were performed with AAV vector [pAAV-hSyn-double floxed hM4Di-mCherry-WPRE (AAV-hM4Di$^{f/f}$), kindly

provided by B. Roth, University of North Carolina]. The AAV was produced by co-transfection of 293T fibroblasts with the transfer vector encoding the hM4Di-mCherry fused protein, the helper plasmid encoding the AAV-DJ *rep*/*cap* genes, and the plasmid encoding adenoviral helper genes, by calcium phosphate co-precipitation. The crude viral lysate was purified by CsCl density gradients two times and titrated with RT-qPCR, as described previously[32].

Purifications of lentivirus and AAV were performed by Dr. K. Kobayashi at the Section of Viral Vector Development in National Institute for Physiological Sciences (Okazaki, Aichi, Japan). All recombinant DNA experiments including the production of viral vectors were approved by the relevant committee of the National Institute for Physiological Sciences, and were performed under biosafety level 2 containment for lentivirus and level 1 for AAV.

Viral infection of the PVH

C57BL/6J mice and CRH-Cre mice pre-implanted a bilateral guide cannula in the PVH were anesthetized with i.p. injection of ketamine (100 mg/kg) and xylazine (10 mg/kg), and 500 nl of the virus stock suspension was infused at a flow rate of 50 nl/min bilaterally into the

PVH through an internal cannula projecting 1 mm below the tip of the pre-implanted guide cannula and with the use of an infusion pump (70-2208; Harvard Apparatus, Holliston, MA).

Lentivirus expressing shCRH or empty virus was injected into the PVH of C57BL/6J mice to produce shCRH and Empty vector mice, respectively. Lentivirus expressing shAMPKs$^{f/f}$ in a Cre-dependent manner or empty virus was injected into the PVH of CRH-Cre mice, to produce CRH-Cre:shAMPKs$^{f/f}$ and CRH-Cre:Empty vector mice, respectively. Lentivirus expressing CA-AMPK$^{f/f}$ or enhanced GFP (EGFP$^{f/f}$) in a Cre-dependent manner was also injected into the PVH of CRH-Cre mice, to produce CRH-Cre:CA-AMPK$^{f/f}$ and CRH-Cre:EGFP$^{f/f}$ mice, respectively. AAV-hM4Di$^{f/f}$ was injected into the PVH of CRH-Cre mice to produce CRH-Cre:hM4Di$^{f/f}$ mice.

Administration of agents into the PVH

Clozapine-N-oxide (CNO), activator of hM4Di, was infused into the PVH in conscious unrestrained CRH-Cre:hM4Di$^{f/f}$ mice (2 pmol in each side dissolved in 100 nl of saline). BIBP3226 (antagonist of Y1R) was infused into the PVH of C57BL/6J mice (20 pmol in each side dissolved in 100 nl of saline). These agents were infused for 2 min through an internal cannula projecting 1 mm below the tip of the pre-implanted guide cannula and with

the use of an infusion pomp (70-2208; Harvard Apparatus). Infusion was performed every 12 hours for CNO and 24 hours for BIBP3226, respectively, and continued from 1 h before social defeat stress to the end of food selection measurement (total 4 days). Same volume of physiological saline was infused in control mice.

Procedure for social defeat stress

An experimental mouse was introduced in the home cage (22×15×12 cm) of an ICR mouse (aggressor) for 5 minutes (Fig. 1A). All experimental mice displayed subordinate posturing within the 5 min-period. The cage was then divided into two equal compartments by a stainless steel partition, and animals were allowed to sensory contact in the same cage for 24 hours (Fig. 1A). Water and LCD were provided ad libitum during the sensory contact period. When experimental mice were observed any injury after physical interaction with ICR mice, they were omitted from the experiments. Control (non-stressed) as well as stressed mice were handled gently before experiments.

Measurement of food selection and locomotor activity

Food selection experiments were performed with the combination of HCD1 (D11071504M:

57% of calories from sucrose) and HFD (D12492: 55% of calories from lard), and that of HCD2 (D1171501: 50% of calories from starch) and HFD (D12492) (Table 1), respectively. Constituents other than carbohydrate and fat were same among HCDs and HFD (Table 1). LCD (Table 1) were purchased form CLEA (Tokyo, Japan) and others were from Research Diet (New Brunswick, NJ). Before experiments, mice were habituated to the monitoring cage for food selection during 2 days with LCD, and then habituated to HFD and HCDs for 1 day, respectively. Food selection and locomotor activity were measured with the use of multifaceted feeding and activity monitoring system (MFD-100M; Shinfactory, Fukuoka, Japan). Food selection and locomotor activity were monitored every minute for 2 days before social defeat stress and for 3 days after the stress, respectively (total 5 days) (Fig 1A). Daily calorie intake of foods and locomotor activity were determined with the cumulated data from 1800 to 1800 hours.

Isolation of medial hypothalamic nuclei

Medial hypothalamic nuclei were isolated as described previously[23]. Each region of the hypothalamus [PVH, arcuate hypothalamus (ARH), ventromedial hypothalamus (VMH), and DMH] and parietal cortex were dissected from 1 mm thick sagittal sections from the

midline of the fresh brain. Coordinates for each hypothalamic region are as follows[23]; PVH: square area with an anterior margin of the border with the posterior region of the anterior commissure, a dorsal margin of the border with the thalamus, a ventral margin 1mm ventral to the border with the thalamus, and a posterior margin of the white matter separating PVH from VMH/DMH; VMH plus DMH: triangular area with an anterior-dorsal margin of the white matter separating PVH and the anterior hypothalamus from VMH-DMH, a ventral margin of the border with ARH, and a posterior margin of the border with the mammillary body; ARH: the ventral portion of the medial hypothalamus with a dorsal margin of the border with the ventral part of VMH-DMH. The accuracy of dissection of hypothalamic nuclei was verified by mRNA expression of neuropeptides and transcriptional factor: CRH mRNA for PVH, steroid factor 1 (SF1) mRNA for VMH plus DMH, AgRP mRNA for ARH.

RNA extraction and RT-qPCR analysis

Total RNA was isolated from hypothalamic tissues with the Trizol reagent (Life Technologies). Portions of the RNA (150 ng) were subjected to reverse transcription (RT) with an oligo(dT) primer and avian myeloblastosis virus reverse transcriptase (Takara, Shiga, Japan). The mRNAs for CRH, SF-1, AgRP, EGFP, FLAG-tagged CA-AMPK and Cre were

detected by PCR with Ex Taq or LA Taq polymerase (Takara) and a Veriti 96-Well Thermal Cycler (Life technologies). The abundance of mRNAs for CRH, α1AMPK, α2AMPK, and NPY was determined by RT-qPCR analysis (StepOne Real-Time PCR system, Life technologies) with SYBR *Premix Ex Taq* (Takara). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA was used as an internal standard. PCR primers (Table 2) were obtained from Sigma Genosys (Ishikari, Japan) or Life Technologies.

Immunoblot analysis

Hypothalamic nuclei for immunoblot analysis were collected at 3 h of food selection with HFD and HCD1 after 24 h-social defeat stress (day 1). Before experiment, mice were habituated to HFD and HCD1 for 1 day, respectively. A group of mice was subjected to the social defeat stress (1800 to 1800 hours). Mice with or without social defeat stress were fasted from 1200 to 1800 hours, and subjected to food selection for HCD1 and HFD (1800 to 2100 hours). Mice were euthanized by cervical dislocation at 2100 hours. Hypothalamic nuclei were immediately collected as described above, and stored at -80 ˚C until analysis.

Hypothalamic nuclei were homogenized on ice in 20 mM Tris (pH 7.4), 5 mM EDTA (pH 8.0), 10 mM sodium pyrophosphate, 1% Triton X, phosphatase inhibitor

(Calbiochem, San Diego, CA) and proteinase inhibitor (Sigma-Aldrich, St. Louis, MO). The homogenates were centrifuged at 14,000 × *g* for 20 min at 4˚C, and the resulting supernatants were fractionated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (8 μg of protein). The separated proteins were transferred to a polyvinylidene difluoride membrane. The membrane was then exposed to 5% dried skim milk in 50 mM Tris-HCl (pH7.5), 150 mM NaCl and 0.1% Tween 20 (TBST) for 2 h, and incubated for 16 h at 4˚C in TBST containing 5% bovine serum albumin and primary antibodies including those for Thr172-phosphorylated AMPK (p-AMPK), total AMPK (t-AMPK), Ser79-phosphorylated acetyl-CoA carboxylase (ACC) (p-ACC), and total ACC (t-ACC) (Cat. 2535, 2532, 3661 and 3676, respectively; Cell Signaling Technology, Danvers, MA). Immune complexes were detected with horseradish peroxidase-conjugated secondary antibodies (Cat. SC-3837; Santa Cruz Biotechnology, Santa Cruz, CA) and enhanced chemiluminescence reagents (GE healthcare, Tokyo, Japan). Protein bands were captured with Ez-Capture MG (ATTO, Tokyo, Japan) and quantified using CS Analyzer 3.0 (ATTO).

Immunohistochemical and fluorescence analysis

To examine the expression of mCherry in PVH-CRH neurons of CRH-Cre mice, mice were

adrenalectomized and subjected to the immunohistochemical and fluoresence analysis. Mice were anesthetized with i.p. injection of ketamine (100 mg/kg) and xylazine (10 mg/kg), and perfused with 10 ml of PBS followed by 20 ml of fixative (4% paraformaldehyde in PBS). The brain was removed, trimmed, immersed for 3 h at 4˚C in the same fixative solution. The tissue was incubated in PBS containing 6.8% (w/v) sucrose for overnight, in PBS containing 18% (w/v) sucrose for 8 h, and then in PBS containing 30% (w/v) sucrose for overnight at 4˚C. The tissue was embedded in OCT compound (Sakura Finetechnical, Tokyo, Japan), immediately frozen in liquid nitrogen, and stored at −80˚C until analysis. Serial 20-μm-thick cryosections were prepared with a cryostat (Microm HM 500; Microm, Wolldorf, Germany), with the brain oriented for sectioning according to the mouse stereotaxic atlas[30]. The sections were treated with acetone for 20 min on ice and exposed to 5% normal goat serum (Santa Cruz Biotechnology) in PBS for 1 h at room temperature. The sections were then incubated with rabbit polyclonal antibodies for CRH (Cat. AB-02, 1:200 dilution; Advanced targeting systems, San Diego, CA) during overnight at 4˚C, and with Alexa 488-labeled goat antibodies for rabbit immunoglobulin G (IgG) (Cat. A11008, 1:200; Life Technologies) for 1 h at room temperature. The fluorescences of mCherry and Alexa 488 were detected with a fluorescence microscope (DMI4000B; Leica Microsystems,

Watzlar, Germany).

To examine p-AMPK in PVH neurons or PVH-CRH neurons, brain samples were prepared at 3 h of food selection experiment after 24 h-social defeat stress (day 1). Similar to that for immunoblot analysis of phosphorylation of AMPK and ACC, mice were habituated to HFD and HCD1 for 1 day, respectively. A group of mice was then subjected to the social defeat stress (1800 to 1800 hours). Mice with or without social defeat stress were fasted from 1200 to 1800 hours, and subjected to food selection for HCD1 and HFD (1800 to 2100 hours). Mice were then anesthetized with i.p. injection of ketamine (100 mg/kg) and xylazine (10 mg/kg) at 2100 hours, and perfused with 10 ml of PBS followed by 20 ml of fixative (4% paraformaldehyde in PBS).

Sections of the brain (6-μm-thick) were embedded in paraffin after fixation in 4% paraformaldehyde and oriented for sectioning according to the mouse stereotaxic atlas[30]. Sections were boiled with 10 mM sodium citrate buffer (pH 6.0) for 10 min, allowed to cool to room temperature, and exposed to 3% H_2O_2 for 10 min at room temperature in order to block endogenous peroxidase activity. Nonspecific staining was prevented by incubation of the sections with 5% normal goat serum (Santa Cruz Biotechnology) in TBST. The sections were incubated with rabbit monoclonal antibodies for p-AMPK (Cat. 2535; Cell Signaling

Technology) at a dilution of 1:50 in TBST containing 5% normal goat serum for overnight at 4˚C, and then with biotinylated secondary antibodies (Vectastain Elite ABC kit; Vector Laboratories, Burlingame, CA) for 1 h at room temperature. Immune complexes were detected with streptavidin-conjugated horseradish peroxidase (Vector Laboratories) and 3,3'-diaminobenzidine tetrahydrochloride (Sigma-Aldrich).

For immunohistochemical and fluorescence analysis of p-AMPK in PVH-CRH neurons, whole hypothalamus collected from CRH-Cre:hM4Di$^{f/f}$ mice was embedded in paraffin after fixation in 4% paraformaldehyde and oriented for sectioning. After rehydration, fluorescence of endogenous mCherry was detected and captured with a fluorescence microscope (DMI4000B; Leica Microsystems). Sections were then boiled with 10 mM sodium citrate buffer (pH 6.0) for 10 min, allowed to cool to room temperature, and incubated in 5% normal goat serum (Santa Cruz Biotechnology) in TBST. The sections were incubated with rabbit monoclonal antibodies for p-AMPK (Cat. 2535; Cell Signaling Technology) at a dilution of 1:50 in TBST containing 5% normal goat serum for overnight at 4˚C, and then with Alexa 488-labeled goat antibodies for rabbit IgG (Cat. A11008, 1:200; Life Technologies) for 1 h at room temperature.

Electrophysiology

To examine the effect of hM4Di DREADD system on neuronal activity of PVH-CRH neurons, slices of hypothalamus (300-μm-thick) were prepared from adult CRH-Cre:hM4Di$^{f/f}$ mice under deep anesthesia with isoflurane, and kept at 33˚C in normal artificial cerebrospinal fluid (ACSF) containing 5 mM glucose and constituents as follows[33]: 126 NaCl, 3 KCl, 1.3 $MgSO_4$, 2.4 $CaCl_2$, 1.2 NaH_2PO_4, 26 $NaHCO_3$ (mM). CRH-positive neurons labeled with mCherry in the PVH were targeted by patch pipettes under fluorescent and infrared differential interference contrast optics (BX51, Olympus, Tokyo, Japan). The patch pipettes (4-6 MΩ) were filled with an internal solution containing the following (mM): 130 K-gluconate, 8 KCl, 1 MgCl2, 0.6 EGTA, 10 HEPES, 3 MgATP, 0.5 Na2GTP 10 Na-phosphocreatine and 0.3% biocytin (pH 7.3 with KOH). Membrane potentials were recorded in the current-clamp mode using a Multiclamp 700B amplifier (Molecular Devices, Sunnyvale, CA). We selected cells with a high seal resistance (> 1 GΩ) and a low series resistance < 30 MΩ. Extracellular recordings were obtained in the cell-attached mode using patch pipettes filled with the modified ACSF. After minimally 5 min of stable recording, 1 μM CNO was bath applied while recording continued. The effect of CNO was assessed at 10-12 min after the bath application.

Measurement of plasma corticosterone concentration

To examine the changes in plasma concentration of corticosterone after social defeat stress, blood samples were collected under the following conditions: (1) after 5 minutes of physical interaction with an ICR mouse, (2) after 24 h of social defeat stress, and (3) without social defeat stress. The plasma corticosterone concentration was measured with a corticosterone enzyme immune assay kit (YK240; Yanaihara Institute Inc., Shizuoka, Japan).

Statistical analysis

Data are presented as means ± s.e.m. Statistical comparisons among multiple groups were performed by analysis of variance (ANOVA) followed by Tukey-Kramer's post hoc test. Statistical analysis between 2 groups was performed by unpaired or paired Student's *t* test (two-tailed). A P value of < 0.05 was considered statistically significant.

RESULTS

Changes in stress markers after social defeat stress

I first examined whether social defeat stress in this study changes stress markers in C57BL/6J mice. Locomotor activity was decreased on the next day after social defeat stress (day 1) (Fig. 1B). CRH mRNA expression in the PVH was significantly increased after 24 h of the sensory contact period (Fig. 1C). Consistent with previous reports[28], plasma concentration of corticosterone was increased after 5 min of physical interaction with ICR mice, and returned to the basal level after 24 h of sensory contact period (Fig. 1D). Thus, the social defeat stress activated HPA axis and suppressed locomotor activity.

Social defeat stress enhances carbohydrate selection

To examine whether social defeat stress changes food selection behavior, I conducted a two diet choice experiment with HFD and HCD1 (Table 1). Before social defeat stress, C57BL/6J mice chose HFD over HCD1. However, after 24 h of social defeat stress, these mice tended to increase selection of HCD1 on the next day (day 1), and significantly increased on day 2 (Fig 2A). HFD intake tended to decrease on day 2 after social defeat stress, but was not statistically significant. Cumulated intake of HCD1 for 2 days (day 1

plus day 2) was significantly increased and that of HFD were decreased after social defeat stress, compared with that before stress. Total calorie intake was not altered by social defeat stress (Fig. 2B). Fig 2C shows the time course of change in HCD1 intake after social defeat stress. Most of mice (10/11) increased selection of HCD1 after stress, but individual mouse changed food selection with different time course. I compared the maximal HCD1 intake during 3 days after social defeat stress with that before stress (day 2). Maximal HCD1 intake after stress increased more than double, compared with that before stress (Fig. 2D). In contrast, HFD intake on the same day was significantly decreased. Total calorie intake was not different before and after stress (Fig. 2E). Previous study showed that a subset of mice have a resilience from social defeat stress[19]. The susceptibility to social defeat stress may cause different time course of food selection. These results suggest that social defeat stress increases selection of a HCD over HFD in C57BL/6J mice.

To examine whether social defeat stress-induced change in carbohydrate selection is specific for the sucrose-based diet, HCD1, I examined food selection with different combination of foods: HFD versus HCD2 (50% of calories from starch, Table 1). Social defeat stress induced small but significant increase in cumulated HCD2 intake and decreased cumulated HFD intake (day 1 plus day 2) (Fig. 3A). Total calorie intake did not

change (Fig. 3B). All mice showed an increase in HCD2 selection after social defeat stress (Fig. 3C). Maximal HCD2 intake during 3 days after stress was approximately two-holds higher than that before stress (day 2) (Fig. 3D). HFD intake on the same day was significantly decreased (Fig. 3D). Total calorie intake did not change (Fig. 3E). These results suggest that social defeat stress increases carbohydrate intake and reduces fat intake in two-diet choice experiments.

