Roles for corticotropin-releasing factor receptor type 1 in energy homeostasis in mice

坂本, 竜一

https://doi.org/10.15017/1500443

出版情報：九州大学, 2014, 博士（医学）, 論文博士
バージョン：
権利関係：やむを得ない事由により本文ファイル非公開（2）

Elsevier Editorial System(tm) for Metabolism
Manuscript Draft

Manuscript Number: METABOLISM-D-13-00243R1

Title: Roles for corticotropin-releasing factor receptor type 1 in energy homeostasis in mice

Article Type: Research Paper

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August 9, 2013

Christos Mantzoros, MD, DSc, PhD hon.
Professor of Medicine, Harvard Medical School
Editor-in-Chief
Metabolism

Dear Professor Mantzoros,

Re: "Roles for corticotropin-releasing factor receptor type 1 in energy homeostasis in mice", an original research paper submitted to Metabolism

Thank you for your letter and for the referees' comments dated 17 May, from which I am glad to know that our research in CRFR1KO mice contributes, in principle, to the research field of the HPA axis.

We have studied the reviewers' comments carefully and have made corrections that we hope meet with your approval. I enclose herewith a revised manuscript that includes a report of additional experiments done at the referees' suggestion.

We feel that overall the questions raised by the reviewers are reasonable, and we have answered all of these questions one by one in the following pages. We look forward to your re-evaluation of the revised version of our manuscript.

We would like to thank the reviewers for their helpful comments and hope that we have now produced a more balanced and better account of our work. We trust that the revised manuscript is acceptable for publication in *Metabolism*.

Yours Sincerely,

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Roles for corticotropin-releasing factor receptor type 1 in energy homeostasis in mice

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Heading: CRFR1 in energy homeostasis

Keywords: Corticotropin-releasing factor receptor, diabetes, glucose homeostasis, hepatic steatosis

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Text word count: 4369

Abstract word count: 236

References: 34

Figures: 6

Disclosure: The authors have no conflict of interest to disclose.

The abbreviations used are:

CRF, corticotrophin-releasing factor; CRFR, CRF receptor; Ucn, urocortin; GTT, glucose tolerance test: ITT, insulin tolerance test; HPA, hypothalamus-pituitary-adrenal; GSIS, glucose stimulated insulin secretion; GLP-1, glucagon-like peptide-1; GIP, glucose-dependent insulinotropic polypeptide

Abstract

Objective. Expression of corticotropin-releasing factor type 1 receptor (CRFR1) has been shown on pancreatic β cells, and its activation potentiates glucose-stimulated insulin secretion (GSIS). However, the roles of CRFR1 in energy metabolism beyond insulin release remain elusive.

Materials/Methods. We characterized the metabolic phenotypes of mice lacking CRFR1 (CRFR1KO mice) under conditions of energy excess.

Results. When fed a normal diet, the glucose profile of CRFR1KO mice in response to a glucose tolerance test was similar to that of wild-type (WT) mice, while serum insulin levels were significantly lower in CRFR1KO mice, reflecting high insulin sensitivity in part due to very low glucocorticoid levels. Histology of the pancreas revealed islet hypoplasia in CRFR1KO mice, suggesting a role of CRFR1 in maintaining the β cell mass in a manner similar to incretins. In response to a high-fat diet, CRFR1KO mice showed insulin resistance, but serum insulin levels during glucose challenge remained at a low level, indicating defective GSIS. In addition, CRFR1KO mice showed resistance to diet-induced obesity and hepatic steatosis. Although total food intake was not

different between CRFR1KO and WT mice, oxygen consumption was significantly increased in CRFR1KO mice. The increased energy expenditure may explain the lean phenotype of CRFR1KO mice under conditions of energy excess.

Conclusions. Our results suggest that CRFR1 plays important roles in whole body energy homeostasis, providing compelling evidence of the close relationship between energy homeostasis and the function of the hypothalamic-pituitary-adrenal axis.

Key words: CRFR1, insulin secretion, β cell mass, obesity

1. Introduction

Energy homeostasis is regulated by various hormones and the nervous system in a coordinated fashion. Insulin and glucagon from pancreatic islets are the major regulators of blood glucose levels through the mechanisms of glucose uptake into muscle and adipose tissues and hepatic glucose output. Dysregulation of blood glucose is known to cause type 2 diabetes. There is increasing evidence

69 that the endocrine output of pancreatic islets is substantially modulated by an
70 intricate network of extracellular signals. The incretins, secreted from
71 neuroendocrine cells within the intestinal tract, potentiate glucose-stimulated
72 insulin secretion (GSIS) and facilitate rapid normalization of post-prandial
73 hyperglycemia (1).
74 The effects of stress on energy homeostasis and the involvement of the
75 neuropeptide corticotropin-releasing factor (CRF) in modulating the anorexia of
76 stress and sympathetic nervous system tone are well recognized (2). CRF,
77 originally characterized as the principal neuroregulator of the
78 hypothalamic-pituitary-adrenal (HPA) axis, has broad central and peripheral
79 distribution and actions. The presence of a receptor system for CRF has been
80 demonstrated in peripheral tissues involved in the regulation of immune,
81 cardiovascular and energy homeostasis (3-5). Activation of the HPA axis by
82 stressful stimuli is known to increase visceral adiposity, and thus lead to the
83 metabolic syndrome (6). Moreover, a relationship between diabetes and altered
84 HPA axis function has been indicated in humans (7, 8).
85 The CRF system consists of two different receptors (CRFR1 and CRFR2) and

four ligands: CRF, urocortin 1 (Ucn1), Ucn2 and Ucn3 (9). CRF has a higher affinity for CRFR1 than for CRFR2, while Ucn1 exhibits higher affinity for and activation of CRFR2 than does CRF. Ucn2 and Ucn3 are selective for CRFR2 with little or no affinity for CRFR1 (10-13). Recently, two members of the CRF family of neuropeptides, CRF and Ucn3, were demonstrated to promote GSIS in an incretin-like manner (14-17). CRF receptors, together with glucagon-like peptide-1 (GLP-1) and glucose-dependent insulinotropic polypeptide (GIP), belong to the family of class B G protein-coupled receptors (14).

A functional CRF receptor system, including both CRFR1 and CRFR2, has also been shown to be expressed in pancreatic islets. Ucn2 and Ucn3, by acting through CRFR2 in peripheral tissues, are well known to be modulators of glucose homeostasis and metabolic function. The role of the CRF-CRFR1 system in energy metabolism has also been the focus of attention. However, the roles of CRFR1 in energy metabolism beyond insulin release remain elusive. In this study, we therefore investigated the metabolic function of CRFR1 using mice lacking CRFR1 (CRFR1KO mice). CRFR1KO mice showed resistance to diet-induced obesity and hepatic steatosis. Metabolic assessment revealed that

energy expenditure in CRFR1KO mice was significantly increased, explaining their lean phenotype. Our results suggest that CRFR1 plays important roles in whole body energy homeostasis through both peripheral and central mechanisms.

2. Materials and Methods

2. 1. Animals

CRFR1KO mice were obtained from the Jackson Laboratory (Bar Harbor, ME, USA). CRFR1KO and WT mice were obtained as littermates from mating pairs of heterozygous parents (CRFR1+/-) and male mice were used for the experiments. All experiments were performed on mice with the C57BL/6 x 129/Sv hybrid genetic background. All animals were maintained on a 12-h light (8:00-20:00)/12-h dark (20:00-8:00) cycle in a temperature and humidity controlled room (22 °C degree, 55.0% humidity). They had free access to tap water and a standard commercial pellet diet with the following composition: 48.9% carbohydrate, 26.8% protein, 5.0% fat, and an energy content of 347.4 kcal/100 g (CLEA Rodent Diet CA-1; Kyudo, Tosu, Japan). Mice were fed a

high-fat diet with the following composition: 54.4% carbohydrate, 24.4% protein, 34% fat, and an energy content of 342.2 kcal/100 g (Oriental Yeast, Suita, Japan) for 12 weeks from 6 to 18 weeks of age. Mouse experiments were performed according to the guidelines of the animal ethics committee of Kyushu University Graduate School of Medicine.

2.2. RT-PCR

Total RNA was isolated from several tissues including cerebrum, pituitary gland, islet, skeletal muscle, subcutaneous white adipose tissue (WAT), interscapular brown adipose tissue (BAT), and liver using ISOGEN (Nippon Gene Co. Ltd, Tokyo, Japan) according to the manufacturer's protocol. Total RNA was subjected to reverse transcription using Superscript III reverse transcriptase (Invitrogen, Carlsbad, CA) primed by Oligo(dT) 15 primers (Promega corporation, Madison, WI, USA). Quantitative analyses of the expression of CRF receptors were performed using a Roche LightCycler (Roche, Mannheim, Germany) based on a florescence monitoring system as previously described (18). Primer sets used in this study were as follows; CRFR1,

5’-GGTGTGCCTTTCCCCATCATT-3’/5’-TGATCAGCAGGACCAGGATCA-3’ (138 bp); CRFR2, 5’-ATGACCTGCATTACCGAATCGC-3’/5’-CCACGCGATGTTTCTCAGAATG -3’ (178 bp); β-actin, 5’-ACCAACTGGGACGACATGGAG-3’/5’-GTGGTGGTGAAGCTGTAGCC-3’ (379 bp); Srebp1c, 5’-TGCGGCTGTTGTCTACCATA-3’/5’-TGCTGGAGCTGACAGAGAAA -3’ (578 bp); Fasn, 5’-CTCCGTGGACCTTATCACTA-3’/5’-CTGGGAGAGGTTGTAGTCAG -3’ (192 bp); Acc, 5’-CCAGGCCATGTTGAGACGCT-3’/5’-ATCACAGAGCGGACGCCATC -3’ (132); Cpt1a, 5’-CCGATCATGGTTAACAGCAA-3’/5’-TGCAGCAGAGATTTGGCATA -3’ (596); leptin, 5’-TGTCCAGGGTTGATCTCACA-3’/5’-GGAACAAAACTCCCCACAGA -3’ (131); adiponectin, 5’-GACGTTACTACAACTGAAGAGC-3’/5’-CATTCTTTTCCTGATACTGGTC-

154 3’ (532). Numbers in parentheses indicate the size of the PCR products for each
155 primer pair. RNA extraction, cDNA preparation and quantitative PCR were all
156 performed in triplicate. The cDNA was used for quantitative real-time PCR
157 using the FastStart DNA Master SYBR Green I kit (Roche, Mannheim,
158 Germany). The PCR conditions were 95 °C for 10 min followed by 40 cycles at
159 95 °C for 15 s and 60 °C for 1 min. Forty cycles of PCR were programmed to
160 ensure the threshold crossing point (cycle number) was attained. Fluorescence
161 emission was monitored continuously during cycling. At the completion of
162 cycling, melting curve analysis was carried out to establish the specificity of the
163 amplicons produced. The level of expression of each mRNA and their estimated
164 crossing points in each sample were determined relative to the standard
165 preparation using LightCycler computer software.
166
167 2.3. Histological analysis and biochemical assays
168 The whole pancreas was excised, weighed and cut into two pieces corresponding
169 to the head (duodenal) and the tail (spleen) parts of the organ. The tissues were
170 separately fixed in 4% (w/v) paraformaldehyde overnight at 4 °C, dehydrated in

100% ethanol, and embedded in paraffin. Each entire pancreatic piece was cut, through its length, into 5 µm thick sections. Every 30th section of each pancreas piece was stained with Mayer's hematoxylin solution for 5 min, washed with water, counterstained with 0.5% eosin alcohol solution for 2 min, dehydrated, and mounted, yielding eleven sections per animal. For immunostaining of insulin, glucagon and pancreatic polypeptide, sections were subjected to antigen retrieval using DAKO Cytomation Target Retrieval Solution (Dako Corporation, Carpinteria, CA, USA). After blocking with BlockAce (Dainippon Sumitomo Pharmaceutical Co. Ltd., Osaka, Japan), sections were incubated with primary antibodies at 4 °C for 12-16 h, followed by 1 h incubation with secondary antibodies. Guinea pig anti-porcine insulin antibody (1:500), rabbit anti-human glucagon antibody (1:500) and rabbit anti-human pancreatic polypeptide antibody (Dako Corporation) were used as the primary antibodies. Anti-guinea pig-HRP conjugated antibody (Dako Corporation) and anti-rabbit-HRP conjugated antibody (Cell Signaling Technology Inc., Danvers, MA, USA) were used as the secondary antibodies. Goat anti-mouse CRF-R1 polyclonal antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) was used as the primary

antibody and florescent-labeled rabbit anti-goat immunoglobulin (Alexa Flour 594, Molecular Probes, Eugene, OR, USA) was used as the secondary antibody. The livers and the epididymal WAT were excised and fixed in 4% (w/v) paraformaldehyde overnight at 4 °C. Paraffin sections were stained with hematoxylin and eosin as described above. Frozen sections of paraformaldehyde-fixed liver were stained with Oil-red O using standard techniques. Staining of the sections was detected using a BioZero laser microscope (Keyence, Osaka, Japan). The insulin content of whole pancreas was determined after extraction with acid-ethanol. Insulin levels were measured using a rat/mouse insulin ELISA kit (Morinaga Institute of Biological Science Inc., Kanagawa, Japan). Plasma corticosterone levels were assayed using a Cayman corticosterone EIA Kit (Cayman Chemical Co., Ann Arbor, MI, USA). Total liver triglyceride was determined using the Folch method. Briefly, 500 mg liver samples were homogenized in methanol: chloroform. Samples were then centrifuged and aliquots from the lower phases were collected and evaporated. Triglyceride levels were measured using the TG E-test (Wako, Osaka, Japan) according to the manufacturer's protocol.

205

206 2.4 Morphometry measurements

207 The pancreatic tissue area was determined by computer-assisted measurements

208 using a Keyence BioZero microscope. The area of each pancreatic islet was

209 measured and the data were subjected to statistical analysis using Excel

210 (Microsoft Japan, Tokyo, Japan). Islets less than 10,000 μm^2 in area were

211 defined as small; those ranging from 10,000–20,000 μm^2 as medium, and those

212 exceeding 20,000 μm^2 as large.

213

214 2.5 Metabolic measurements, insulin, and glucose tolerance tests

215 Mice were weighed weekly and food intake was measured every 4 min for 3 days

216 from individually housed mice using a food consumption monitor system

217 (NeuroScience Idea. Co., Ltd., Osaka, Japan). Spontaneous physical activity was

218 measured using a Letica infrared system (Panlab, Barcelona, Spain) as

219 previously described (19). Mice were fed regular chow, maintained at a constant

220 room temperature and humidity (25 °C degree, 55.0% humidity), and subjected

221 to oxygen consumption measurements at 20 weeks of age using a

computer-controlled open-circuit indirect calorimeter (Oxymax; Columbus Instruments, Columbus, OH, USA). Mice were housed individually in metabolic chambers (10 x 20 cm^2) and had free access to food and water. After a 1 h adaptation to the chamber, VO_2 was assessed at 4 min intervals for 24 h. All sample data were analyzed using Oxymax Windows software. Mice were fasted overnight and baseline blood glucose levels were measured in tail-vein blood using a Glutest Sensor (Sanwa Kagaku Inc., Nagoya, Japan). Glucose (1 mg dextrose/g body weight) in sterile phosphate buffered saline was injected intraperitoneally, and blood glucose levels were measured 15, 30, 60, 90, and 120 min after injection. To measure plasma insulin levels, ~50 µl of blood was collected from the tail vein before, and 15 and 30 min after, intraperitoneal injection with glucose. For the insulin tolerance test, mice were fasted for 4 h and intraperitoneally injected with regular insulin (0.5 units/kg body weight). Blood glucose levels were measured prior to, and 15, 30, 60 min after, injection.