I examined the amount of HFD and HCD1 intake after social defeat stress when each diet was presented alone. Social defeat stress did not change HCD1 intake (Fig. 4A). Calorie intake of HCD1 before and after stress was about 15 kcal/day, respectively, which was same to the total calorie intake in two-diet choice experiments (Fig. 2B and 3B). Thus, 15 kcal/day of HCD1 intake may be sufficient for carbohydrate intake after social defeat stress. In addition, when HFD was presented alone, intake of HFD was not different either with or without stress (Fig. 4B). HFD intake was increased after 24 h-feeding of LCD without stress, similar to that after stress (Fig. 4B). The results suggest that increased HFD intake when presented alone is due to the switching from LCD to HFD but not social defeat stress. Thus, social defeat stress enhances carbohydrate selection without change in total calorie intake or fat preference.

Inhibition of CRH expression in the PVH blunts stress-induced carbohydrate selection

I examined the effect of inhibition of CRH expression in the PVH on stress-induced alteration of food selection behavior. Expression of shCRH in the PVH of C57BL/6J mice (shCRH mice) decreased CRH mRNA expression by 50% in basal condition and to less than 50% after social defeat stress, compared with empty vector-infected control mice (Empty vector mice) (Fig. 5A). Suppression of CRH mRNA expression in the PVH decreased stress-induced increase in plasma corticosterone concentration after 5 min of physical interaction with ICR mice, while it did not affect the concentration before stress (Fig. 5B). In contrast, locomotor activity decreased both in Empty vector and shCRH mice after social defeat stress (Fig. 5C). These results suggest that suppression of CRH mRNA expression in the PVH inhibits social defeat stress-induced activation of HPA axis but not suppression of locomotor activity.

A two-diet choice experiment revealed that social defeat stress increased selection of HCD1 on day 2, and decreased selection of HFD on the same day (Fig. 5D). Cumulated intake for 2 days (day 1 plus day 2) also revealed increased selection of HCD1 and decreased that of HFD (Fig. 5D). In contrast, social defeat stress-induced change in food selection was suppressed in shCRH mice (Fig. 5D). Total calorie intake did not change both in Empty

vector mice and shCRH mice (Fig. 5E). These results suggest that stress-induced carbohydrate selection is mediated by action of CRH released from PVH-CRH neurons.

Inhibition of PVH-CRH neuron activity suppresses stress-induced alteration of food selection behavior

To examine the effect of inhibition of neural activity of PVH-CRH neurons on stress-induced change in food selection behavior. I forcibly expressed hM4Di DREADD, an engineered Gi protein-coupled receptor that is activated by CNO, in PVH-CRH neurons, by infecting AAV encoding hM4Di-mCherry fused protein into the PVH of CRH-Cre mice (CRH-Cre:hM4Di$^{f/f}$ mice). The hM4Di-mCherry protein was expressed by Cre-mediated removal of LoxP sequences. The PVH and parietal cortex preferentially expressed CRH, and the ARH and VMH plus DMH expressed AgRP and SF-1, respectively (Fig. 6A). Consistent with the CRH expression, Cre recombinase preferentially expressed in the PVH and parietal cortex, but not in other hypothalamic nuclei of CRH-Cre mice (Fig. 6A). Immunohistochemical analysis showed that mCherry was expressed in almost CRH-positive neurons in the PVH of CRH-Cre:hM4Di$^{f/f}$ mice, although the expression level was varied in individual cell (Fig. 6B). Electrophysiological experiments revealed that

application of CNO suppressed spontaneous firing (Fig. 6C and D) and decreased membrane potential (Fig. 6C and E) in PVH-CRH neurons.

Injection of CNO but not of saline into the PVH suppressed stress-induced change in food selection in CRH-Cre:hM4Di$^{f/f}$ mice (Fig. 6F). Neither CNO nor saline injection affected total calorie intake (Fig. 6G). These data suggest that activation of PVH-CRH neurons is necessary for social defeat stress-induced change in food selection between carbohydrate and fat diets.

Social defeat stress activates AMPK in PVH-CRH neurons

I next examined whether social defeat stress activates AMPK in PVH-CRH neurons. Immunoblot analysis revealed that social defeat stress increased Thr172 phosphorylation of AMPK (p-AMPK) in the PVH (Fig. 7A). Ser79 phosphorylation of ACC, which is a target of AMPK, also increased in the PVH after social defeat stress (Fig. 7A). Immunohistochemical analysis revealed that social defeat stress increased p-AMPK-positive cells in the PVH (Fig. 7B). Immunofluorescence analysis showed that 30% (20/67) of CRH neurons was positive with p-AMPK after social defeat stress in rostral region of the PVH of CRH-Cre:hM4Di$^{f/f}$ mice (Fig. 7C). p-AMPK-positive CRH neurons was abundant

in rostral region of the PVH, compared with that in caudal region of the PVH (Fig. 7C). These results suggest that social defeat stress activates AMPK in a subset of CRH neurons, especially in rostral part of the PVH.

Inhibition of AMPK in PVH-CRH neurons abolishes stress-induced carbohydrate selection without changes in stress markers

I next examined the effect of specific inhibition of AMPK expression in PVH-CRH neurons on food selection behavior after social defeat stress. To examine the Cre-driven expression of shAMPKs$^{f/f}$, I introduced the lentivirus expressing shAMPKs$^{f/f}$ into C2C12 cells that expressed with or without Cre. Infection of the lentivirus reduced both amounts of α1- and α2AMPK mRNAs in C2C12 cells expressing Cre by 40%, respectively, compared with that in intact C2C12 cells (data not shown). The abundance of α1- and α2 AMPK mRNAs in the PVH also reduced in CRH-Cre:shAMPKs$^{f/f}$ mice, compared with CRH-Cre:Empty vector mice (Fig. 8A).

I examined whether suppression of AMPK expression in PVH-CRH neurons affects stress markers after social defeat stress. CRH-Cre:shAMPKs$^{f/f}$ mice did not change social defeat stress-induced increase in CRH mRNA abundance (Fig. 8B) and plasma

corticosterone concentration (Fig. 8C) and decrease of locomotor activity (Fig. 8D), compared with those in CRH-Cre:Empty vector mice. These results suggest that changes in the stress markers after social defeat stress are independent of AMPK activity in PVH-CRH neurons.

I examined food selection behavior of CRH-Cre:shAMPKs$^{f/f}$ mice after social defeat stress. A two-diet choice experiment revealed that change in food selection between HFD and HCD1 after social defeat stress was completely inhibited in CRH-Cre:shAMPKs$^{f/f}$ mice but not in CRH-Cre:Empty vector mice (Fig. 8E). Total calorie intake did not change in both groups of mice after social defeat stress (Fig. 8F). Thus, I concluded that AMPK in PVH-CRH neurons is necessary for social defeat stress-induced change in food selection behavior, but not activation of HPA axis or suppression of locomotor activity.

Constitutive activation of AMPK in PVH-CRH neurons mimics stress-induced carbohydrate selection without affecting stress markers

To examine whether activation of AMPK in PVH-CRH neurons increases carbohydrate selection in two-diet choice experiment, a lentivirus containing a double-floxed construct for FLAG-tagged CA-AMPK or EGFP was infected into the PVH of CRH-Cre mice (to

yield CRH-Cre:CA-AMPK$^{f/f}$ and CRH-Cre:EGFP$^{f/f}$ mice, respectively). Consistent with Cre mRNA expression, CRH-Cre:CA-AMPK$^{f/f}$ and CRH-Cre:EGFP$^{f/f}$ mice expressed CA-AMPK and EGFP mRNAs in the PVH, respectively (Fig. 9A).

I examined stress markers in CRH-Cre:CA-AMPK$^{f/f}$ mice. Similar to the results of CRH-Cre:shAMPKs$^{f/f}$ mice, CRH-Cre:CA-AMPK$^{f/f}$ mice did not change locomotor activity, CRH mRNA expression in the PVH, or plasma corticosterone level (Fig. 9B-D).

I next examined food selection behavior of CRH-Cre:CA-AMPK$^{f/f}$ mice in a two-diet choice experiment. CRH-Cre:CA-AMPK$^{f/f}$ mice increased selection of HCD1 and reduced that of HFD, compared to those of CRH-Cre:EGFP$^{f/f}$ mice (Fig. 9E). Total calorie intake of HFD and HCD1 in a two-diet choice experiment (Fig. 9F) as well as calorie intake when each diet was presented alone (Fig. 9G) did not differ between groups. These results suggest that AMPK in PVH-CRH neuron is necessary and sufficient for social defeat stress-induced change in HCD-HFD selection, but not for total calorie intake or stress markers.

Administration of NPY Y1 receptor antagonist into the PVH partially suppresses stress-induced carbohydrate selection

NPY signal in the PVH is a potential regulator of food selection after social defeat stress,

because 1) NPY injection into the PVH increases carbohydrate intake[12,13], 2) NPY expressing neurons (NPY neurons) in the DMH and ARH directly projects to the PVH[34], and 3) activation of HPA axis by psychological stress is prevented by inhibition of neuronal activity of the DMH with type A γ-amino butyric acid (GABA) receptor ($GABA_AR$) agonist muscimol[35]. I examined the role of NPY signal in the PVH in stress-induced alteration of food selection behavior. RT-qPCR analysis revealed that NPY mRNA expression in the DMH but not ARH significantly increased after social defeat stress (Fig. 10A).

NPY injection into the PVH enhances carbohydrate intake through activation of Y1R[36]. I examined whether infusion of BIBP3226 (BIBP), a selective Y1R antagonist, into the PVH inhibits social defeat stress-induced alteration of food selection behavior. Although social defeat stress slightly increased HCD1 intake even after daily injection of BIBP into the PVH of C57BL/6J mice, BIBP largely decreased the social defeat stress-induced change in food selection behavior (Fig. 10B). Total calorie intake was not affected by BIBP or saline injection (Fig. 10C). These data suggest that stress-induced alteration of food selection behavior is mediated by NPY signal in the PVH at least in part. NPY neurons in the DMH may be involved in the stress-induced change in food selection behavior.

DISCUSSION

My data indicate that AMPK in PVH-CRH neurons plays a key role in regulation of food selection behavior regarding the choice between fat and carbohydrate diet after social defeat stress. I found that social defeat stress activates AMPK in PVH-CRH neurons and enhanced carbohydrate selection in a two-diet choice experiment between a HCD and HFD. This alteration of food selection was completely suppressed by inhibition of AMPK expression in PVH-CRH neurons. Furthermore, carbohydrate selection was induced by expression of CA-AMPK in PVH-CRH neurons. These findings clearly show that AMPK activation in PVH-CRH neurons is necessary and sufficient for alteration of food selection behavior in response to social defeat stress. In contrast, AMPK in CRH neurons in the PVH did not affect total calorie intake in two-diet choice experiments. Social defeat stress did not change preference for fat diet. These results suggest that AMPK in PVH-CRH neurons regulates selection of carbohydrate and fat diet, keeping fat preference.

I found that stress-induced carbohydrate selection is mediated by action of CRH and activation of CRH neurons in the PVH. Inhibition of CRH expression or its neuronal activity blunted stress-induced change in food selection behavior. Recent study in our laboratory suggested that activation of AMPK increases $[Ca^{2+}]_i$ in a subset of PVH-CRH

neurons (in submission). AMPK activation thus likely increases CRH secretion from PVH-CRH neurons. Together, I propose that social defeat stress enhances carbohydrate selection by activation of AMPK in a subset of PVH-CRH neurons and enhancement of CRH secretion (Fig. 11).

My results also showed that social defeat stress-induced activation of HPA axis is independent of AMPK in PVH-CRH neurons (Fig. 11). Inhibition of AMPK expression or CA-AMPK expression in PVH-CRH neurons did not change CRH mRNA amount in the PVH or plasma corticosterone levels. Social defeat stress activated AMPK in a subset of PVH-CRH neurons in rostral part of the PVH. CRH neurons that regulate ACTH secretion from pituitary are mostly located in the medial to caudal part of parvocellular region, but few in the rostral of the PVH[37]. Thus, distinct populations of PVH-CRH neurons may regulate food selection and HPA axis, respectively. CRH neurons in the PVH is unlikely involved in the regulation of locomotor activity. Previous studies suggest that other brain regions such as amygdala regulate locomotor activity after stress[38].

NPY is a possible candidate for stress-induced activation of PVH-CRH neurons. Present results showed that social defeat stress increased NPY mRNA expression in the DMH but not ARH and that administration of Y1R antagonist into the PVH partially

inhibited social defeat stress-induced carbohydrate selection. Previous study showed that activation of HPA axis by psychological stress was prevented by inhibition of neuronal activity of the DMH with $GABA_AR$ agonist muscimol[35]. CRH neurons in the PVH are activated by NPY[39]. Recent study in our laboratory also showed that NPY injection into the PVH activates AMPK in the PVH (in submission). These results suggest that NPY activates CRH neurons in the PVH via AMPK activation in response to stress. However, infusion of Y1R antagonist still remained stress-induced carbohydrate selection. Other mechanisms may exist for change in food selection after social defeat stress. NPY Y5 receptor (Y5R) is expressed in the PVH and regulates feeding behavior[40]. Thus, stress-induced activation of AMPK in PVH-CRH neurons and food selection behavior might be mediated by both Y1R and Y5R. In addition, another possible regulator of PVH-CRH neurons for food selection behavior is noradrenalin (NA). NA containing neurons (NA neurons) in the A2 region in the dorsomedial medulla are activated by conditioned fear stimuli[41]. NA neurons in this region directly innervate the parvocellular division of the PVH, a region known to contain majority of CRH neurons[42]. NA injection into the PVH also increases carbohydrate intake via activation of α2 adrenoceptor (α2AdR)[36]. Y1R, Y5R and α2AdR are coupled to inhibitory G (Gi or Go) proteins[43,44]. A subset of PVH-CRH neurons is innervated by GABA-

containing neurons[45]. NPY inhibits $GABA_AR$-mediated synaptic transmission in the PVH[46], and α2AdR also inhibits GABA release[47]. Thus, NPY and NA may activate PVH-CRH neurons for food selection behavior by inhibiting GABAergic transmission.

Pathophysiological role of stress-induced carbohydrate intake has still been under investigation, although many studies show that stress leads carbohydrate craving in human as well as animals[7–11,48]. A previous report showed that adrenalectomized rats preferred sucrose more than saccharin, and ingestion of sucrose suppressed CRH mRNA expression in the PVH[49]. The study suggests that sucrose might act similarly to glucocorticoid in the negative feedback for the HPA axis. Thus, stress-induced carbohydrate selection may hasten end of the stress responses by inhibiting CRH mRNA expression in the PVH. Further investigation is necessary to clarify the effects of carbohydrate feeding on neuronal activity of PVH-CRH neurons.

Collectively, the present data show that social defeat stress enhances carbohydrate selection via activation of AMPK in a subset of PVH-CRH neurons. In contrast, social defeat stress-induced activation of HPA axis is not mediated by AMPK in PVH-CRH neurons. NPY Y1R in the PVH involves social defeat stress-induced carbohydrate selection in part, and NPY neurons in the DMH likely mediate the alteration of food selection in

response to social defeat stress. To conclude, AMPK in PVH-CRH neurons is a principal regulator of social defeat stress-induced alteration of food selection behavior. This study provides important insight into the regulation of food selection under stress condition and a new model of stress-induced carbohydrate craving.

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REFERENCES

1. Macht, M. How emotions affect eating: a five-way model. *Appetite* **50,** 1–11 (2007).

2. Harris, R. Chronic and acute effects of stress on energy balance: are there appropriate animal models? *Am J Physiol-Regul Integr Comp Physiol* **308,** R250–R265 (2015).

3. Vallès, A., Martí, O., García, A. & Armario, A. Single exposure to stressors causes long-lasting, stress-dependent reduction of food intake in rats. *Am J Physiol-Regul Integr Comp Physiol* **279,** R1138–R1144 (2000).

4. Rybkin, I. *et al.* Effect of restraint stress on food intake and body weight is determined by time of day. *Am J Physiol* **273,** R1612–1622 (1997).

5. Moles, A. *et al.* Psychosocial stress affects energy balance in mice: modulation by social status. *Psychoneuroendocrinology* **31,** 623–633 (2006).

6. Lutter, M. *et al.* The orexigenic hormone ghrelin defends against depressive symptoms of chronic stress. *Nat Neurosci* **11,** 752–753 (2008).

7. Patterson, Z., Khazall, R., Mackay, H., Anisman, H. & Abizaid, A. Central ghrelin signaling mediates the metabolic response of C57BL/6 male mice to chronic social

defeat stress. *Endocrinology* **154,** 1080–1091 (2013).

8. Oliver, G., Wardle, J. & Gibson, E. Stress and Food Choice: A Laboratory Study. *Psychosom Med* **62,** 853-865 (2000).

9. Roberts, C., Campbell, I. & Troop, N. Increases in Weight during Chronic Stress are Partially Associated with a Switch in Food Choice towards Increased Carbohydrate and Saturated Fat Intake. *Eur Eat Disord Rev* **22,** 77–82 (2014).

10. Rutters, F., Nieuwenhuizen, A., Lemmens, S., Born, J. & Westerterp−Plantenga, M. Acute Stress−related Changes in Eating in the Absence of Hunger. *Obesity* **17,** 72–77 (2009).

11. Hitze, B. *et al.* How the selfish brain organizes its supply and demand. *Front Neuroenergetics* **2,** 7 (2010).

12. Stanley, B., Daniel, D., Chin, A. & Leibowitz, S. Paraventricular nucleus injections of peptide YY and neuropeptide Y preferentially enhance carbohydrate ingestion. *Peptides* **6,** 1205–1211 (1985).

13. Morley, J., Levine, A., Gosnell, B., Kneip, J. & Grace, M. Effect of neuropeptide Y on ingestive behaviors in the rat. *Am J Physiol* **252,** R599–R609 (1987).

14. Naleid, A., Grace, M., Chimukangara, M., Billington, C. & Levine, A.

Paraventricular opioids alter intake of high-fat but not high-sucrose diet depending on diet preference in a binge model of feeding. *Am J Physiol-Regul Integr Comp Physiol* **293,** R99–R105 (2007).

15. Vale, W., Spiess, J., Rivier, C. & Rivier, J. Characterization of a 41-residue ovine hypothalamic peptide that stimulates secretion of corticotropin and beta-endorphin. *Science* **213,** 1394–1397 (1981).

16. Tsigos, C. & Chrousos, G. Hypothalamic–pituitary–adrenal axis, neuroendocrine factors and stress. *J Psychosom Res* **53,** 865– 871 (2002).