2.6 Analysis of Pancreatic Islets

Pancreatic islets of adult non-fasted CRFR1KO and WT mice (4–6 months old)

were isolated as described (20), using digestion with collagenase type IV (Worthington Biochemical Corp., Lakewood, NJ, USA). In brief, mice were euthanized and collagenase in Hank's solution (Life Technologies, Carlsbad, CA, USA) was injected into the biliary tract. The pancreas was then excised and incubated for 13 min at 37 °C. After incubation and agitation, the suspension was transferred to centrifuge tubes and washed three times in Hanks' solution followed by centrifugation for 1 min at 150 × g. Islets were obtained by density gradient with HISTOPAQE-1077 and -1119 (Sigma Aldrich, Tokyo, Japan), and picked under a stereomicroscope.

For measurement of insulin secretion from islets, pancreatic islets from 4- to 6-month-old mice were pooled and pre-cultured in RPMI 1640 medium (Sigma, Tokyo, Japan) containing 10% FBS at 37 °C and under 5% CO_2 for 16 h. Islets were then transferred to Krebs-Ringer bicarbonate-4-(2-hydroxyethyl)-1-piperazine ethanesulfonic acid (HEPES) buffer (KRB; 140 mM NaCl, 3.6 mM KCl, 0.5 mM NaH2PO4, 0.5 mM MgSO4, 1.5 mM CaCl2, 2 mM NaHCO3, 10 mM HEPES, and 0.1% BSA) containing 2.8 mM glucose for stabilization. After 60 min incubation, islets were stimulated

256 with 11.2 or 22.4 mM glucose for 60 min followed by the measurement of
257 insulin secretion using a Rat/Mouse insulin ELISA kit (Morinaga Institute of
258 Biological Science Inc.). For the stimulation study, 100 nM CRF (Peptide
259 Institute, Osaka, Japan) was added to the KRB buffer.
260
261 2.7 Statistics
262 All experiments were carried out at least three times. Data are described as the
263 mean ± SEM and were analyzed by ANOVA. The level of statistical significance
264 were taken as $P<.05$.
265
266 **3. Results**
267 3.1 CRF receptor expression in peripheral tissues
268 mRNA expression of the CRF receptors was analyzed in central and peripheral
269 tissues of WT mice (Fig.1). CRFR1 mRNA was predominantly expressed in the
270 cerebrum, but it was also observed in pancreatic islets and the pituitary gland. In
271 contrast, CRFR2 mRNA was expressed in skeletal muscle, brown adipose tissue
272 and liver, consistent with previous reports (14, 21-23). Our results again

273 confirm the differential expression of CRFRs, suggesting a functional diversity
274 between CRFR1 and CRFR2. CRFR1 is well known to be involved in the HPA
275 axis, activation of which may lead to visceral adiposity and thus metabolic
276 syndrome and diabetes (6-8). Recently, CRFR1 has been shown to be expressed
277 in pancreatic β cells and involved in insulin secretion (14). Therefore, CRFR1
278 may control energy homeostasis at both central and peripheral levels. To clarify
279 the role of CRFR1 in energy homeostasis, we have characterized the metabolic
280 aspects of CRFR1 function using CRFR1KO mice. It is noted that CRFR2
281 expression was not changed in islets from CRFR1KO mice (data not shown),
282 suggesting that there is no compensatory mechanism between CRFR1 and
283 CRFR2 expression in pancreatic β cells.
284
285 3.2 Enhanced insulin sensitivity in CRFR1KO mice
286 The body weight of CRFR1KO mice was comparable to that of WT mice (Fig.
287 2A). As shown in Fig. 2B, blood glucose levels in response to *ad libitum* feeding
288 showed no statistical difference between CRFR1KO and WT mice. However,
289 plasma insulin levels of CRFR1KO mice were significantly lower than those of

290 WT mice (P<.01). The insulin tolerance test (ITT) clearly showed that
291 CRFR1KO mice were less insulin resistant compare to control mice (P<.05, Fig.
292 2C). Accordingly, the glucose challenge showed improved glucose tolerance,
293 although plasma insulin levels were lower in CRFR1KO mice (Fig. 2D and 2E).
294 In order to address the reason for enhanced insulin sensitivity, plasma
295 corticosterone concentration was measured. As shown in Fig. 2F, the
296 corticosterone level in CRFR1KO mice was significantly lower than that in WT
297 mice. In addition, a disappearance of circadian rhythms in the secretion of
298 corticosterone was observed in CRFR1KO mice, explaining improved insulin
299 sensitivity, and thus less insulin secretion during glucose challenge (24).
300
301 3.3 Islet morphology of CRFR1KO mice.
302 Expression of CRFR1 in pancreatic β cells prompted us to analyze the
303 morphology of the pancreatic islets. The functional units of the endocrine
304 pancreas are the islets of Langerhans, which are nested within the exocrine
305 tissue of the pancreas and are composed of α, β, δ, and PP cells. Pancreatic β
306 cells produce insulin and form the core of the islet, whereas α, δ, and PP cells

307 are arranged at the periphery of the islet and secrete glucagon, somatostatin, and
308 pancreatic polypeptide, respectively. As shown in Fig. 3A,
309 immunohistochemical (IHC) studies revealed that insulin-positive cells were
310 nested in the core of the islet while glucagon and pancreatic
311 polypeptide-positive cells were arranged at the periphery of the islet in both
312 CRFR1KO and WT mice. This suggests that CRFR1 is not essential for the
313 architectural formation of the islets. IHC studies using an antibody against
314 CRFR1 confirmed its expression in WT mice, and a lack of expression in
315 CRFR1KO islets. Although primary β cells express CRFR1 in islets, it is not
316 possible to exclude the possibility that the other cells such as α, δ, and PP cells
317 express CRFR1.
318
319 3.4 Inactivation of CRFR1 in adult mice results in pancreatic islet hypoplasia
320 Histological and morphometric analyses of pancreatic islets in CRFR1KO mice
321 revealed a statistical difference in average islet size between WT (11,467 ± 485
322 μm^2) and CRFR1KO mice (9,348 ± 594 μm^2) at 40 weeks of age, as shown in Fig.
323 3B. Hematoxylin and eosin (HE) staining of the pancreas of WT and CRFR1KO

mice at 40 weeks of age are shown. Distribution of small (<10,000 μm^2), medium (10,000-20,000 μm^2), or large (>20,000 μm^2) islet areas is shown in Fig. 3B as a percentage of islets measured. In CRFR1KO mice, there was an increased proportion of small islets and a decreased proportion of large islets compared to WT mice. As shown in Fig. 3C, whole pancreas insulin content of CRFR1KO mice was significantly decreased compared with WT mice, although there was no difference in their pancreas weights, reflecting the islet hypoplasia observed in the histology of CRFR1KO mice. It is noted that there was no statistical difference in islet morphology at 8 weeks of age (data not shown), indicating that islet hypoplasia is not defined by genetic loss of CRFR1.

3.5 Impaired GSIS in islets isolated from CRFR1KO mice

To assess the insulin-secreting ability of β cells, islets were isolated from CRFR1KO and WT mice. GSIS from CRFR1KO islets was defective, showing significantly lower insulin secretion than WT islets in response to both 11.2 mM and 22.4 mM glucose (P<.01; Fig. 4A). To address whether activation of CRFR1 potentiates GSIS, the effect of CRF stimulation on WT islets was examined. As

shown in Fig. 4B, CRF exhibited no effect on GSIS from CRFR1KO islets, while it significantly enhanced GSIS from WT islets at the higher glucose concentration, indicating that CRF signals through CRFR1 and potentiates GSIS.

3.6 CRF1KO mice are resistant to high-fat diet induced obesity

We hypothesized that CRFR1 potentiates insulin secretion in response to high glucose concentrations (such as in diabetes) in a similar manner to GLP-1. To test this hypothesis *in vivo*, CRFR1KO mice were fed a high-fat diet for 12 weeks from 6 to 18 weeks of age to induce diabetes. Body weight of the CRFR1KO mice was significantly lower than that of WT mice (Fig. 5A). As shown in Fig. 5B, blood glucose levels under *ad libitum*-fed condition showed no statistical difference between CRFR1KO and control mice. However, plasma insulin levels of CRFR1KO mice were significantly lower than those of control mice ($P<.01$). As revealed by ITT and shown in Fig. 5C, CRFR1KO mice showed a slight increase in insulin resistance due to the high-fat diet compared with the normal diet (Fig. 2C); however, they still showed high insulin

358 sensitivity compared to the WT mice. Consistent with this, glucose challenge
359 showed a better glucose profile with less plasma insulin secretion in CRFR1KO
360 mice (Fig. 5D and 5E). Although CRFR1KO showed insulin resistance on the
361 high fat diet, insulin secretion during glucose challenge was equivalent to the
362 level observed with the normal diet (Fig. 2E), suggesting defective insulin
363 secretion in response to high glucose. While the WT mice exhibited obesity and
364 fatty liver as shown in Fig. 5F, CRFR1KO mice were resistant to diet-induced
365 obesity. After 12 weeks of high fat diet (starting at 6 weeks of age), CRFR1KO
366 mice attained an average body weight of 35.6 ± 2.28 g compared with 44.55 ±
367 3.34 g for control mice (Figure 5A). The weights of liver (1.71 ± 0.21 g versus
368 1.19 ± 0.29 g) and epididymal white adipose tissue (2.45 ± 0.23 g versus 1.18 ±
369 0.42 g) were significantly lower than those of control mice (Figure 5F).
370 However, there was no significant difference in the weights of other tissues,
371 including kidney, brain, and heart between control and CRFR1KO mice (data not
372 shown). In addition, there was no significant difference in the body length.
373 Histological analysis with HE and oil red O staining revealed lipid accumulation
374 in the livers of the control mice, whereas less accumulation was observed in the

375 livers of CRFR1KO mice. Consistent with this, triglyceride content in the liver
376 of CRFR1KO mice was significantly lower than that of WT mice. Consistent
377 with this, the average size of adipocytes in the epididymal fat of CRFR1KO
378 mice was smaller than that of the control mice (Fig. 5G). To investigate the
379 mechanisms underlying resistance to diet-induced hapatic steatosis, the
380 expression of mRNA encoding key lipogenic enzymes was quantified in the liver
381 after high fat diet for 8 weeks (Fig. 5H). The mRNA levels involved in
382 lipogenesis such as Srebp1c and fatty acid synthetase (Fasn) were significantly
383 lower in CRFR1KO mice than WT mice. Acetyl-CoA carboxylase (ACC) was
384 also decreased, although this was not significant. In addition, carnitine
385 palmitoyltransferase Ia (CPT1a), catalyzing an essential step in the β-oxidation
386 of long chain fatty acids, was significantly increased. Collectively, lipogenesis
387 was decreased while lipid oxidation was increased in CRFR1KO mice.
388 We next analyzed the expression of adipocytokines such as leptin and
389 adiponectin in WAT. As shown in Fig. 5I, the expression level of leptin in
390 CRFR1KO mice was lower than that in WT mice, while adiponectin was
391 significantly increased in CRFR1KO mice. These results suggest that CRFR1

392 plays important roles in energy homeostasis through central and peripheral
393 actions. A further investigation is proposed to better understand these effects.
394
395 3.7 Metabolic measurements of CRFR1KO mice
396 The concept of energy balance, which comprises both energy intake and energy
397 expenditure, is the key to understanding body weight control (25). To address
398 the mechanisms underlying the lean phenotype in CRFR1KO mice, we first
399 measured food intake, and found that total *ad libitum* food intake was unchanged
400 between CRFR1KO and WT mice, as shown in Fig. 6A. However, there was a
401 significant disruption of the circadian distribution of food intake. CRFR1KO
402 mice consumed significantly more food during the light period (26). We then
403 measured physical activity of mice at 20 weeks of age. As shown in Fig. 6B, the
404 locomotor activity of CRFR1KO mice during the active dark cycle was
405 significantly higher than that of WT mice as previously described (2). Using an
406 open circuit calorimeter, oxygen consumption (Vo_2) and carbon dioxide
407 production (Vco_2) were measured (Fig. 6C). Respiratory quotient (RQ) was
408 calculated as Vco_2 /Vo_2. The Vo_2 and Vco_2 were significantly increased during

409 both dark and light cycles in CRFR1KO mice. There was no significant
410 difference in RQ, indicating that energy substrate utilization was not altered in
411 CRFR1KO mice. The lean phenotype on the high-fat diet can thus be explained
412 by hyperactivity and greater energy expenditure in CRFR1KO mice.
413
414 **4. Discussion**
415 In this study, we have shown that impaired GSIS was observed in islets
416 isolated from CRFR1KO mice, consistent with a previous observation that
417 CRFR1 activation potentiates GSIS (14). In the absence of glucocorticoids,
418 CRFR1KO mice maintained higher insulin sensitivity, enabling enhanced
419 glucose tolerance despite lower fasting and glucose-stimulated insulin levels.
420 The altered overall metabolic status of CRFR1KO mice precludes their use in
421 the study of specific contributions of pancreatic CRFR1 *in vivo*. In our study, a
422 high-fat diet was introduced to CRFR1KO mice to induce obesity and insulin
423 resistance. Indeed, the body weight of CRFR1KO mice on the high-fat diet was
424 increased compared with mice on the normal diet, and they developed slight
425 insulin resistance as assessed by ITT. Consistent with this, glucose challenge

426 revealed that impaired glucose tolerance was found in CRFR1KO mice.
427 However, insulin secretion was not increased, indicating a defect of insulin
428 secretion in response to high glucose in CRFR1KO mice.
429 GLP-1 is released from the intestinal enteroendocrine L cells in response to
430 nutrient ingestion and exerts pleiotropic actions in pancreatic islets, including
431 potentiating GSIS from β cells, suppressing glucagon release from α cells,
432 enhancing β cell proliferation, and preventing β cell apoptosis (27, 28).
433 Interestingly, CRFR1 activation has been shown to stimulate proliferation of
434 primary rat neonatal β cells (14). Because of this, we performed morphometric
435 measurement of islets of CRFR1KO mice in the current study. We found that the
436 islet size of CRFR1KO mice was significantly smaller than that of WT mice. It
437 has been shown that islets adapt to insulin resistance through β cell expansion
438 (29). A reduced requirement for insulin due to higher insulin sensitivity may
439 explain, in part, the islet hypoplasia in CRFR1KO mice. Further analysis is
440 required to clarify the role of CRFR1 in the proliferation of β cells.
441 Collectively, the actions of CRF on pancreatic β cells are similar to those of
442 GLP-1 (30), which is now widely used for the treatment of type 2 diabetes.