17. Muglia, L., Jacobson, L., Dikkes, P. & Majzoub, J. Corticotropin-releasing hormone deficiency reveals major fetal but not adult glucocorticoid need. *Nature* **373,** 427–432 (1995).

18. Müller, M. *et al.* Limbic corticotropin-releasing hormone receptor 1 mediates anxiety-related behavior and hormonal adaptation to stress. *Nat Neurosci* **6,** 1100–1107 (2003).

19. Elliott, E., Ezra-Nevo, G., Regev, L., Neufeld-Cohen, A. & Chen, A. Resilience to social stress coincides with functional DNA methylation of the Crf gene in adult mice. *Nat Neurosci* **13,** 1351–1353 (2010).

20. Arase, K., York, D., Shimizu, H., Shargill, N. & Bray, G. Effects of corticotropin-releasing factor on food intake and brown adipose tissue thermogenesis in rats. *Am J Physiol* **255,** E255–E259 (1988).

21. Weninger, S., Muglia, L., Jacobson, L. & Majzoub, J. CRH-deficient mice have a normal anorectic response to chronic stress. *Regul Pept* **84,** 69–74 (1999).

22. Hardie, D. AMP-activated/SNF1 protein kinases: conserved guardians of cellular energy. *Nat Rev Mol Cell Biol* **8,** 774–785 (2007).

23. Minokoshi, Y. *et al.* AMP-kinase regulates food intake by responding to hormonal and nutrient signals in the hypothalamus. *Nature* **428,** 569–574 (2004).

24. Xue, B. & Kahn, B. AMPK integrates nutrient and hormonal signals to regulate food intake and energy balance through effects in the hypothalamus and peripheral tissues. *J Physiol* **574,** 73–83 (2006).

25. Meerlo, P., Sgoifo, A., De Boer, S. & Koolhaas, J. Long-lasting consequences of a social conflict in rats: behavior during the interaction predicts subsequent changes in daily rhythms of heart rate, temperature, and activity. *Behav. Neurosci.* **113,** 1283–1290 (1999).

26. Meerlo, P., Sgoifo, A. & Turek, F. The effects of social defeat and other stressors

on the expression of circadian rhythms. *Stress* **5,** 15–22 (2002).

27. Keeney, A. *et al.* Differential Effects of Acute and Chronic Social Defeat Stress on Hypothalamic−Pituitary−Adrenal Axis Function and Hippocampal Serotonin Release in Mice. *J Neuroendocrinol* **18,** 330–338 (2006).

28. Sgoifo, A., Boer, S., Haller, J. & Koolhaas, J. Individual differences in plasma catecholamine and corticosterone stress responses of wild-type rats: relationship with aggression. *Physiol Behav* **60,** 1403–1407 (1996).

29. Taniguchi, H. *et al.* A resource of Cre driver lines for genetic targeting of GABAergic neurons in cerebral cortex. *Neuron* **71,** 995–1013 (2011).

30. Paxinos, G. & Franklin, K. *The mouse brain in stereotaxic coordinates*, vol. 2 (Adacemic press, 1997).

31. Woods, A. *et al.* Characterization of the role of AMP-activated protein kinase in the regulation of glucose-activated gene expression using constitutively active and dominant negative forms of the kinase. *Mol Cell Biol* **20,** 6704–6711 (2000).

32. Yagi, H. *et al.* Complete restoration of phenylalanine oxidation in phenylketonuria mouse by a self-complementary adeno-associated virus vector. *J Gene Med* **13,** 114–122 (2011).

33. Ishikawa, A., Komatsu, Y. & Yoshimura, Y. Experience-dependent emergence of fine-scale networks in visual cortex. *J Neurosci* **34,** 12576–12586 (2014).

34. Li, C, Chen, P & Smith, M. Neuropeptide Y (NPY) neurons in the arcuate nucleus (ARH) and dorsomedial nucleus (DMH), areas activated during lactation, project to the paraventricular nucleus. *Regul pept* **75,** 93– 100 (1998).

35. DiMicco, J., Samuels, B., Zaretskaia, M. & Zaretsky, D. The dorsomedial hypothalamus and the response to stress: part renaissance, part revolution. *Pharmacol Biochem Behav* **71,** 469–480 (2002).

36. Tempel, D. & Leibowitz, S. Adrenal Steroid Receptors: Interactions with Brain Neuropeptide Systems in Relation to Nutrient Intake and Metabolism. *J Neuroendocrinol* **6,** 479–501 (1994).

37. Simmons, D. & Swanson, L. Comparison of the spatial distribution of seven types of neuroendocrine neurons in the rat paraventricular nucleus: Toward a global 3D model. *J Comp Neurol* **516,** 423–441 (2009).

38. Goldstein, L., Rasmusson, A., Bunney, B. & Roth, R. Role of the amygdala in the coordination of behavioral, neuroendocrine, and prefrontal cortical monoamine responses to psychological stress in the rat. *J Neurosci* **16,** 4787–4798 (1996).

39. Dimitrov, E., DeJoseph, M., Brownfield, M. & Urban, J. Involvement of neuropeptide Y Y1 receptors in the regulation of neuroendocrine corticotropin-releasing hormone neuronal activity. *Endocrinology* **148,** 3666–3673 (2007).

40. Gerald, C. *et al.* A receptor subtype involved in neuropeptide-Y-induced food intake. *Nature* **382,** 168–171 (1996).

41. Zhu, L. & Onaka, T. Facilitative role of prolactin-releasing peptide neurons in oxytocin cell activation after conditioned-fear stimuli. *Neuroscience* **118,** 1045–1053 (2003).

42. Cunningham, E. & Sawchenko, P. Anatomical specificity of noradrenergic inputs to the paraventricular and supraoptic nuclei of the rat hypothalamus. *J Comp Neurol* **274,** 60–76 (1988).

43. Michel, M. *et al.* XVI. International Union of Pharmacology recommendations for the nomenclature of neuropeptide Y, peptide YY, and pancreatic polypeptide receptors. *Pharmacol Rev* **50,** 143–150 (1998).

44. Hein, L. & Kobilka, B. Adrenergic receptor signal transduction and regulation. *Neuropharmacology* **34,** 357–366 (1995).

45. Miklós, I. & Kovács, K. GABAergic innervation of corticotropin-releasing

hormone (CRH)-secreting parvocellular neurons and its plasticity as demonstrated by quantitative immunoelectron microscopy. *Neuroscience* **113,** 581–592 (2002).

46. Cowley, M. *et al.* Integration of NPY, AGRP, and Melanocortin Signals in the Hypothalamic Paraventricular Nucleus Evidence of a Cellular Basis for the Adipostat. *Neuron* **24,** 155–163 (1999).

47. Alachkar, A., Brotchie, J. & Jones, O. T. alpha2-Adrenoceptor-mediated modulation of the release of GABA and noradrenaline in the rat substantia nigra pars reticulata. *Neurosci Lett* **395,** 138–142 (2006).

48. Willner, P. *et al.* 'Depression' increases 'craving' for sweet rewards in animal and human models of depression and craving. *Psychopharmacology* **136,** 272–283 (1998).

49. Laugero, K., Bell, M., Bhatnagar, S., Soriano, L. & Dallman, M. Sucrose ingestion normalizes central expression of corticotropin-releasing-factor messenger ribonucleic acid and energy balance in adrenalectomized rats: a glucocorticoid-metabolic-brain axis? *Endocrinology* **142,** 2796–2804 (2001).

Table 1. Composition of diets

Percentage of total calories		LCD	HFD (D12492)	HCD1 (D11071504M)	HCD2 (D1171501)
	Protein	24.9 [1)]	20	20	20
	Carbohydrate	70.5 [2)]	20	69	69
	Fat	4.6 [3)]	60	11	11
	Ingredients (kcal%)	100	100	100	100
Total kilo-calories per gram		3.4	5.2	3.87	3.87
Protein	Casein	0	200	200	200
	L-cystine	NI	3	3	3
Carbohydrate	Maltodextrin 10	0	125	125	125
	Corn starch	(+)	0	0	**495**
	Sucrose	0	68.8	**563.8**	68.8
Fat	Lard	0	**245**	25	25
	Soybean oil	(+)	25	25	25
Fiber		(+) [4)]	50 [5)]	50 [5)]	50 [5)]
Minerals		(+)	10 [6)]	10 [6)]	10 [6)]
Calcium biphosphate		NI	13	13	13
Calcium carbonate		NI	5.5	5.5	5.5
Potassium citrate･H_2O		NI	16.5	16.5	16.5
Vitamins		(+)	10 [7)]	10 [7)]	10 [7)]
Choline bitartrate		(+)	2	2	2

The lab chow diet (LCD, Rodent Diet CE-2) was from CLEA (Tokyo, Japan) and other diets were from Research Diets (New Brunswick, NJ). [1)]Soy bean cake, white fish meal and yeast. [2)]Corn starch and wheat flour. [3)]Soybean oil. [4)]Mostly Fusuma paper and rice bran. [5)]Cellulose. [6)]Mineral mix S10001 (Research Diets). [7)]Vitamin mix V10001 (Research Diets). NI, no information.

Table 2. Primer sequences for PCR

Target		Sequence (5' → 3')
CRH	Fwd:	GCTAACTTTTTCCGCGTGTT
	Rev:	GGTGGAAGGTGAGATCCAGA
GAPDH	Fwd:	AACTTTGGCATTGTGGAAGG
	Rev:	ACACATTGGGGGTAGGAACA
AgRP	Fwd:	CCAGAGTTCCCAGGTCTAAG
	Rev:	AGGCATTGAAGAAGCGGCAG
NPY	Fwd:	CTAGGTAACAAGCGAATGGG
	Rev:	AATCAGTGTCTCAGGGCT
SF-1	Fwd:	GCCAGGAGTTCGTCTGTCTC
	Rev:	ACCTCCACCAGGCACAATAG
α1AMPK	Fwd:	AGAGGGCCGCAATAAAAGAT
	Rev:	TGTTGTACAGGCAGCTGAGG
α2AMPK	Fwd:	ACAGCGCCATGCATATTCCTC
	Rev:	ATGTCACACGCTTTGCTCTG
EGFP	Fwd:	CACATGAAGCAGCACGACTTCT
	Rev:	AACTCCAGCAGGACCATGTGAT
CA-AMPK (FLAG tag)	Fwd:	GATTACAAGGACGATGACGACAAG
	Rev:	ATTTAGTACAGGCAGCTGAGGA
Cre recombinase	Fwd:	CTGATTTCGACCAGGTTCGTTC
	Rev:	CGCTCGACCAGTTTAGTTACCC
CRH-Cre genome	Fwd:	CAATGTATCTTATCATGTCTGGATCC
	Rev:	CTTACACATTTCGTCCTAGCC

FIGURE LEGENDS

Figure 1. Changes in locomotor activity, CRH mRNA expression in the PVH and plasma corticosterone level after social defeat stress. (A) Experimental protocol for social defeat stress and food selection measurement. (B) Locomotor activity for 24 h before (day 2) and after (day 1) social defeat stress of same C57BL/6J mice (n = 11). (C) RT-qPCR analysis of CRH mRNA amounts in the PVH of stressed mice [stress (+), after 24 h-social defeat stress] (n = 6) and non-stressed mice [stress (−)] (n = 4). (D) Plasma corticosterone levels of stressed mice [stress (+), after 5 min of direct interaction and 24 h of sensory contact period] (n = 6) and non-stressed mice [stress (−), on day 2 before stress] (n = 8). All data are means ± s.e.m. $^{*}P < 0.05$ versus before stress or stress (−).

Figure 2. Change in food selection between HFD and HCD1 after social defeat stress. (A, B) Daily food selection (HFD versus HCD2) (A) and total calorie intake (B) of C57BL/6J mice in a two-diet choice experiment (n = 11) before and after 24 h-social defeat stress (left) and cumulated intake for 2 days (day 1 plus day 2) before and after social defeat stress (right). (C) Change in HCD1 intake (ΔHCD) of individual mouse. (D, E) Food selection (D) and total calorie intake (E) on day 2 before stress and on a day showing

maximal HCD1 intake during 3 days after stress. All data except (C) are means ± s.e.m. *P < 0.05 versus corresponding HFD value, †P < 0.05 versus corresponding value before stress on day 2 or cumulated intake before stress.

Figure 3. Change in food selection between HFD and HCD2 after social defeat stress. (A, B) Daily food selection (HFD versus HCD2) (A) and total calorie intake (B) of C57BL/6J mice in a two-diet choice experiment (n = 10) before and after 24 h-social defeat stress (left) and cumulated intake for 2 days (day 1 plus day 2) before and after social defeat stress (right). (C) Change in HCD2 intake (ΔHCD) of individual mouse. (D, E) Food selection (D) and total calorie intake (E) on day 2 before stress and on a day showing maximal HCD1 intake during 3 days after stress. All data except (C) are means ± s.e.m. *P < 0.05 versus corresponding HFD value, †P < 0.05 versus corresponding value before stress on day 2 or cumulated intake before stress.

Figure 4. Changes in intake of HFD or HCD after social defeat stress. (A) Food intake of C57BL/6J mice when fed HCD1 alone before (day 2) and after social defeat stress (day 1) (n =4). (B) Food intake of C57BL/6J mice when fed HFD alone (n = 4). HFD intake

before (day 2) and after (day 1) stress (left). HFD intake when fed ad libitum and after 1 day of LCD feeding (without stress). All data are means ± s.e.m. $*P < 0.05$ versus before stress or ad libitum.

Figure 5. Effects of shCRH expression in the PVH on stress-induced alteration of food selection behavior and stress markers. (A) RT-qPCR analysis of CRH mRNA in the PVH of control (Empty vector) and shCRH mice with or without stress (stressed mice: $n = 6$ for Empty vector, $n = 8$ for shCRH; non-stressed mice: $n = 4$ for Empty vector, $n = 5$ for shCRH). (B) Plasma corticosterone levels of control (Empty vector) and shCRH mice with or without stress (stressed mice: $n = 4$ for Empty vector, $n = 5$ for shCRH; non-stressed mice: $n = 4$ for Empty vector, $n = 5$ for shCRH). (C) Locomotor activity of control (Empty vector) and shCRH mice ($n = 4$) before (day 2) and after (day 1) social defeat stress. $*P < 0.05$ versus corresponding value of non-stressed mice [stress (−)] or before stress, $†P < 0.05$ versus corresponding value of control (Empty vector) mice. (D, E) Daily food selection (HFD versus HCD1) (D) and total calorie intake (E) in control (Empty vector) ($n = 6$) and shCRH mice ($n = 6$) in a two-diet choice experiment before and after social defeat stress (left) and cumulated intake for 2 days (day 1 plus day 2) before and after 24 h-social defeat stress

(right). *$P < 0.05$ versus corresponding HFD value, †$P < 0.05$ versus corresponding value before stress on day 2 or cumulated intake before stress. All data are means ± s.e.m.

Figure 6. Effect of inhibition of PVH-CRH neurons on stress-induced alteration of food selection behavior. (A) PCR analysis of mRNA expression of Cre recombinase and specific markers for ARH, VMH and PVH in dissected hypothalamic samples of CRH-Cre mice and wild-type (WT) littermates. (B) Representative images of immunohistofluorescence staining of CRH (green) and intrinsic fluorescence of mCherry (red) in the PVH of CRH-Cre:hM4Di$^{f/f}$ mice. Higher magnification views of the boxed region are shown in bottom panels. Scale bars, 50 μm, 5 μm, respectively. (C) Representative traces of extracellular current recording (left) and whole cell voltage recording (right) from a mCherry positive PVH neuron of CRH-Cre:hM4Di$^{f/f}$ mouse. (D, E) Changes in the firing rate (D) and the membrane potential (E) of individual mCherry positive PVH neuron before and after CNO treatment. (F, G) Daily food selection (HFD versus HCD1) (F) and total calorie intake (G) of CRH-Cre:hM4Di$^{f/f}$ mice in a two-diet choice experiment before and after social defeat stress (left) and cumulated intake for 2 days (day 1 plus day 2) before and after 24 h-social defeat stress (right) ($n = 4$). Saline or CNO was administered into the PVH every 12 h for

last 4 days. Data in (F) and (G) are means ± s.e.m. $^{*}P < 0.05$ versus corresponding HFD value, †$P < 0.05$ versus corresponding value before stress on day 2 or cumulated intake before stress.

Figure 7. Changes in AMPK activity in PVH-CRH neurons after social defeat stress. (A) Immunoblot analysis of the phosphorylation of AMPK on Thr172 and ACC on Ser79 in the PVH of C57BL/6J mice at 3 h after 24 h of social defeat stress. Representative blots are shown in the upper panels, and quantitative data (means ± s.e.m.) are shown in the lower panels ($n = 5$). The amounts of phosphorylated proteins were normalized by those of total proteins. $^{*}P < 0.05$ versus stress (−). (B) Representative image of Immunohistochemical analysis of p-AMPK in the PVH of C57BL/6J mice 3 h after 24 h of social defeat stress. Higher magnification views of the boxed region are shown in right of each panel. Scale bars, 200 μm, 50 μm, respectively. (C) Immunohistofluorescence analysis of p-AMPK (green) and intrinsic fluorescence of mChery (red) (marker for CRH neurons) in the rostral and caudal portions of the PVH in CRH-Cre:hM4Di$^{f/f}$ mice. Arrowheads indicate neurons positive for both p-AMPK and mCherry. The numbers of neurons positive for p-AMPK, mCherry, or both in the one side of the PVH from one image are shown below the images.

Scale bar, 25μm.

Figure 8. Effects of preferential inhibition of AMPK expression in PVH-CRH neurons on stress-induced alteration of food selection behavior and stress markers. (A) RT-qPCR analysis of α1- and α2 AMPK mRNA amounts in the PVH of CRH-Cre:Empty vector and CRH-Cre:shAMPKs$^{f/f}$ mice (n = 7). $^{*}P < 0.05$ versus corresponding value of CRH-Cre:Empty vector mice. (B) RT-qPCR analysis of CRH mRNA in the PVH of stressed and non-stressed CRH-Cre:Empty vector and CRH-Cre:shAMPKs$^{f/f}$ mice (n = 4). (C) Plasma corticosterone levels for stressed and non-stressed CRH-Cre:Empty vector and CRH-Cre:shAMPKs$^{f/f}$ mice (n = 4). (D) Locomotor activity of CRH-Cre:Empty vector and CRH-Cre: shAMPKs$^{f/f}$ mice before (day 2) and after (day 1) social defeat stress (n = 7 for CRH-Cre:Empty vector; n = 8 for CRH-Cre:shAMPKs$^{f/f}$). $^{*}P < 0.05$ versus corresponding value of non-stressed mice [stress (−)] or before stress. (E, F) Daily food selection (HFD versus HCD1) (E) and total calorie intake (F) in a two-diet choice experiment with CRH-Cre:Empty vector mice (n = 7) and CRH-Cre:shAMPKs$^{f/f}$ mice (n = 8) before and after 24 h-social defeat stress (left) and cumulated intake for 2 days (day 1 plus day 2) before and after social defeat stress (right). $^{*}P < 0.05$ versus corresponding HFD value, †$P < 0.05$

versus corresponding value before stress on day 2 or cumulated intake before stress. All data are means ± s.e.m.