443 Activation of CRFR1 in the anterior pituitary releases glucocorticoids, leading
444 to insulin resistance in peripheral tissues. Therefore, the clinical application of a
445 CRFR1 agonist for treatment of diabetes may require selective delivery to the
446 pancreatic islets.

447 Although several studies, including the present study, have shown that
448 activation of CRFR1 by CRF potentiates insulin secretion from β cells, whether
449 or not CRF produced by central or peripheral tissues acts as an endogenous
450 ligand for CRFR1 in β cells remains to be clarified. CRF immunohistochemistry
451 of paraffin-embedded pancreatic sections and RT-PCR using total RNA from
452 isolated islets failed to show expression of CRF in the islet (data not shown).
453 However, the presence of CRF immunoreactivity in islet α cells has been
454 reported (31), suggesting the possibility of local production of CRF or a
455 CRF-like molecule acting in a paracrine manner. It is also possible that Ucn1
456 produced in β cells acts in an autocrine or paracrine manner. Alternatively,
457 endogenous ligands including unknown factor(s) might be delivered to the islets
458 either through the blood stream or via neuronal innervation (32).

459 The CRF system interacting with other neuropeptides is directly implicated in

460 the regulation of energy balance and may participate in the pathophysiology of
461 obesity (33). Exogenous administration of CRF, or members of the Ucn family,
462 promotes negative energy balance, both by increasing energy expenditure and by
463 decreasing food intake. These anorexic effects are mainly mediated by
464 interacting with CRFR2 in peripheral tissues including muscle (34) and adipose
465 tissue (5). In this study, we have provided compelling evidence that CRFR1 is
466 also involved in energy homeostasis. On a high-fat diet, CRFR1KO mice were
467 resistant to diet-induced obesity and fatty liver formation. There may be an
468 alteration of neuropeptides due to the lack of CRFR1. It has been reported that
469 diabetes patients show higher cortisol levels compared with normal subjects,
470 suggesting altered function of the HPA axis and altered stress responses (7).
471 Further analysis will be required to understand the mechanisms underlying the
472 alteration of energy expenditure. In conclusion, the complex interactions
473 between the HPA axis and the endocrine pancreas are exemplified by the
474 phenotype of CRFR1KO mice.
475

476 **Acknowledgments**

We thank Y. Hamaguchi and A. Nakano for technical assistance, and acknowledge technical support from the Research Support Center, Kyushu University Graduate School of Medical Sciences.

Funding

This work was supported in part by Japan Society for the Promotion of Science (JSPS) Grants-in-Aid for Scientific Research (C) 23591356 to M.N. and (B) 23390247 to R.T., and Grants-in-Aid for Research Fellowship for Young Science Foundation and Banyu Science Foundation to L.W. The work of M.N. was supported by a grant from the Medical Research Encouragement Prize of The Japan Medical Association.

Conflicts of interest

The authors declare no conflicts of interest.

Author contributions

RS, EM, MN, LW and YK conducted data collection; TY, HN and RT contributed

494 to designing the experiment and analysis of data. RS and MN designed

495 experiments, analyzed the data and wrote the manuscript.

496

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590 substrate cycling between de novo lipogenesis and lipid oxidation.
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592

Figure legends

Figure 1. Expression of CRF receptor mRNA

Quantitative RT-PCR was performed using total RNA extracted from the tissues indicated from 20-week-old WT mice (*n*=5). CRFR1 mRNA was expressed in pancreatic islets, as well as in the cerebrum and pituitary gland. CRFR2 mRNA was highly expressed in skeletal muscle and weakly expressed in brown adipose tissue and liver. β-actin was used as an internal control.

Figure 2. Enhanced insulin sensitivity in CRFR1KO mice

(A) Growth curves of CRFR1KO mice (*n*=12) and WT littermates (*n*=10). (B) Blood glucose and insulin levels in CRFR1KO (*n*=8) and WT (*n*=8) mice at 12-14 weeks of age under *ad libitum*-fed conditions. (C) Insulin tolerance test in 16-week-old male mice (*n*=8 per group). (D, E) Glucose tolerance test in 14-week-old male mice (*n*=8 per group). Blood glucose (D) and serum insulin (E) levels in CRFR1KO and control WT mice after glucose injection. (F) Plasma corticosterone levels at 8:00 and 20:00 in 18-week-old CRFR1KO and WT mice (*n*=8 per group). **P*<.05. ***P*<.01.

610

611 Figure 3. Islet hypoplasia in CRFR1KO mice

612 (A) Immunostaining for insulin, glucagon, and pancreatic polypeptide,

613 showing normal islet architecture in 8-week-old WT and CRFR1KO mice.

614 Immunostaining for CRFR1 IHC with antibody against CRFR1 confirmed its

615 expression in WT islets and the lack of expression in CRFR1KO islets. (B)

616 Morphometric analysis of islet area in pancreata from 40-week-old WT and

617 CRFR1KO mice. For WT, *n*=219; CRFR1KO, *n*=189. Distribution of islet areas

618 <10,000, 10,000-20,000, or >20,000 μm2 is shown as a percentage of islets

619 measured. Representative photographs show islet hypoplasia in CRFR1KO mice.

620 Tissues were stained by hematoxylin and eosin (HE). Scale bars = 25 μm. (C)

621 Insulin content in whole pancreases from the mice in each group (*n*=5). **P*<.05.

622

623 Figure 4. Defective glucose-stimulated insulin secretion in islets from

624 CRFR1KO mice

625 (A) Glucose-stimulated insulin secretion in islets isolated from CRFR1KO and

626 WT mice. ***P*<.01. (B) CRF potentiates GSIS in WT islets in response to 11.2

627 mM glucose. *P<.05.

628

629 Figure 5. CRFR1KO mice show resistance to diet-induced obesity.

630 Male CRFR1KO and WT mice (n=8 per each group) were fed with a high-fat

631 diet for 12 weeks from 6 weeks of age. All experiments were carried out at 19

632 weeks of age. (A) Growth curves of CRFR1 mice (n=8) and WT mice (n=8) fed a

633 high-fat diet from 6 weeks of age. (B) Blood glucose and serum insulin levels in

634 CRFR1KO (n=8) and WT (n=8) mice at 19 weeks of age under *ad libitum*-fed

635 conditions. (C) Insulin tolerance test in 19-week-old male mice. (D, E) Glucose

636 tolerance test in 19-week-old male mice. Blood glucose (D) and serum insulin

637 (E) levels in CRFR1KO and WT mice after glucose injection. (F) Appearance of

638 the mice (upper panel) and their intra-abdominal organs (lower panel). Tissue

639 weights of liver and epididymal white adipose tissue (eWAT) at 19 weeks of age.

640 (G) Histological analysis of liver sections from CRFR1KO and WT mice by HE

641 and Oil red-O staining. Liver lipid deposits can be seen as red droplets in the Oil

642 red-O images. HE staining of epididymal WAT sections reveals the smaller

643 diameter of fat cells in CRFR1KO mice. Scale bars = 50 μm. Liver triglyceride

644 contents are shown on the right. (H) Relative expression of genes involved in

645 lipid metabolism in the liver. (I) Relative expression of leptin and adiponectin

646 mRNA in WAT. *P<.05. **P<.01.