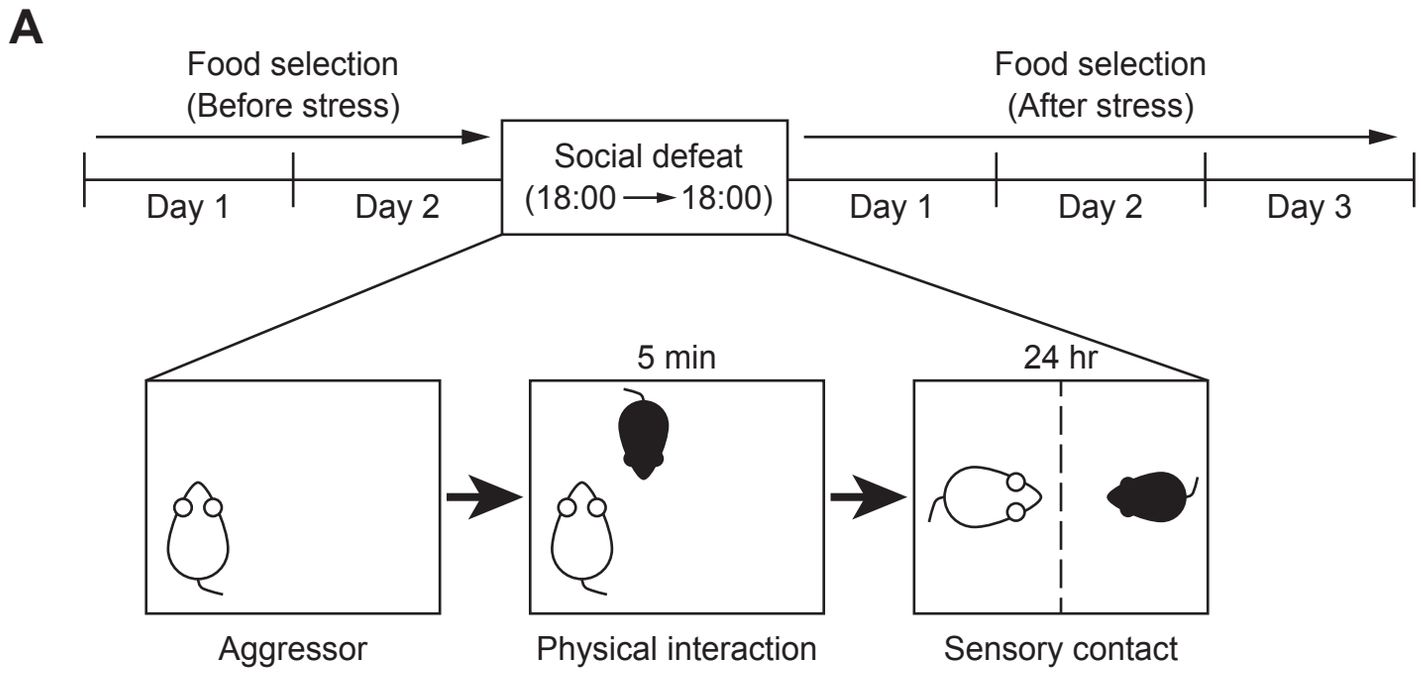
Figure 9. Effects of preferential expression of CA-AMPK in PVH-CRH neurons on food selection behavior and stress markers. (A) PCR analysis of EGFP and FLAG tagged CA-AMPK mRNAs in dissected hypothalamic samples of CRH-Cre:EGFP$^{f/f}$ mice and CRH-Cre:CA-AMPK$^{f/f}$ mice. (B) RT-qPCR analysis of CRH mRNA in the PVH of CRH-Cre:EGFP$^{f/f}$ (n = 6) and CRH-Cre:CA-AMPK$^{f/f}$ mice (n = 5). (C) Plasma corticosterone levels for CRH-Cre:EGFP$^{f/f}$ (n = 6) and CRH-Cre:CA-AMPK$^{f/f}$ mice (n = 5). (D) Locomotor activity for 24 h of CRH-Cre:EGFP$^{f/f}$ (n = 6) and CRH-Cre:CA-AMPK$^{f/f}$ mice (n = 5). (E, F) Daily food selection (HFD versus HCD1) (E) and total calorie intake (F) of CRH-Cre:EGFP$^{f/f}$ (n = 6) and CRH-Cre:CA-AMPK$^{f/f}$ mice (n = 5) in a two-diet choice experiment. (G) Daily food intake for HFD or HCD1 of CRH-Cre:EGFP$^{f/f}$ (n = 6) and CRH-Cre:CA-AMPK$^{f/f}$ mice (n = 5) when each diet was presented alone. Data from (D) to (G) are averages of 3 days measurement. All quantitative data are shown as means ± s.e.m. $*P < 0.05$ versus corresponding HFD value, $\dagger P < 0.05$ versus corresponding value of CRH-Cre:EGFP$^{f/f}$ mice.

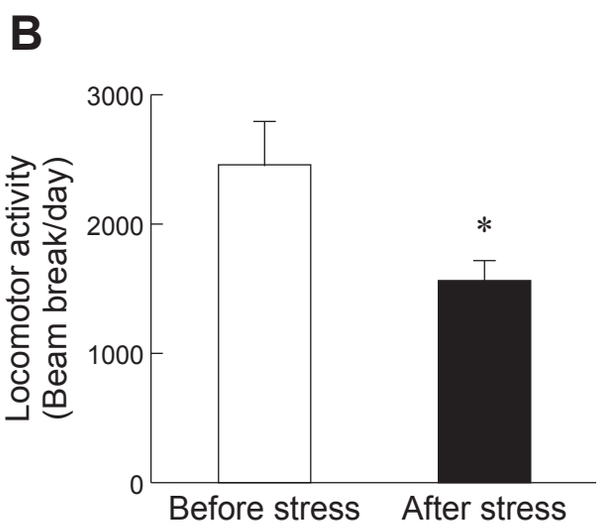
Figure 10. Effects of BIBP3226 administration into the PVH on stress-induced alteration of food selection behavior. (A) RT-qPCR analysis of NPY mRNA amount in the DMH or ARH of stressed and non-stressed C57BL/6J mice (stressed mice: $n = 3$ for DMH, $n = 4$ for ARH; non-stressed mice: $n = 3$ for DMH, $n = 6$ for ARH). $^{*}P < 0.05$ versus non-stressed mice [stress (−)]. (B, C) Daily food selection (HFD versus HCD1) (B) and total calorie intake (C) in a two-diet choice experiment with C57BL/6J mice before and after 24 h-social defeat stress (left) and cumulated intake for 2 days (day 1 plus day 2) before and after stress (right) ($n = 6$ for saline; $n = 4$ for BIBP3226). Saline or BIBP3226 was administered into the PVH every 24 h for last 4 days. $^{*}P < 0.05$ versus corresponding HFD value, $\dagger P < 0.05$ versus before stress on day 2 or cumulated food intake before stress. All data are means ± s.e.m.

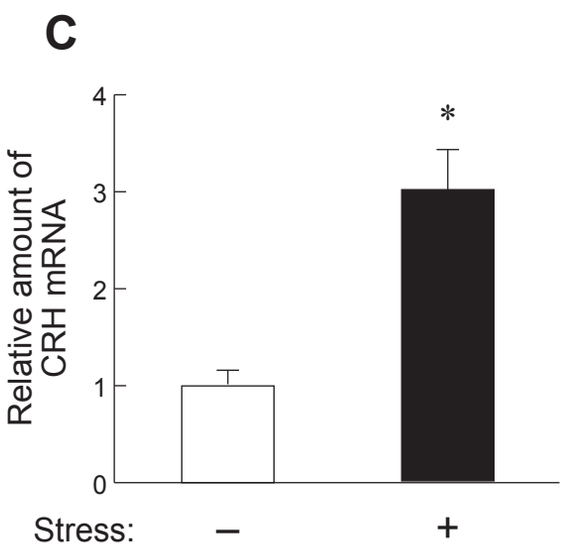
Figure 11. Model for social defeat stress-induced carbohydrate selection. Social defeat stress activates AMPK in a subset of CRH neurons in rostral part of the PVH. The activation of AMPK increases neuronal activity of the CRH neurons and release of CRH, and stimulates carbohydrate diet selection. In contrast, stress-induced activation of HPA axis is independent of AMPK activity in PVH-CRH neurons. Distinct populations of PVH-CRH

neurons may regulate food selection and HPA axis.

Figure 1


A
Food selection
(Before stress)
Social defeat
(18:00 → 18:00)
Food selection
(After stress)
Day 1
Day 2
Day 1
Day 2
Day 3
5 min
24 hr
Aggressor
Physical interaction
Sensory contact


B
Locomotor activity
(Beam break/day)
3000
2000
1000
0
*
Before stress
After stress


C
Relative amount of
CRH mRNA
4
3
2
1
0
*
Stress:
–
+

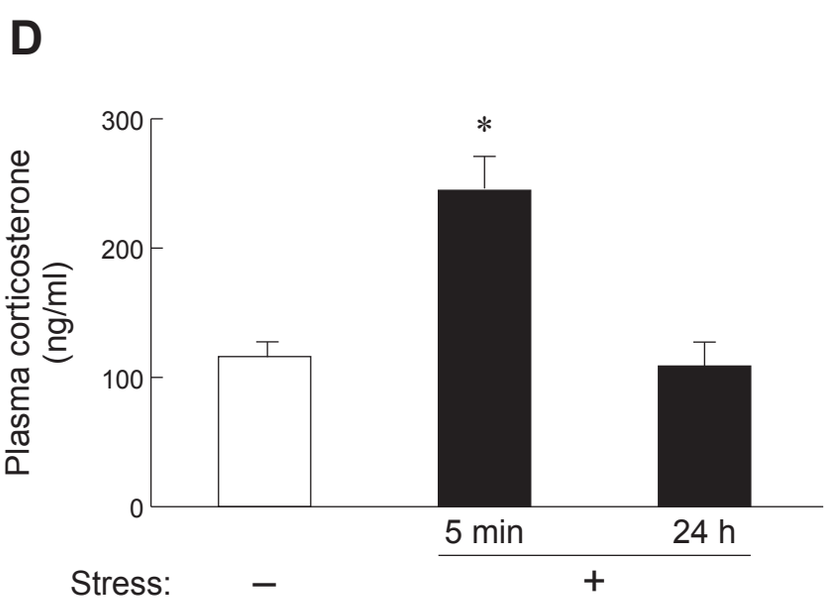

D
Plasma corticosterone
(ng/ml)
300
200
100
0
*
5 min
24 h
Stress:
–
+

Figure 2

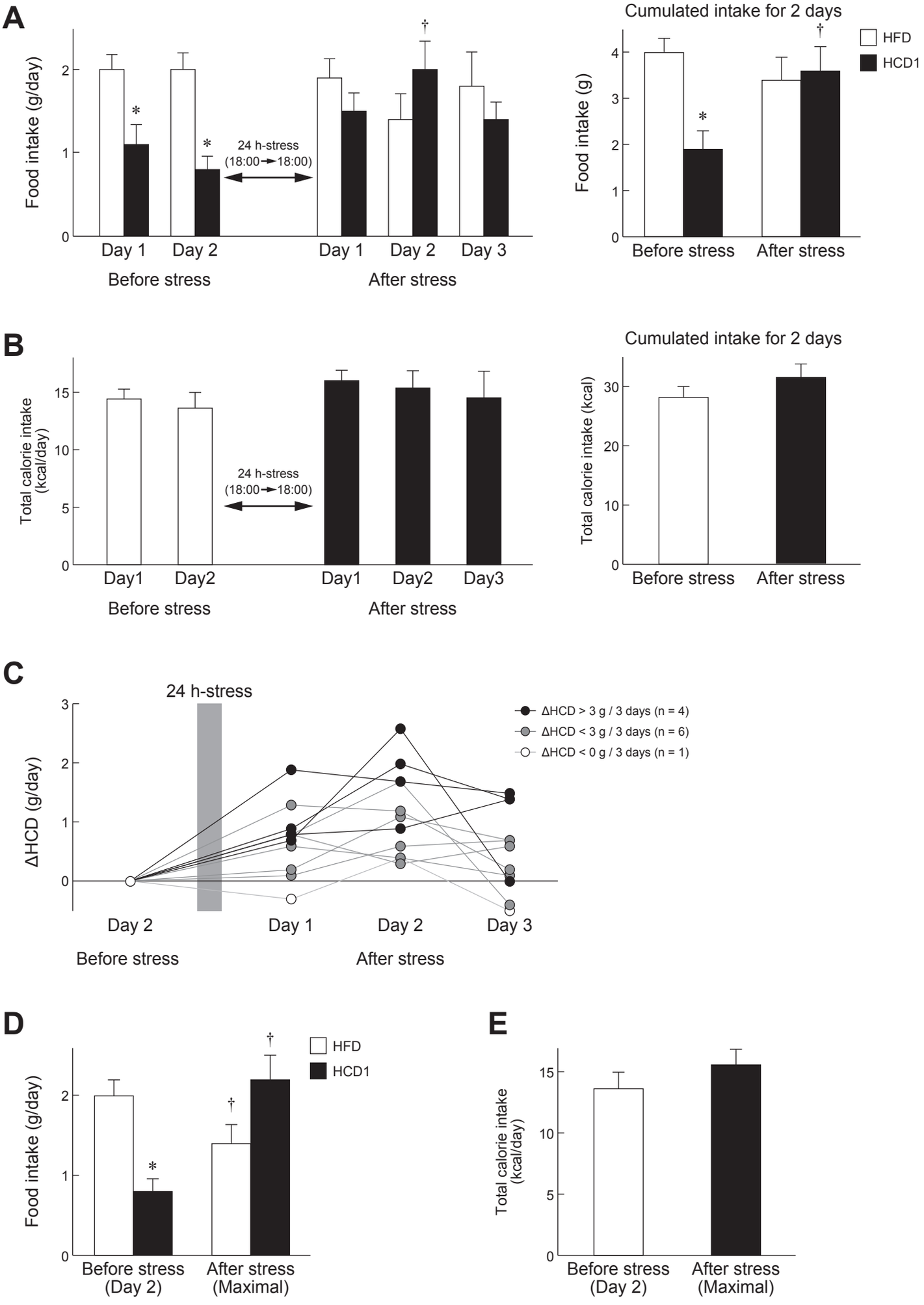

A
Food intake (g/day)
24 h-stress
(18:00→18:00)
Day 1
Day 2
Day 1
Day 2
Day 3
Before stress
After stress
Cumulated intake for 2 days
HFD
HCD1
Food intake (g)
Before stress
After stress
B
Total calorie intake (kcal/day)
24 h-stress
(18:00→18:00)
Day1
Day2
Day1
Day2
Day3
Before stress
After stress
Cumulated intake for 2 days
Total calorie intake (kcal)
Before stress
After stress
C
24 h-stress
ΔHCD (g/day)
ΔHCD > 3 g / 3 days (n = 4)
ΔHCD < 3 g / 3 days (n = 6)
ΔHCD < 0 g / 3 days (n = 1)
Day 2
Day 1
Day 2
Day 3
Before stress
After stress
D
Food intake (g/day)
HFD
HCD1
Before stress
(Day 2)
After stress
(Maximal)
E
Total calorie intake
(kcal/day)
Before stress
(Day 2)
After stress
(Maximal)

Figure 3

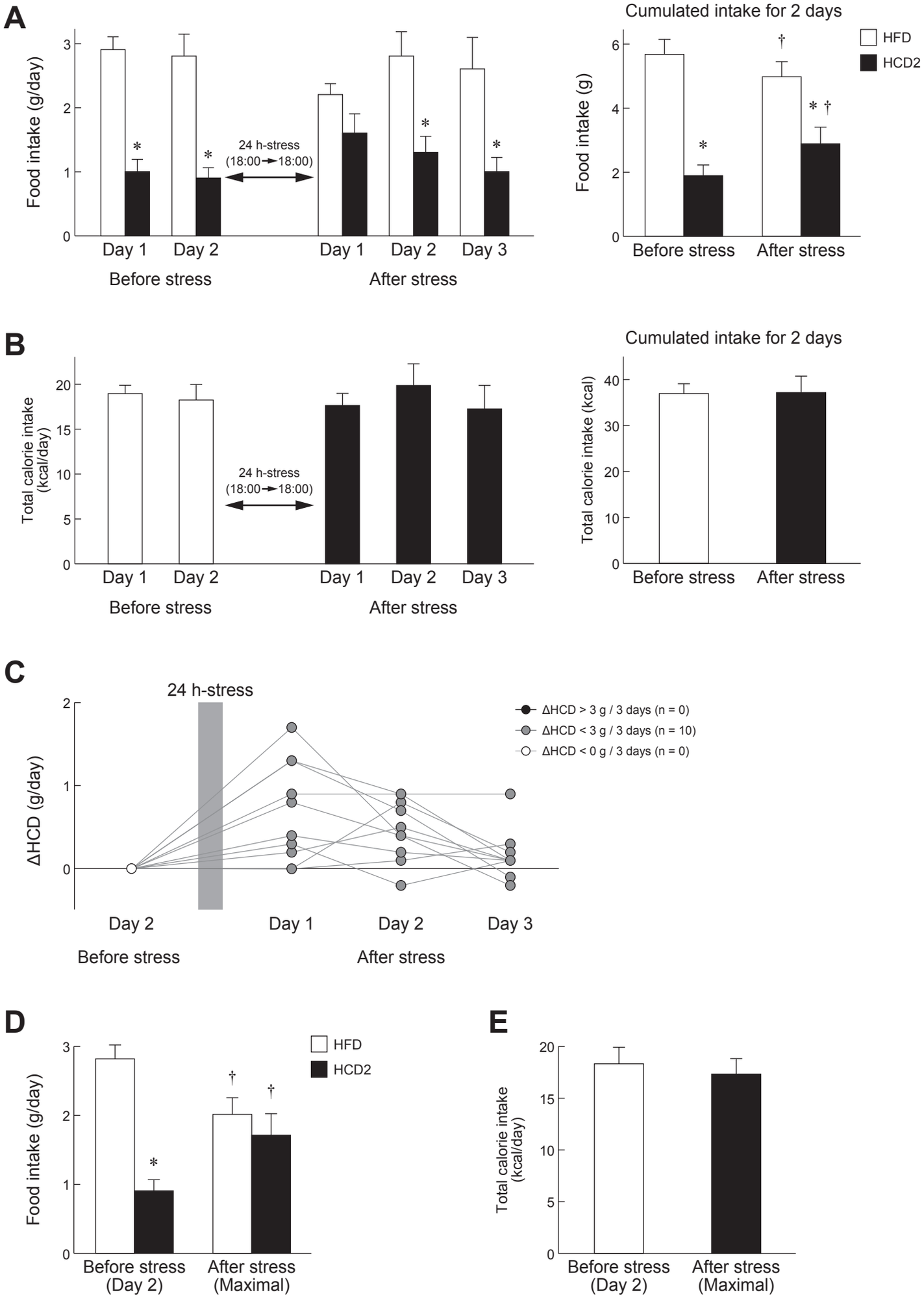

A
Food intake (g/day)
24 h-stress
(18:00→18:00)
Day 1
Day 2
Day 1
Day 2
Day 3
Before stress
After stress
Cumulated intake for 2 days
HFD
HCD2
Food intake (g)
Before stress
After stress
B
Total calorie intake (kcal/day)
24 h-stress
(18:00→18:00)
Day 1
Day 2
Day 1
Day 2
Day 3
Before stress
After stress
Cumulated intake for 2 days
Total calorie intake (kcal)
Before stress
After stress
C
24 h-stress
ΔHCD > 3 g / 3 days (n = 0)
ΔHCD < 3 g / 3 days (n = 10)
ΔHCD < 0 g / 3 days (n = 0)
ΔHCD (g/day)
Day 2
Day 1
Day 2
Day 3
Before stress
After stress
D
HFD
HCD2
Food intake (g/day)
Before stress
(Day 2)
After stress
(Maximal)
E
Total calorie intake
(kcal/day)
Before stress
(Day 2)
After stress
(Maximal)

Figure 4

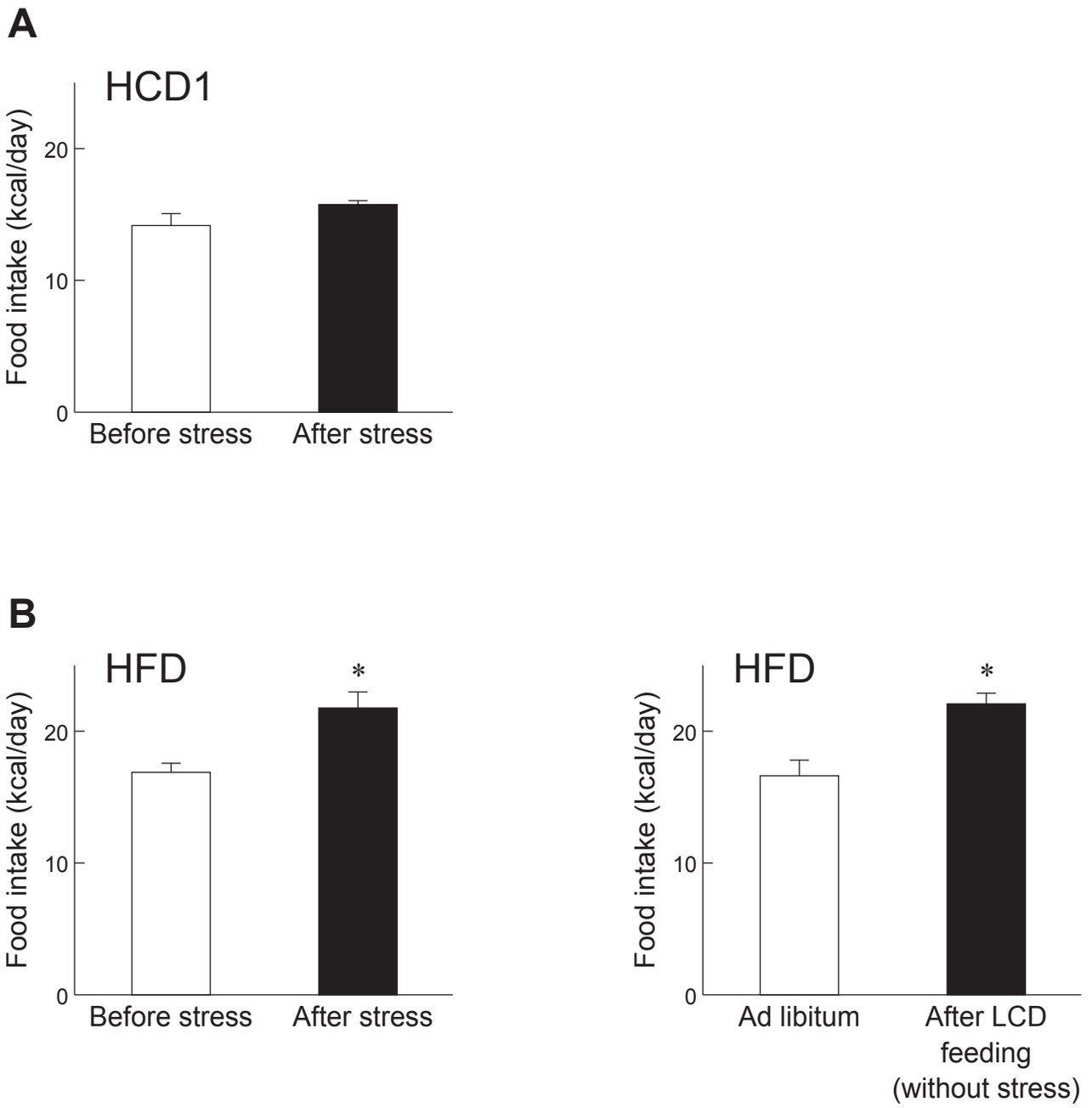

A
HCD1
Food intake (kcal/day)
20
10
0
Before stress
After stress
B
HFD
*
Food intake (kcal/day)
20
10
0
Before stress
After stress
HFD
*
Food intake (kcal/day)
20
10
0
Ad libitum
After LCD feeding (without stress)

Figure 5

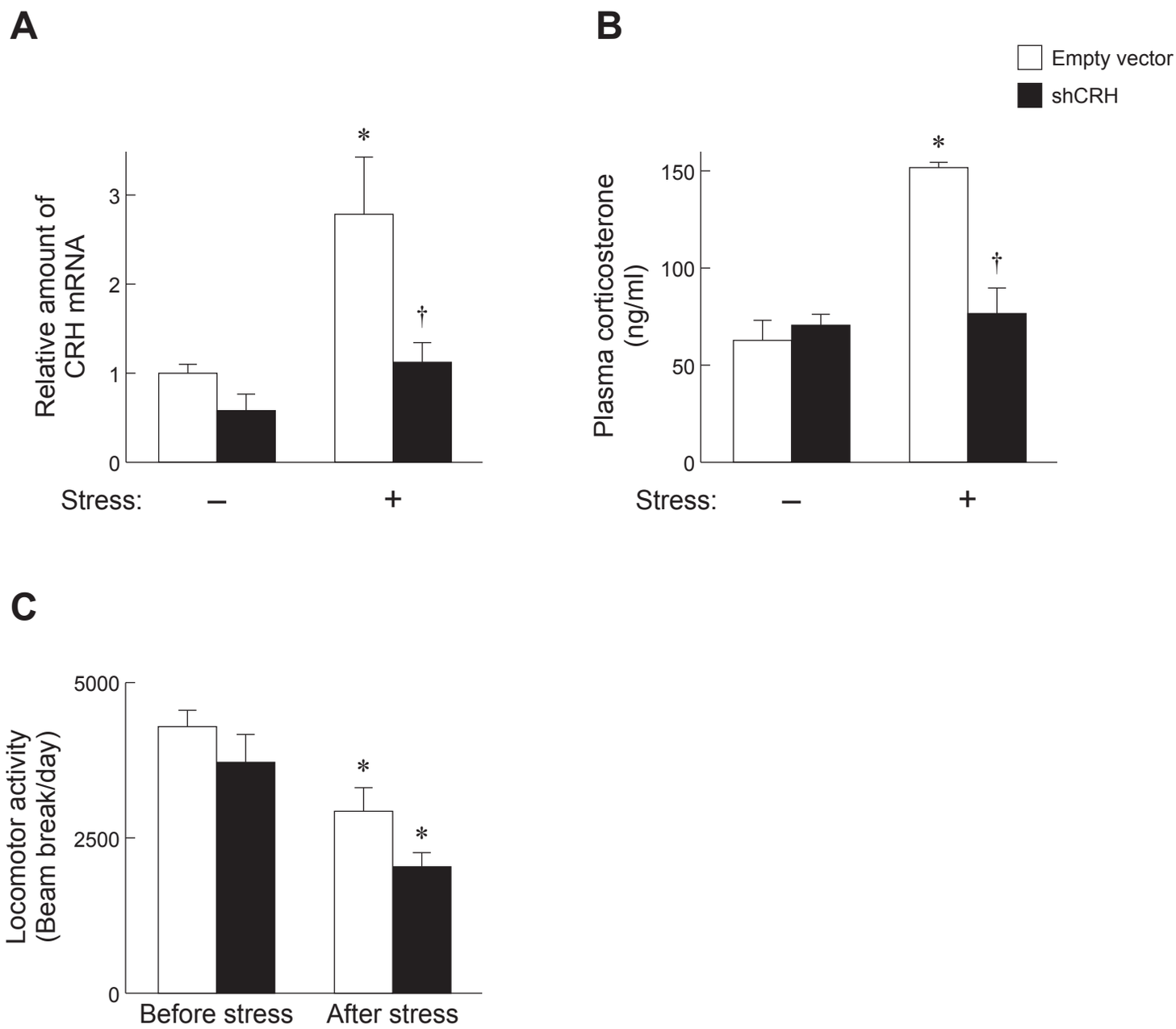
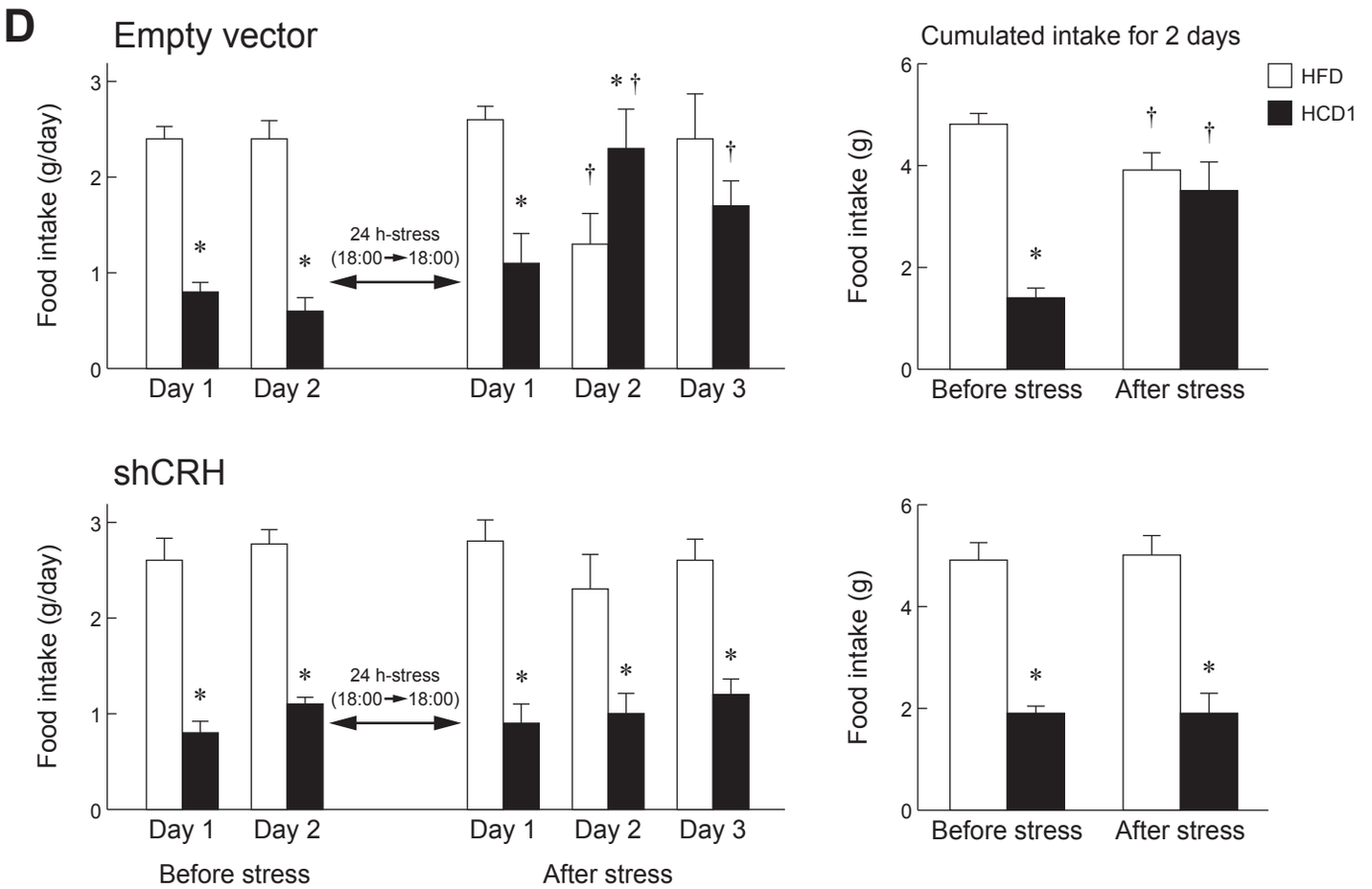

A
Relative amount of CRH mRNA
0
1
2
3
*
†
Stress:
–
+
B
Empty vector
shCRH
Plasma corticosterone (ng/ml)
0
50
100
150
*
†
Stress:
–
+
C
Locomotor activity (Beam break/day)
0
2500
5000
*
*
Before stress
After stress

Figure 5 (continued)