647

648 Figure 6. Assessment of metabolic parameters

649 (A) CRFR1KO and WT mice (n=6 in each group) showed no difference in total

650 food intake, however there was a significant disruption in the circadian rhythm

651 of food intake in CRFR1KO mice. (B) Locomotive activity of mice at 20 weeks

652 of age. CRFR1KO mice were more active than WT mice, as shown by increased

653 exploratory activity in an open field (P<.05). (C) Metabolic rate assessments in

654 CRFR1KO and WT mice. CRFR1KO mice consumed ~20% more oxygen than

655 WT mice as assessed by the average mean oxygen consumption ($V\mathrm{o}_2$). *P<.05.

Figure 1 Sakamoto et al.

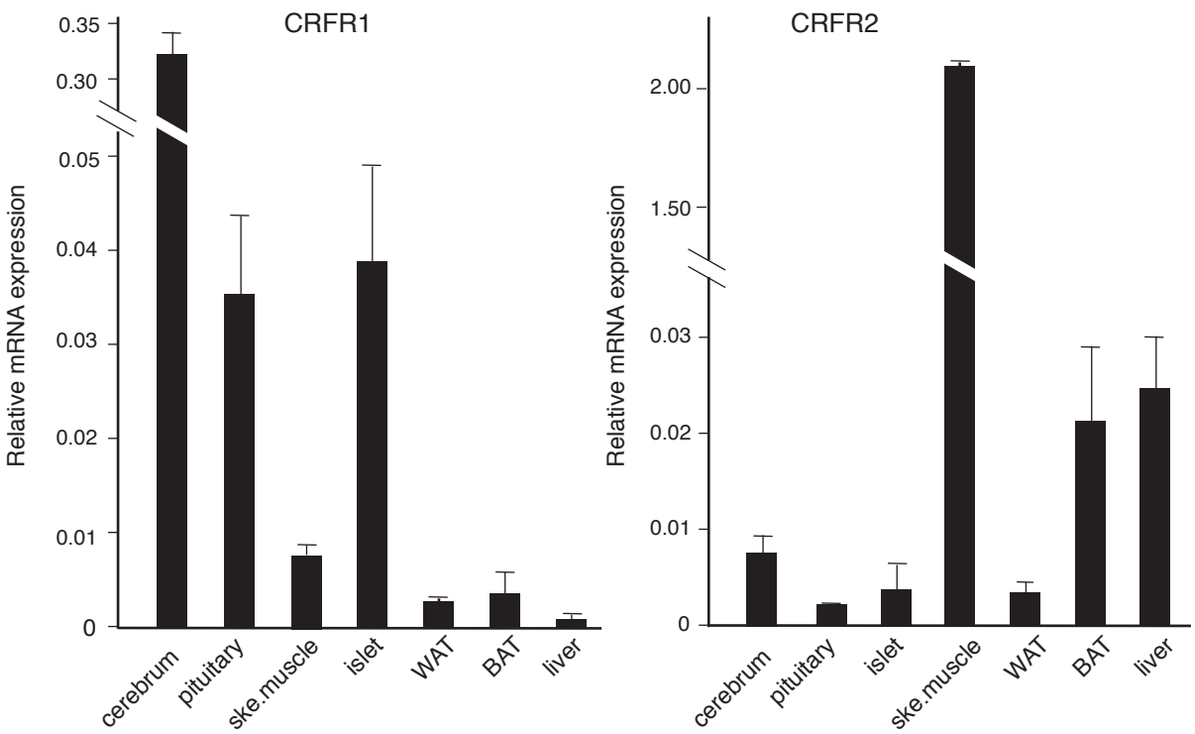

Fig.1 Sakamoto et al.

Figure 2 Sakamoto et al.

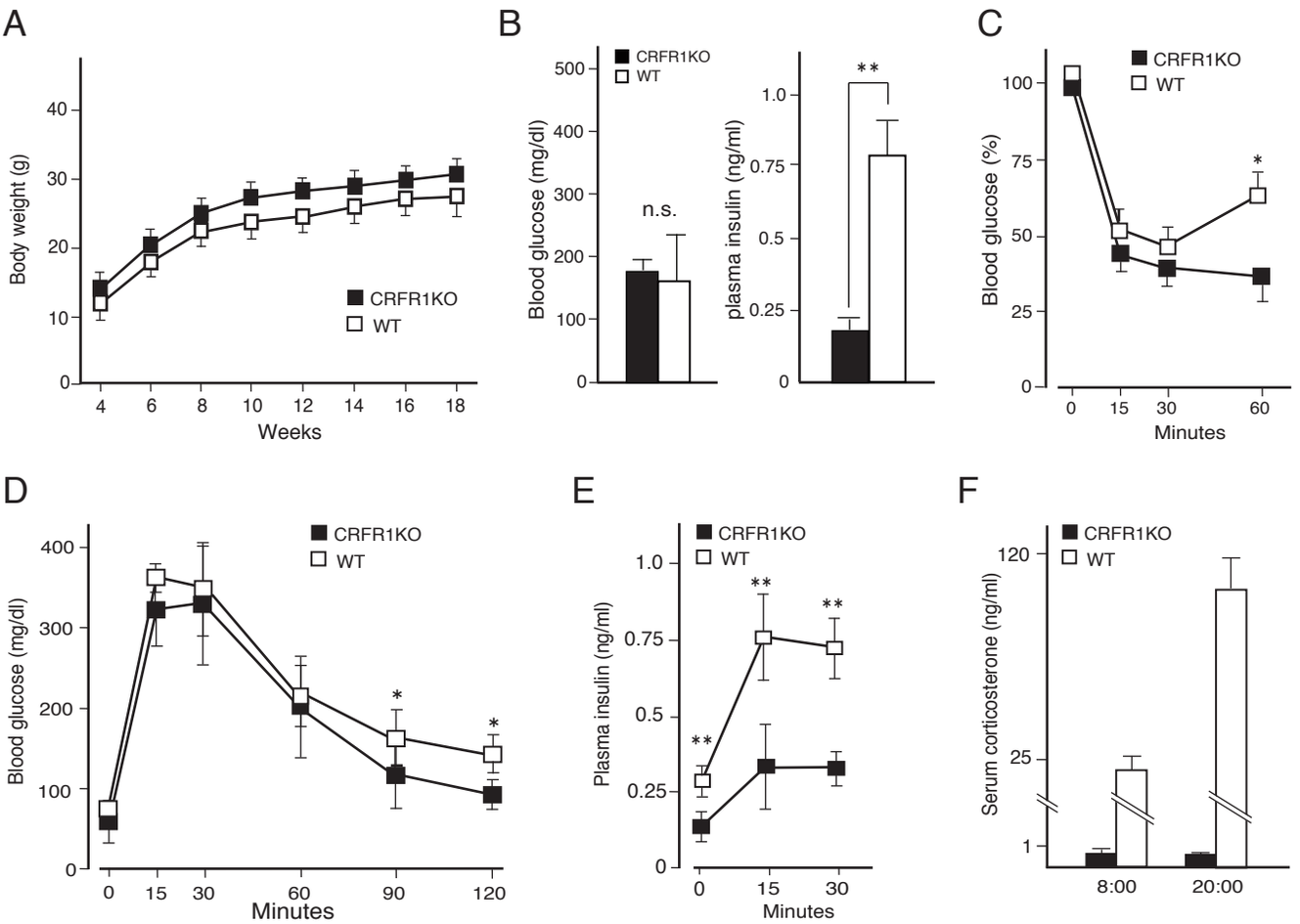

Fig.2 Sakamoto et al.

Figure 3 Sakamoto et al.

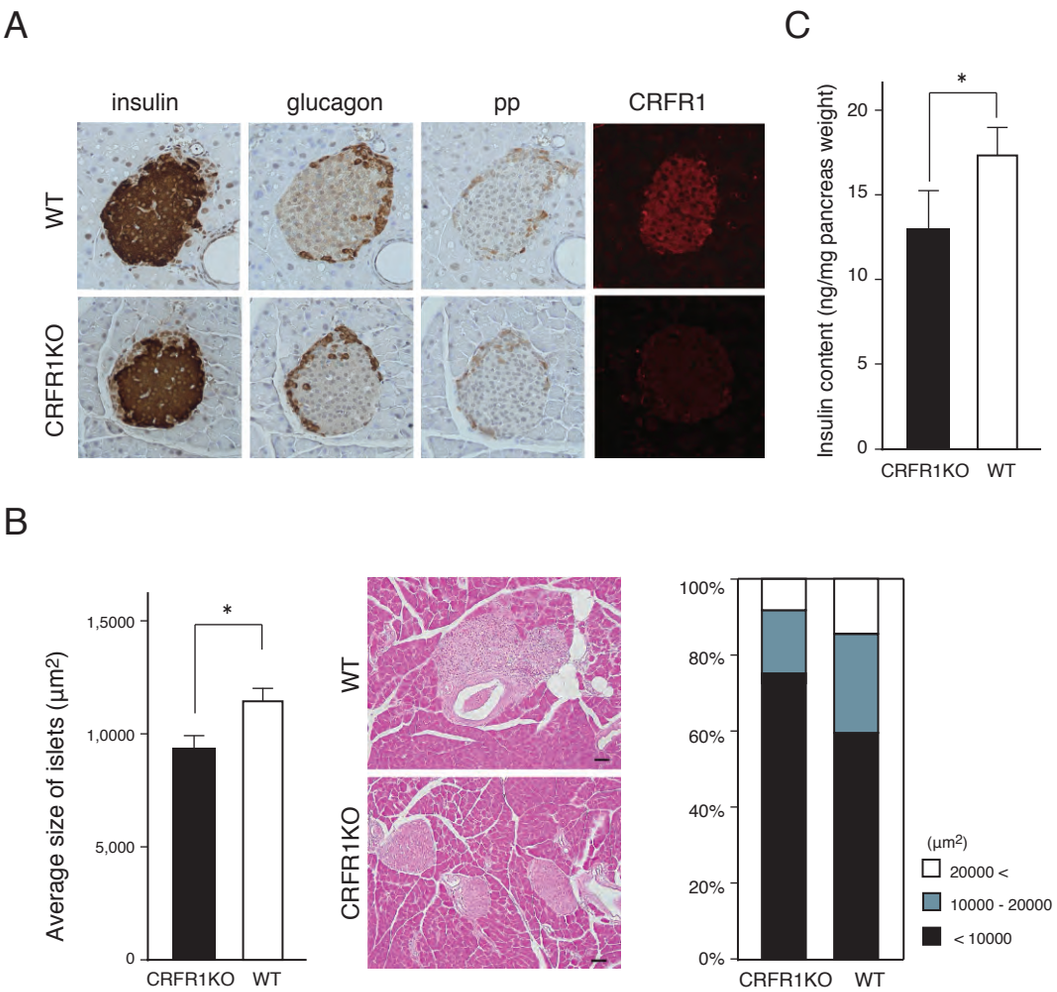

Fig.3 Sakamoto et al.

Figure 4 Sakamoto et al.

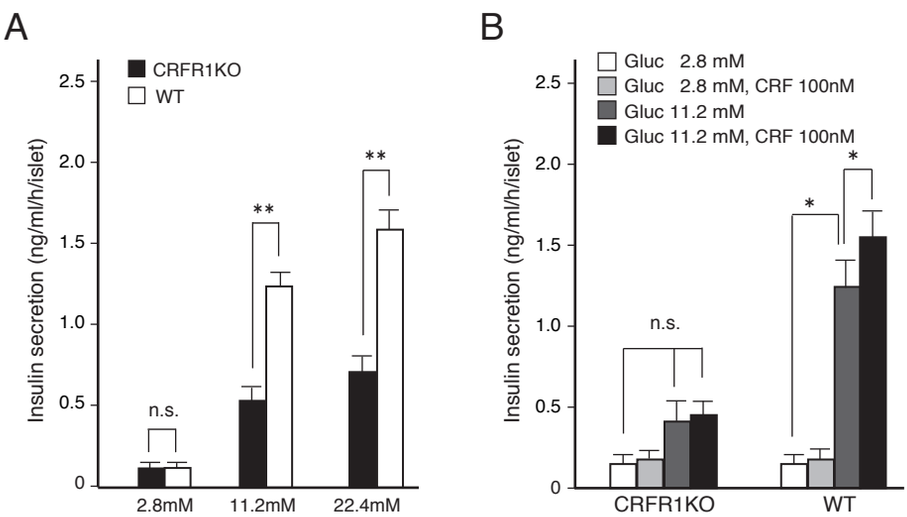

Fig.4 Sakamoto et al.

Figure 5 Sakamoto et al.

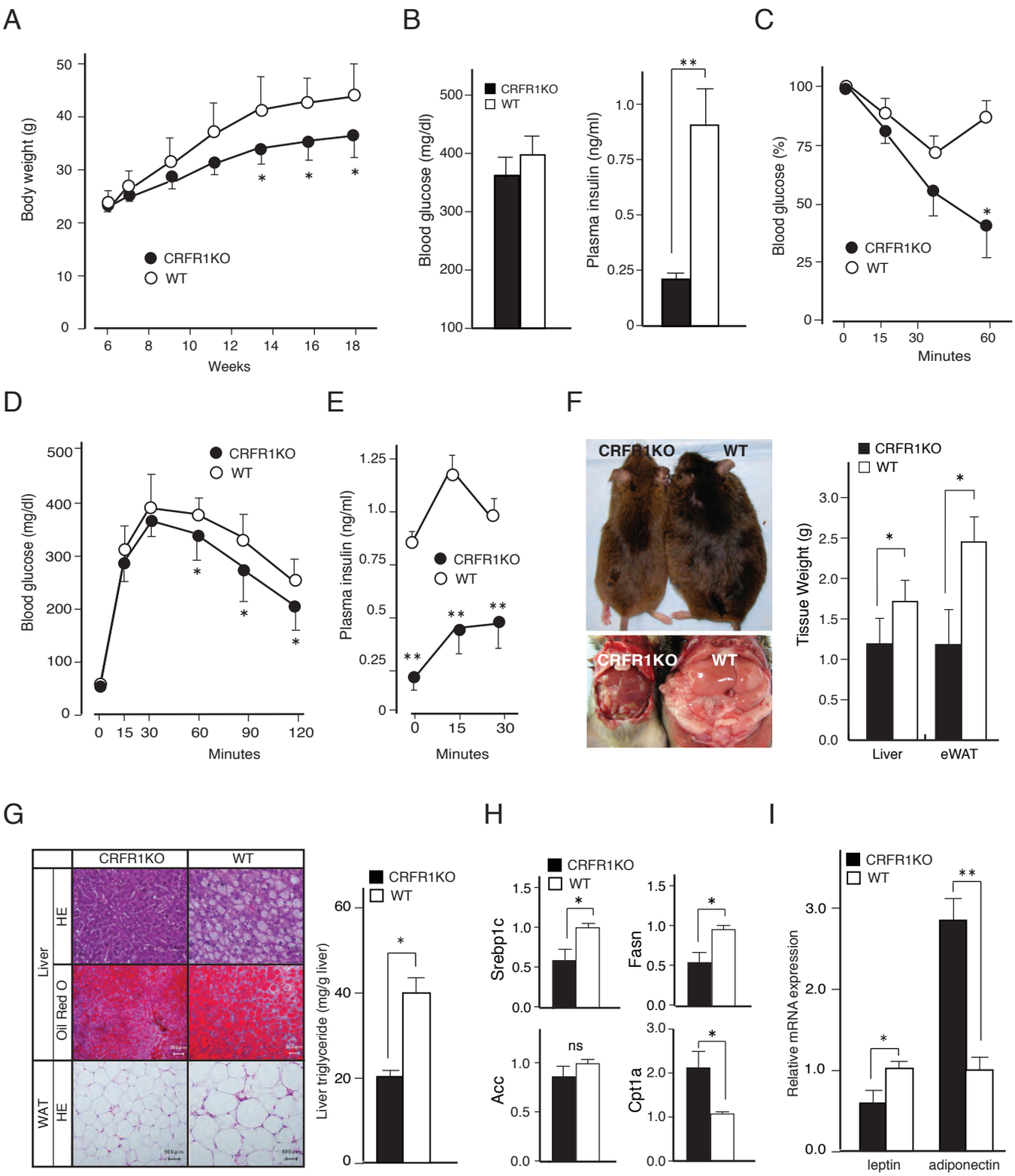

Fig.5 Sakamoto et al.