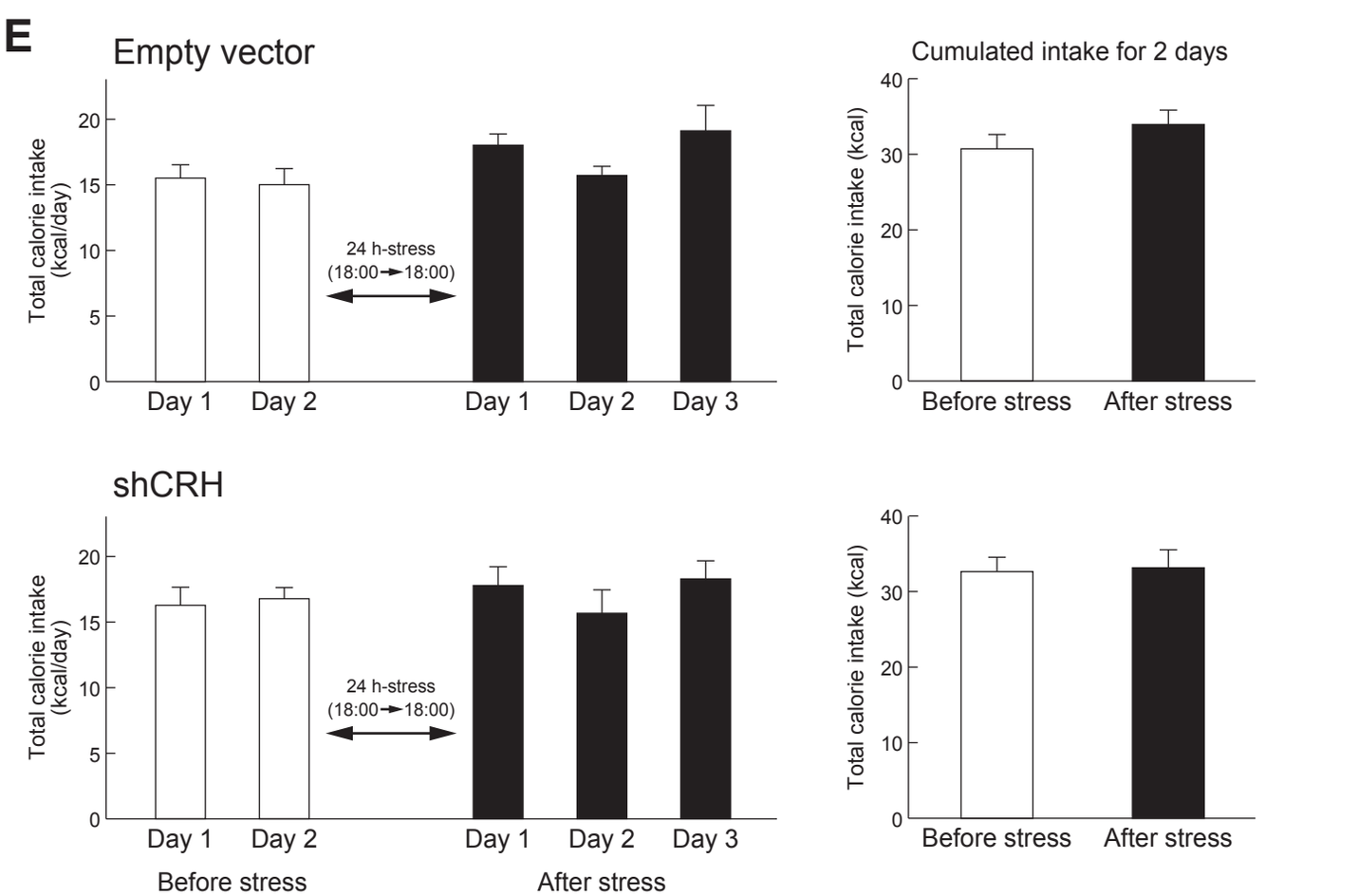


Figure 6

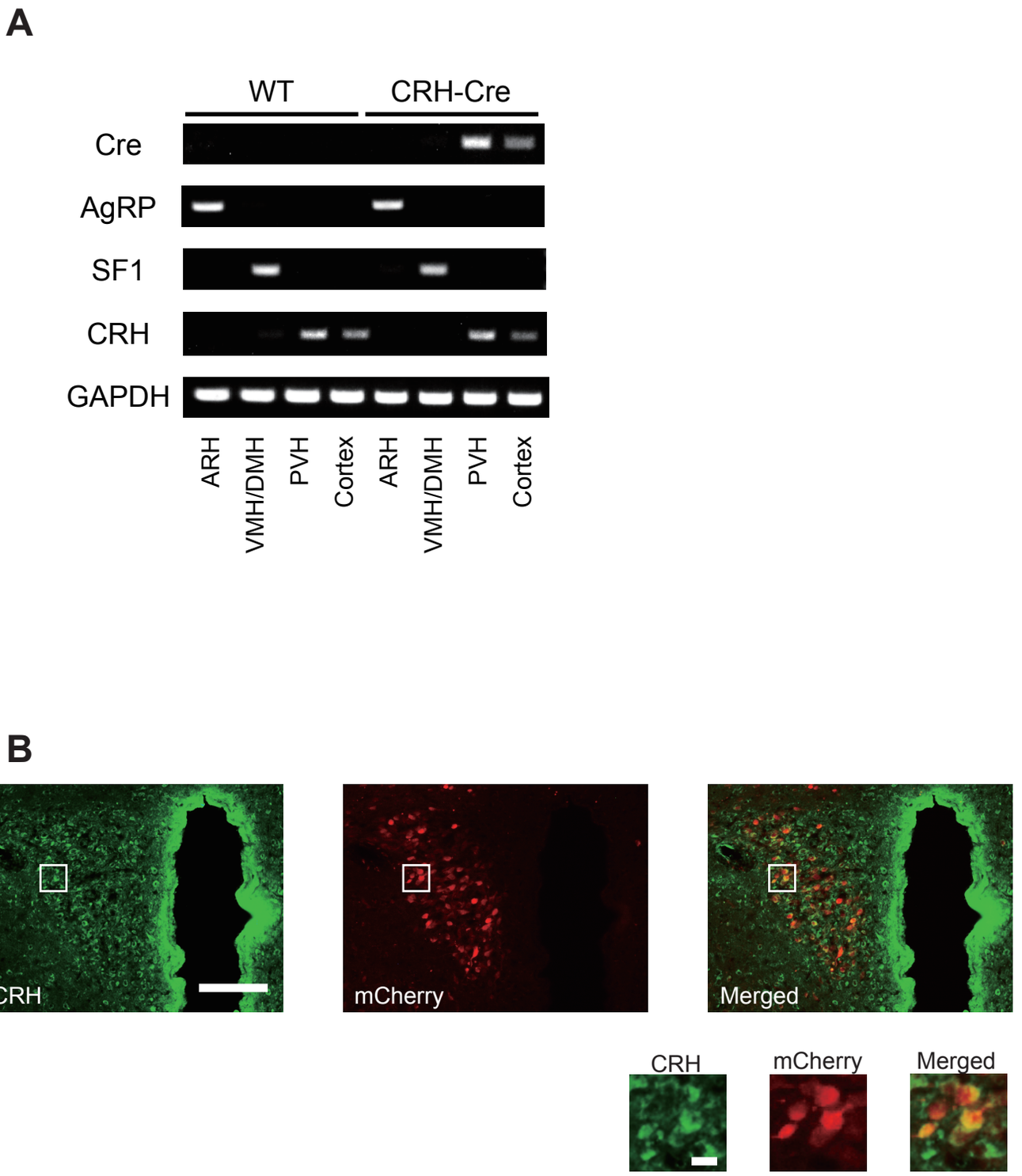
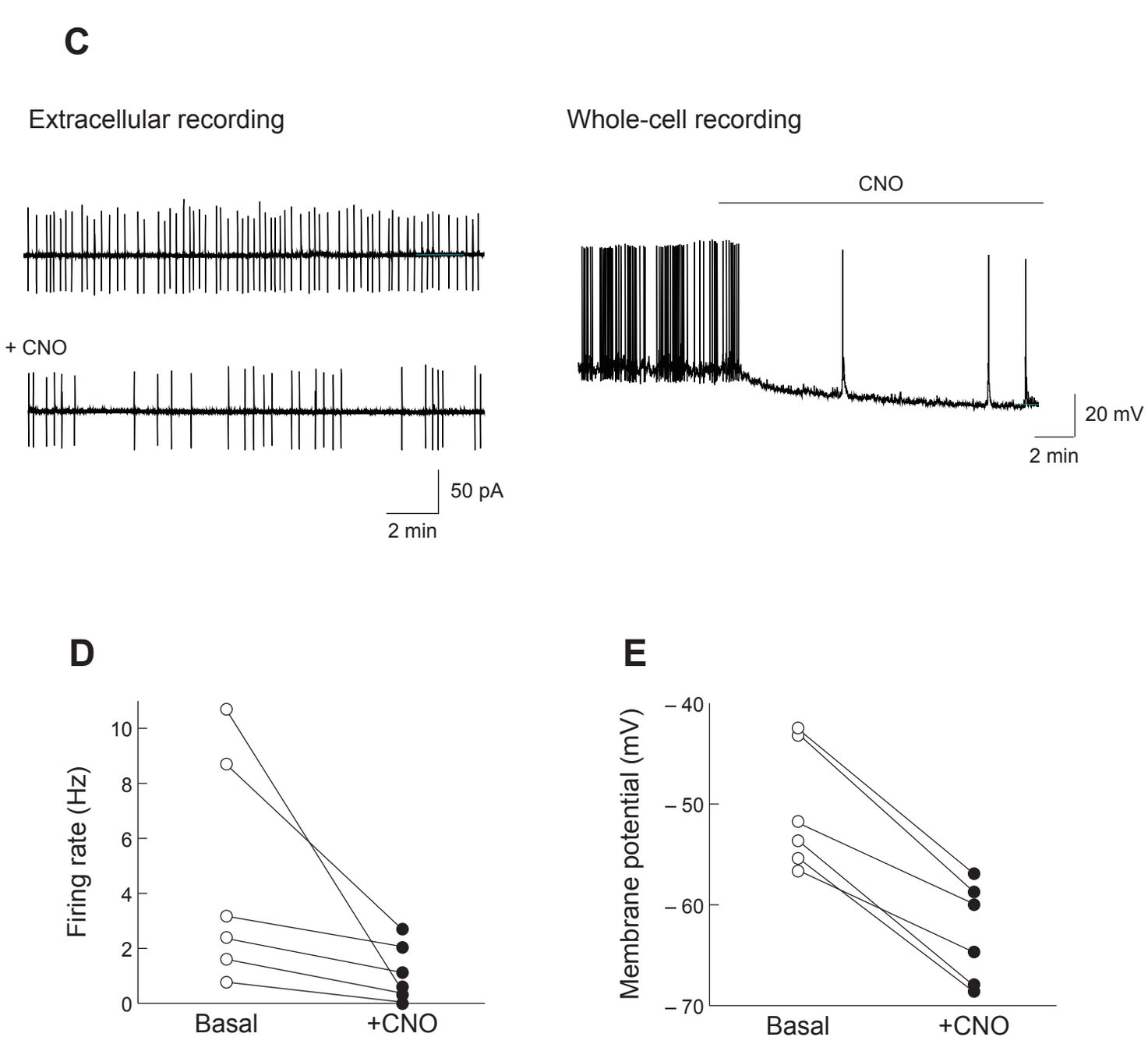

A
WT
CRH-Cre
Cre
AgRP
SF1
CRH
GAPDH
ARH
VMH/DMH
PVH
Cortex
ARH
VMH/DMH
PVH
Cortex
B
CRH
mCherry
Merged
CRH
mCherry
Merged

Figure 6 (continued)



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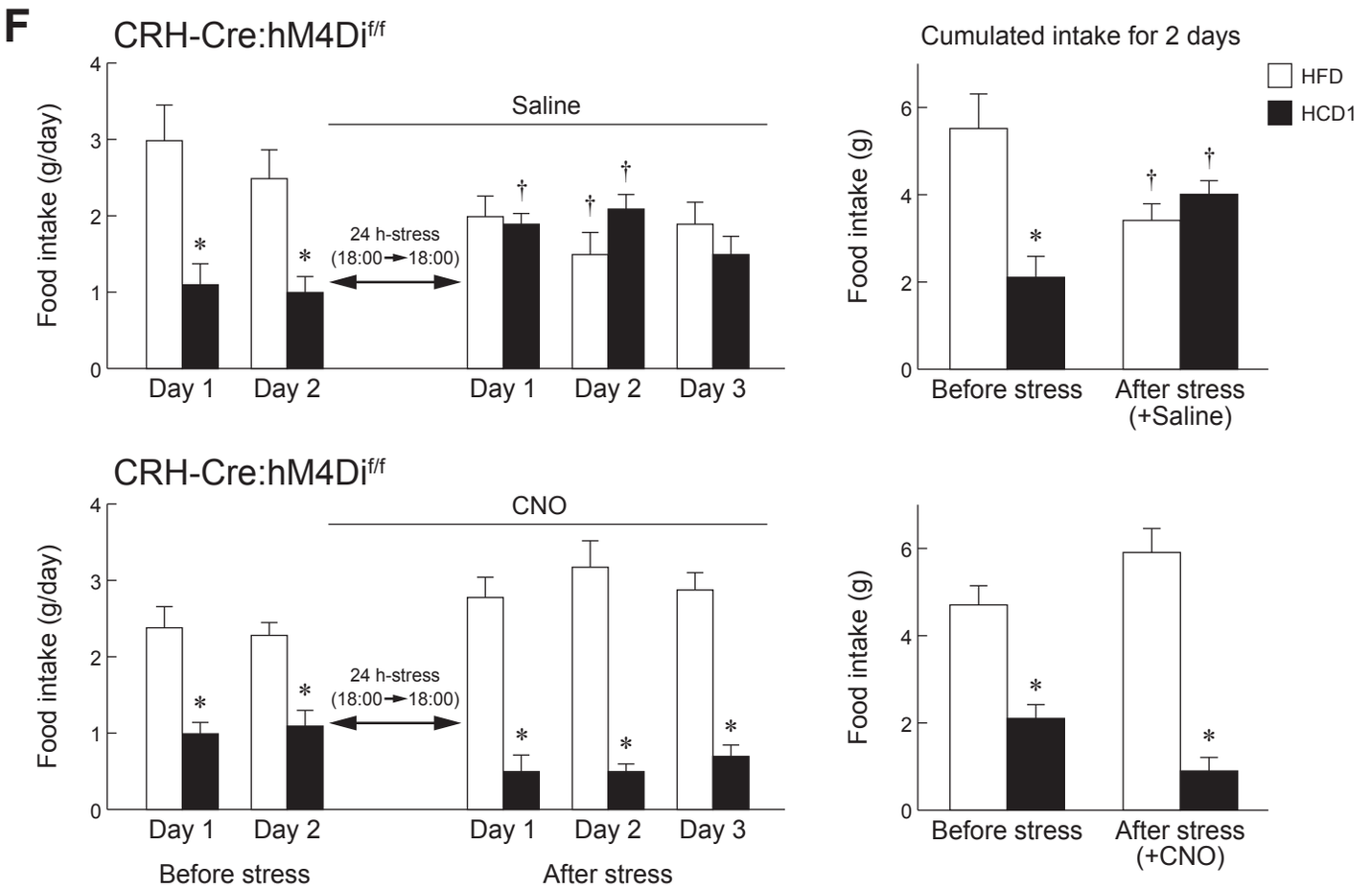


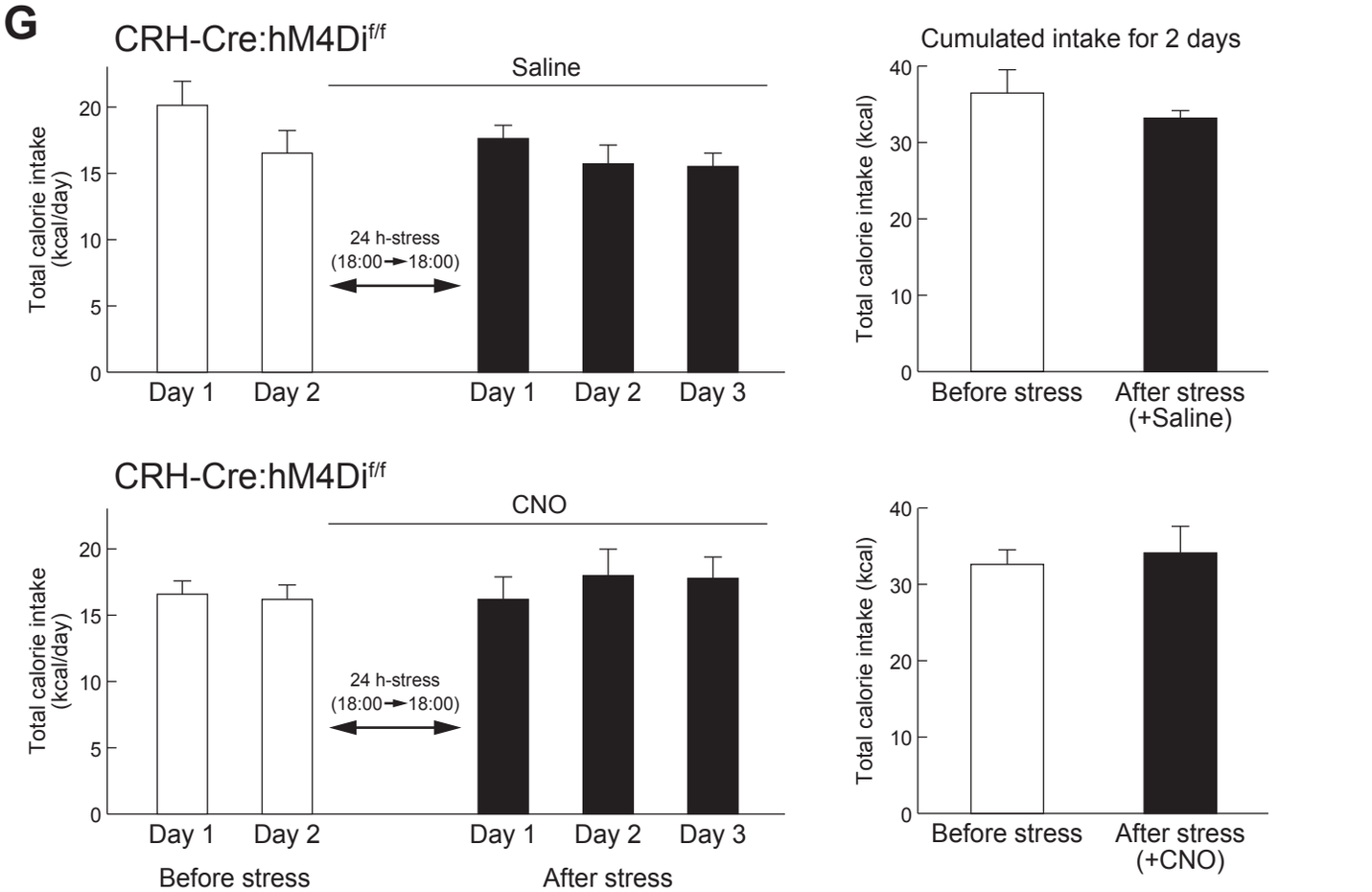
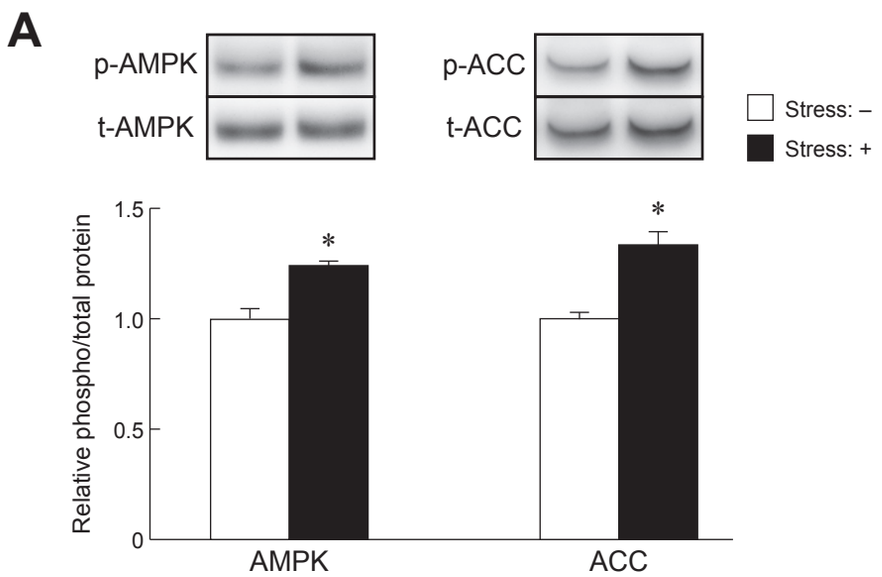
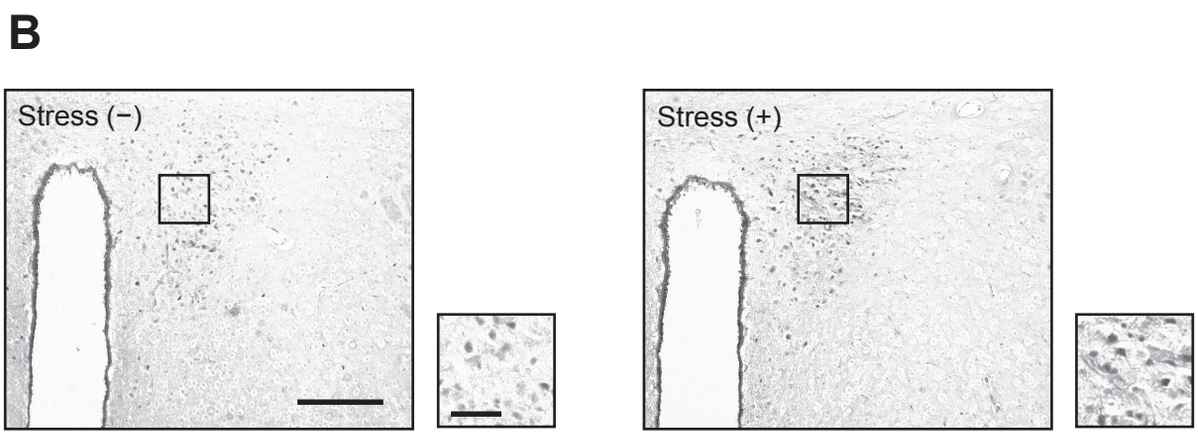


Figure 7


A
p-AMPK
t-AMPK
p-ACC
t-ACC
Stress: –
Stress: +
Relative phospho/total protein
1.5
1.0
0.5
0
*
*
AMPK
ACC


B
Stress (−)
Stress (+)

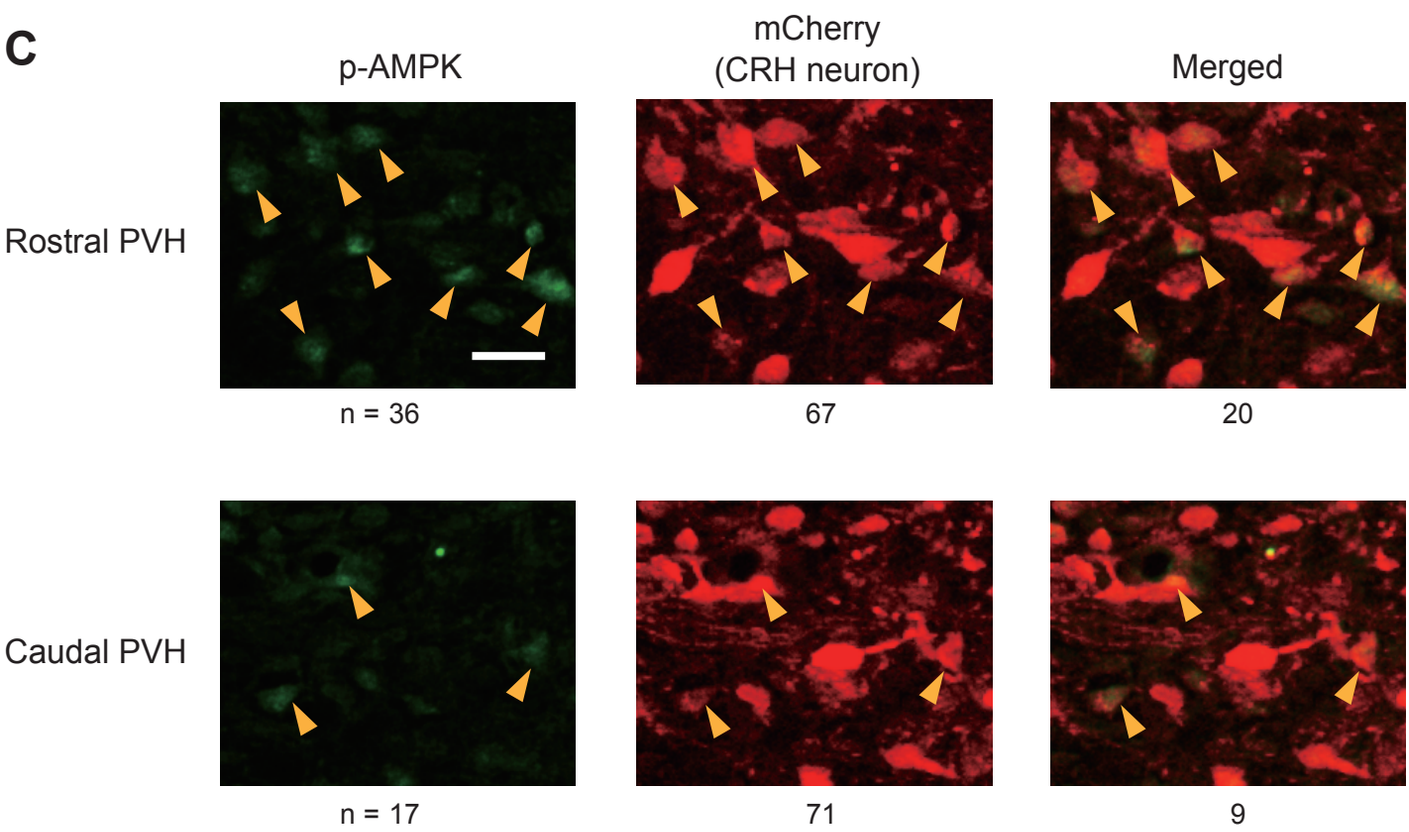

C
p-AMPK
mCherry
(CRH neuron)
Merged
Rostral PVH
n = 36
67
20
Caudal PVH
n = 17
71
9

Figure 8

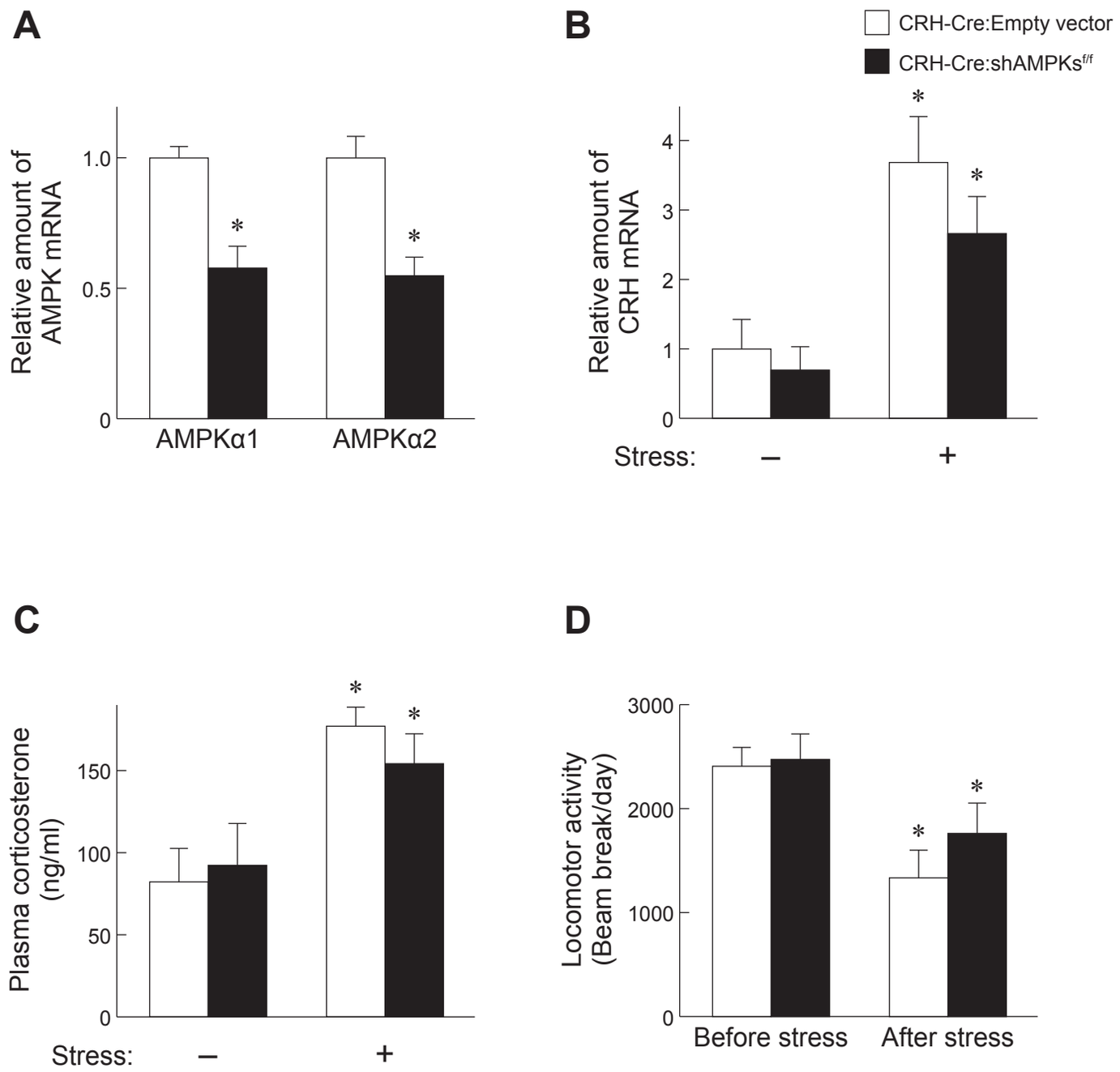
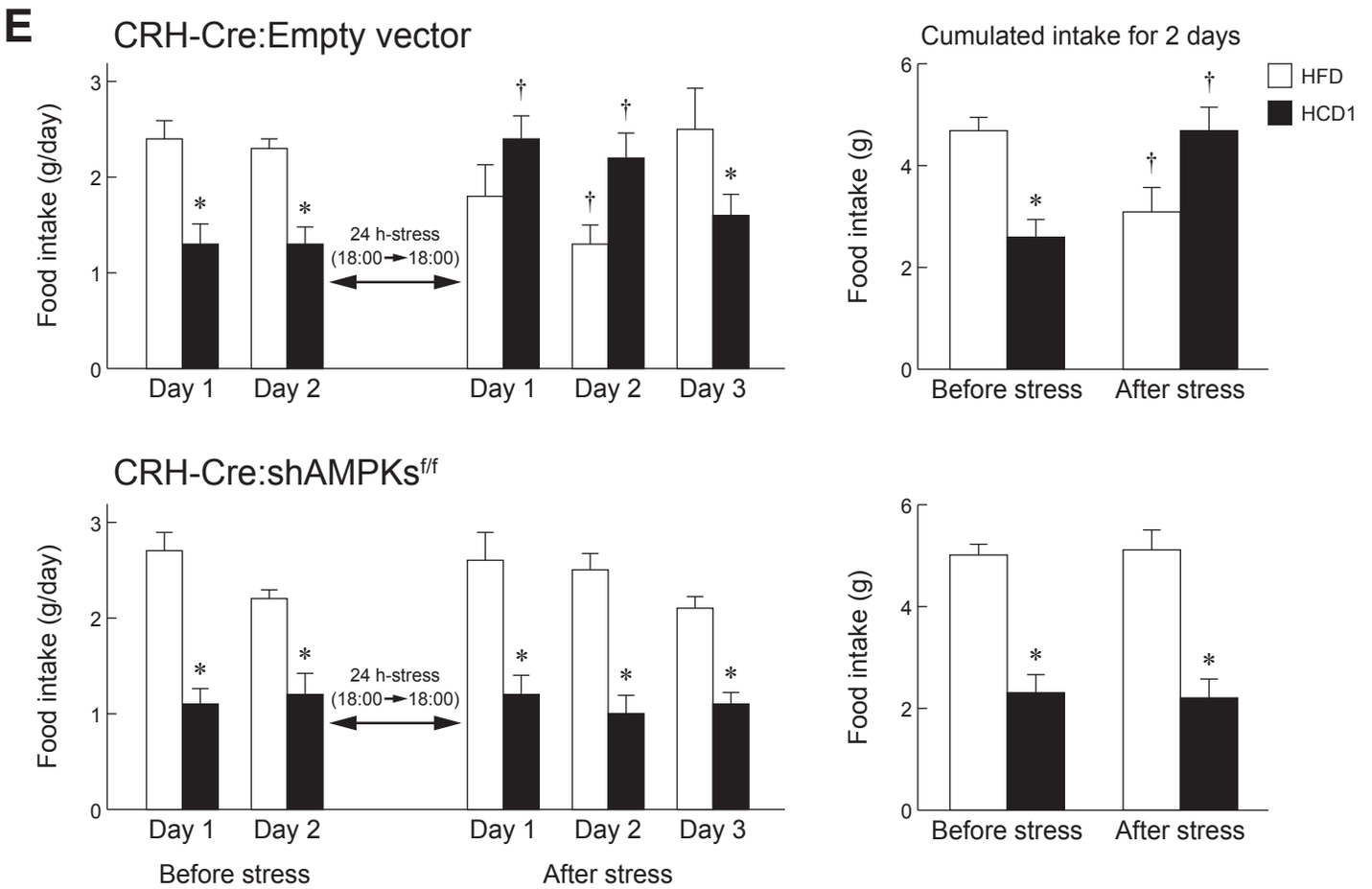

A
Relative amount of AMPK mRNA
1.0
0.5
0
*
*
AMPKα1
AMPKα2
B
CRH-Cre:Empty vector
CRH-Cre:shAMPKsf/f
Relative amount of CRH mRNA
4
3
2
1
0
*
*
Stress:
–
+
C
Plasma corticosterone (ng/ml)
150
100
50
0
*
*
Stress:
–
+
D
Locomotor activity (Beam break/day)
3000
2000
1000
0
*
*
Before stress
After stress

Figure 8 (continued)



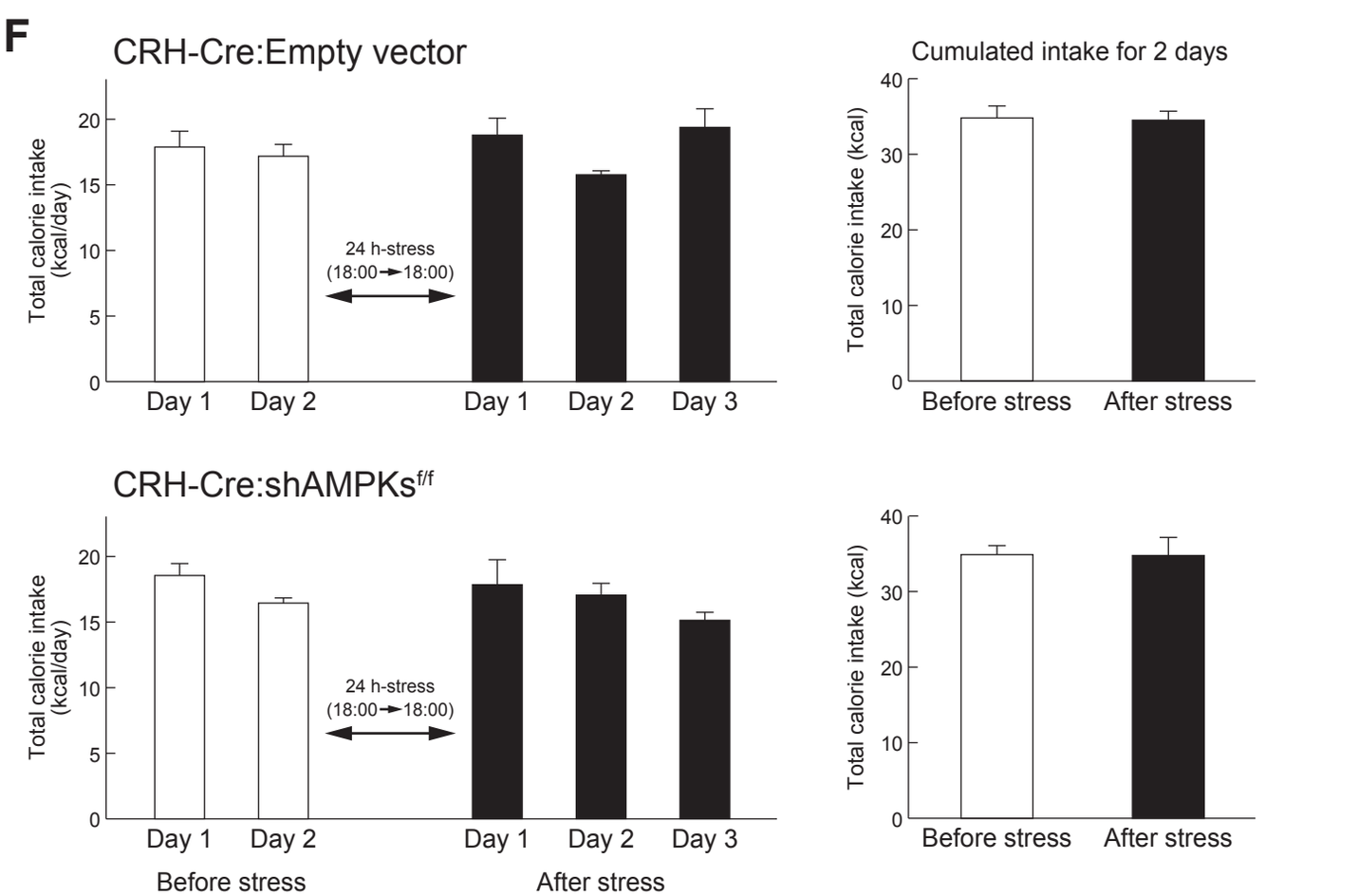


Figure 9

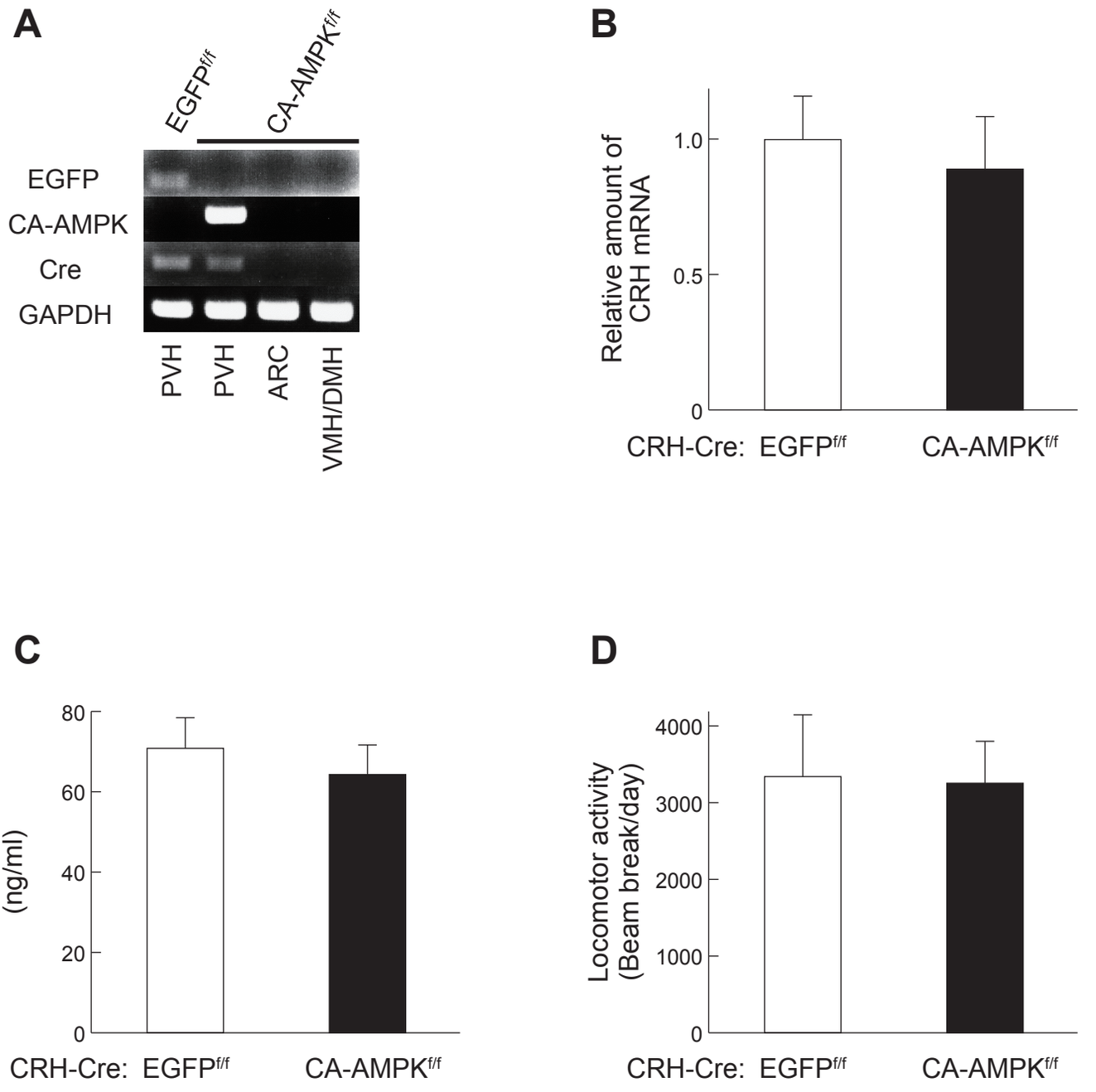

A
EGFP$^{f/f}$
CA-AMPK$^{f/f}$
EGFP
CA-AMPK
Cre
GAPDH
PVH
PVH
ARC
VMH/DMH
B
Relative amount of CRH mRNA
1.0
0.5
0
CRH-Cre: EGFP$^{f/f}$
CA-AMPK$^{f/f}$
C
Plasma corticosterone (ng/ml)
80
60
40
20
0
CRH-Cre: EGFP$^{f/f}$
CA-AMPK$^{f/f}$
D
Locomotor activity (Beam break/day)
4000
3000
2000
1000
0
CRH-Cre: EGFP$^{f/f}$
CA-AMPK$^{f/f}$

Figure 9 (continued)

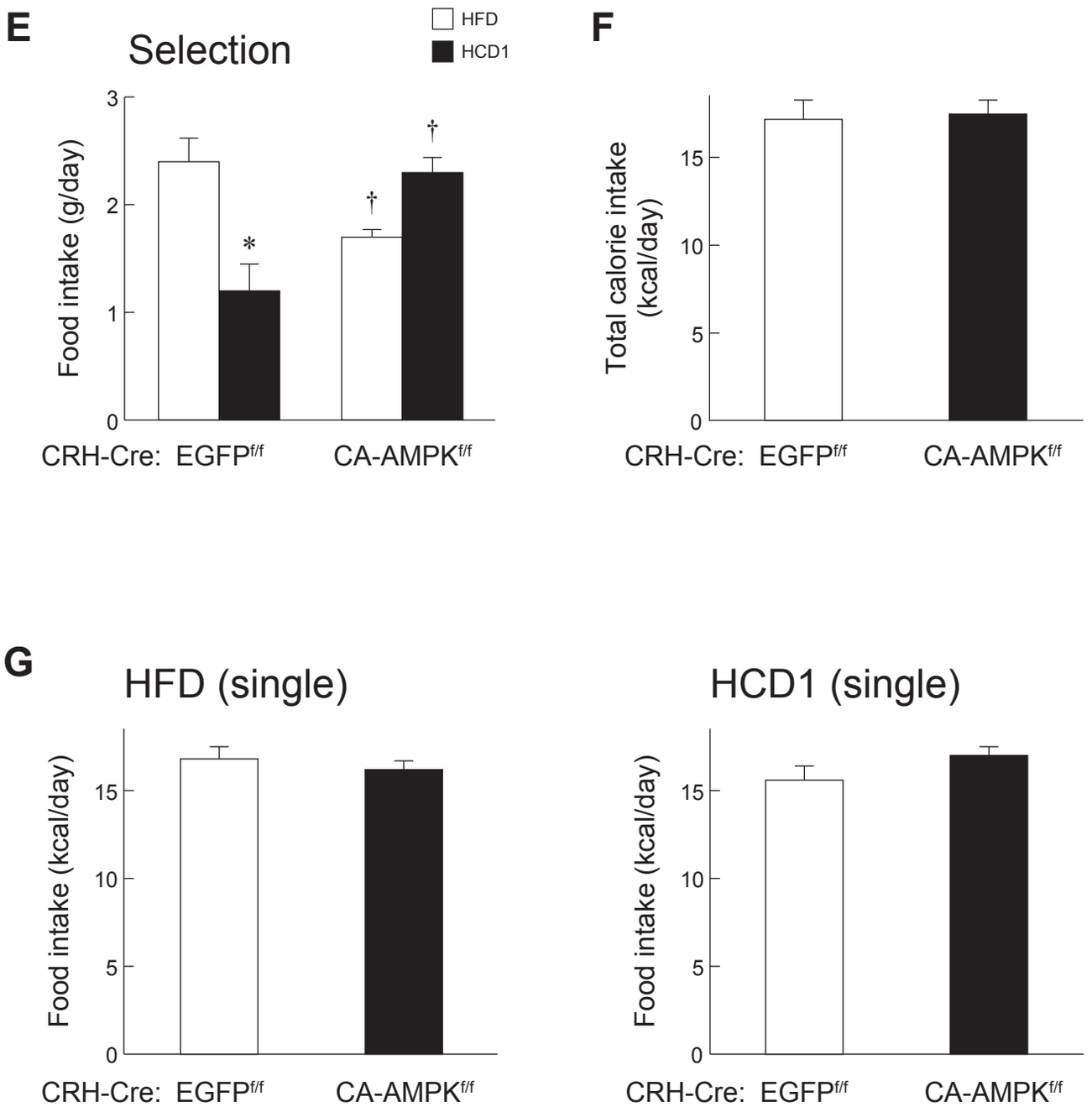


Figure 10

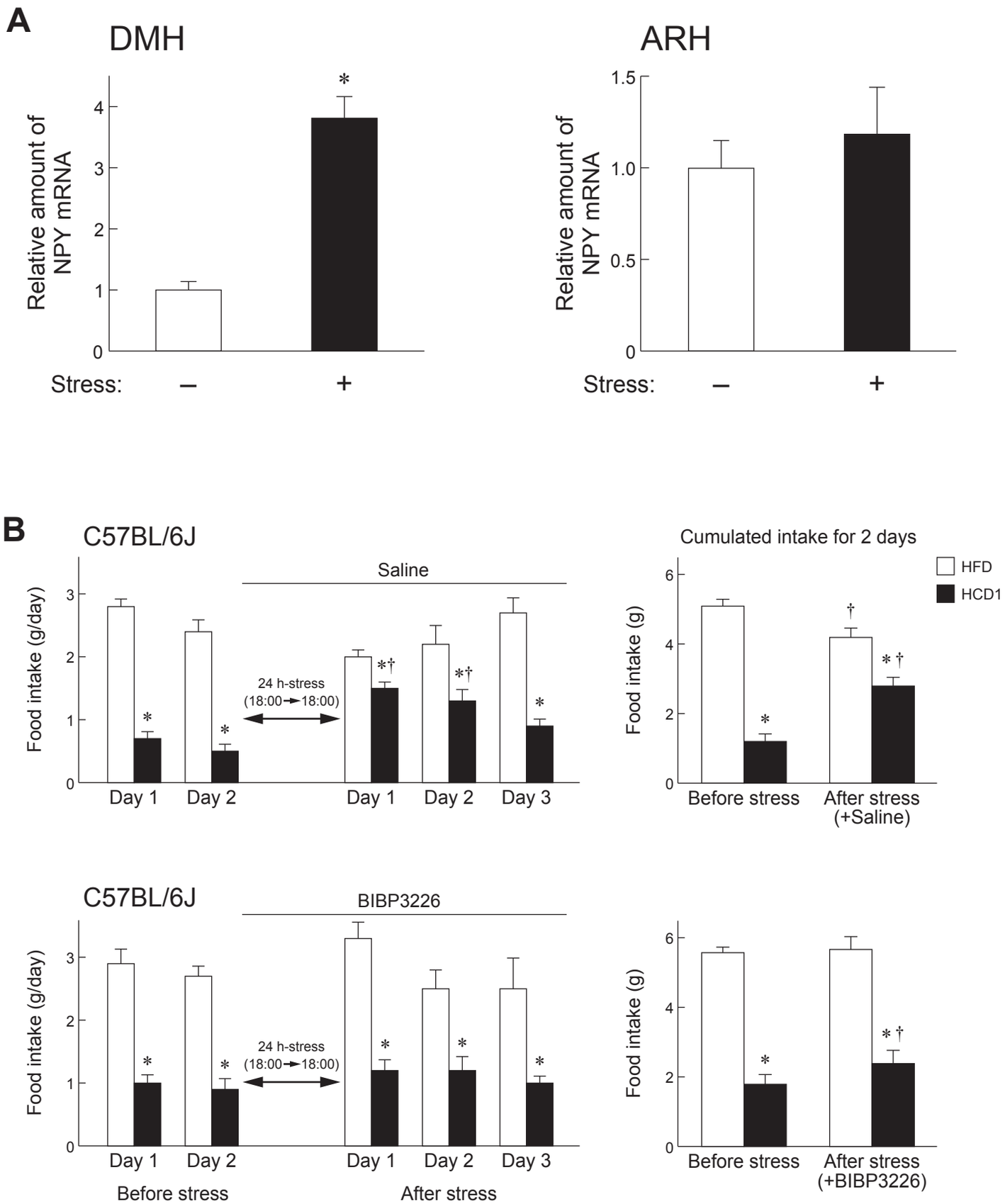

A
DMH
Relative amount of NPY mRNA
Stress:
ARH
Relative amount of NPY mRNA
Stress:
B
C57BL/6J
Saline
Food intake (g/day)
24 h-stress (18:00→18:00)
Day 1
Day 2
Day 1
Day 2
Day 3
Cumulated intake for 2 days
HFD
HCD1
Food intake (g)
Before stress
After stress (+Saline)
C57BL/6J
BIBP3226
Food intake (g/day)
24 h-stress (18:00→18:00)
Day 1
Day 2
Day 1
Day 2
Day 3
Before stress
After stress
Food intake (g)
Before stress
After stress (+BIBP3226)

Figure 10 (continued)

C

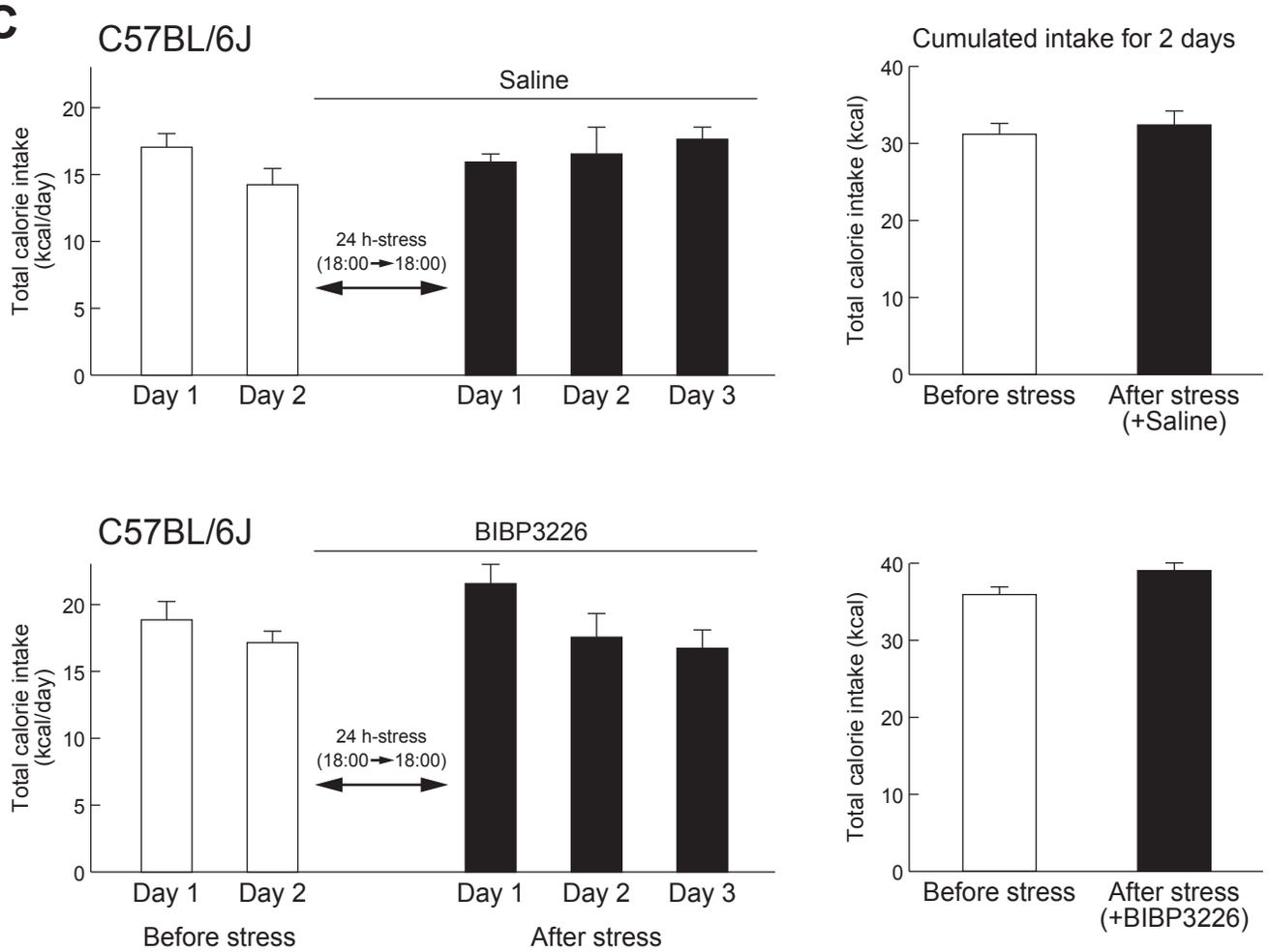
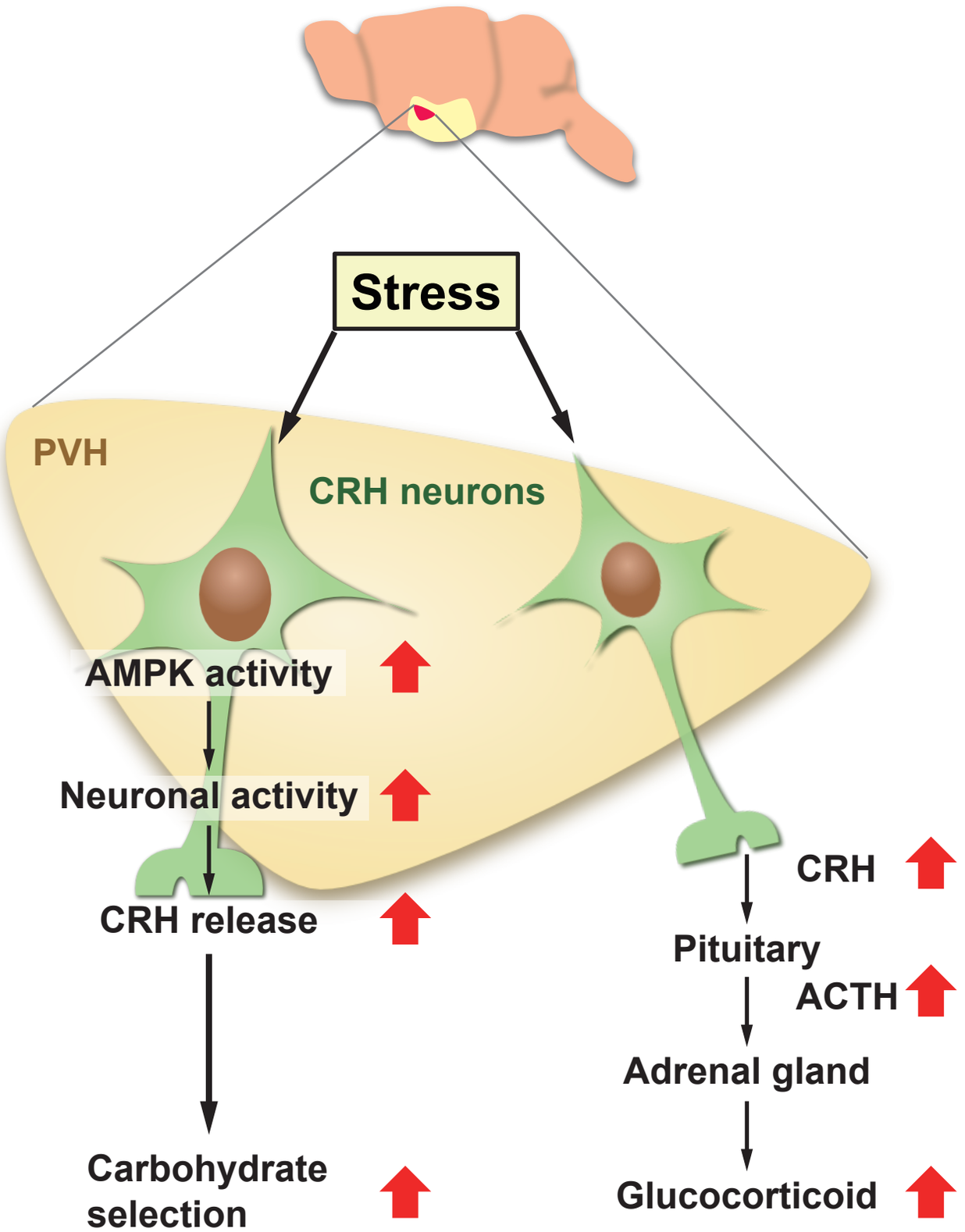


Figure 11


Stress
PVH
CRH neurons
AMPK activity
Neuronal activity
CRH release
Carbohydrate selection
CRH
Pituitary
ACTH
Adrenal gland
Glucocorticoid