Figure 6 Sakamoto et al.

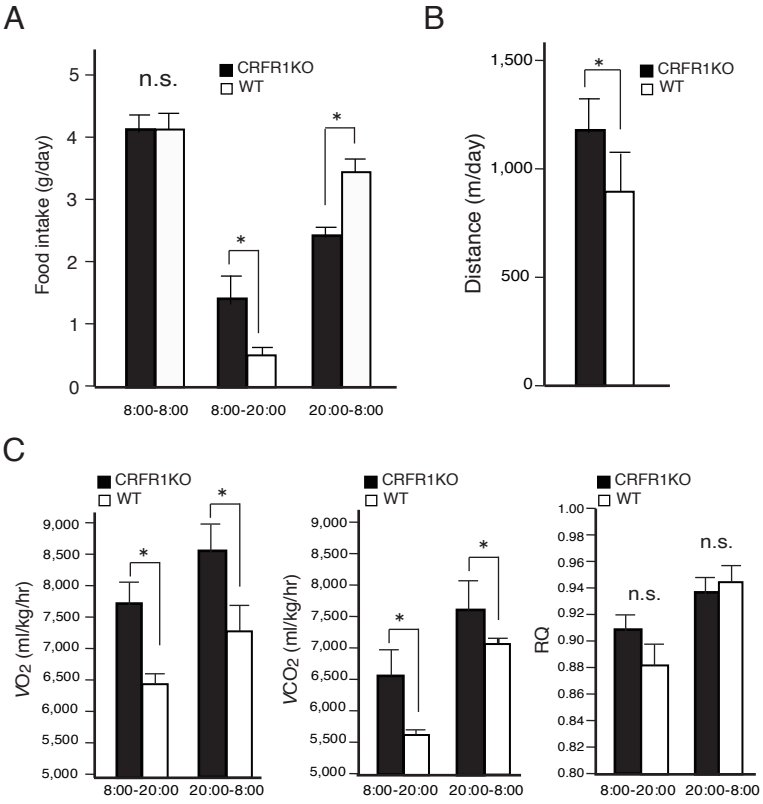

Fig.6 Sakamoto et al.

Response to the comments

Editorial comments: Members of the editorial board and invited external reviewers have evaluated the submitted manuscript. Although they felt that the current data are solid and could have an impact in the field in terms of better understanding glucocorticoid action in the context of insulin sensitivity, several minor concerns are raised by the reviewers.
Editor's comment
1. Specifically, the authors need to explain in more detail methodology and experimental conditions in each setting.
Author response: In accordance with the reviewer's comment, the materials and methods section, and figure legends, have been revised as described in this document.

2. Additionally, it would be informative if the authors could provide more data on liver physiology in CRFR1 KO mice, including gene expression of lipogenetic key enzymes and lipid metabolism. These data will provide physiological mechanisms of how deletion of CRFR1 protects against diet-induced hepatic steatosis.
Author response: In response to this comment, liver transcript levels for genes involved in lipid homeostasis have been analyzed. The data have been added to Figure 5H. In addition, data on liver triglyceride content have been added to Figure 5G. The corresponding information has been added to the Materials and methods and results sections of the revised manuscript (P9, lines 142-150; P23, lines 378-387).

3. Have the authors measured body composition? What is the relative fat mass / lean mass of these mice?
Author response: The authors have measured body composition of the mice. After 12 weeks of high fat diet (start at 6 weeks of age), CRFR1KO mice attained an average body weight of 32.6 ± 2.28 g compared with 44.55 ± 3.34 g for control mice (Figure 5A). The weights of liver (1.71 ± 0.21% versus 1.19 ± 0.29%) and epididymal adipose tissue (2.45 ± 0.23% versus 1.18 ± 0.42%) were significantly lower than those of control mice. However, there was no significant difference in the weights of other tissues, including kidney, brain, and heart between control and CRFR1KO mice. In addition, there was no significant difference in the body length. The authors have added these data to the results section (P22, lines 365-372) and Figure 5F has been revised.

4. Do the authors have measurements of leptin, adiponectin? Hormones related to altered insulin secretion such as GLP-1 levels?

Author response: In response to this comment, the expression of leptin and adiponectin in white adipose tissues has been analyzed. The authors have added these new data as Figure 5I. As shown in Figure 5I, the expression of leptin was decreased and that of adiponectin was increased in WAT of CRFR1KO mice, consistent with their lean phenotype (P23, lines 388-391). The authors have measured GLP1 levels of male mice at 18-20 weeks of age during OGTT (before and 20 min after injection of glucose). However, the GLP1 values varied too much to confidently evaluate, as shown in the reference figure 1A. The authors thus failed to obtain a robust result in this case. This may be due to technical problems, but this variation may also be due to the nature of the GLP1 protein, which is characterized by rapid degradation. Another explanation could be the lower quality of the ELISA for GLP1. Nonetheless, the question of whether GLP1 secretion is altered in CRFR1KO mice is an interesting one. Further experiments are planned to elucidate this further.

5. Can the authors provide more data (LH, FSH etc) and expand their discussion of altered testosterone in these mice?

Author response: The possible influence of CRF on testosterone production is a new field. In response to the editor's comment, we have measured serum testosterone level of male mice at 18-20 weeks of age. However, as shown in the reference figure 1B, a statistical difference was not observed, although there was a trend suggesting an increase of testosterone in CRFR1KO mice. At this time, these results of testosterone measurements are incomplete. Further experiments will be required to clarify the role of CRFR1 on the HPT axis. CRF has been shown to enhance testosterone production in mouse fetal leydig cells (McDowell EN et al. PLOS One 7: e47359, 2012). Fetal testis steroidogenesis plays an important role in the reproductive development of male fetus. Therefore, we have measured ano-genital distance. However, no significant difference in ano-genital distance was found between CRFR1KO and WT mice. In addition, CRFR1KO male mice are fertile. Collectively, these observations suggest that a lack of CRFR1 in mice did not show any apparent phenotype of hypogonadism.

Reviewers' comments:

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Reviewer #1: This article describes studies to investigate the metabolic function of CRFR1 using mice lacking CRFR1 (CRFR1KO mice). The main findings presented include that GSIS of CRFR1KO mice was down-regulated because of high insulin sensitivity, due to very low level of glucocorticoid in this model and the islet size of CRFR1KO mice was small, reflecting a reduced requirement for insulin due to higher insulin sensitivity. CRFR1KO mice showed resistance to diet-induced obesity and hepatic steatosis and the mice kept a relative lean phenotype by hyper-activity and high energy expenditure. Although the concept is interesting, the manuscript is quite incomplete. Several concerns regarding these studies are listed below.

Comments:

1. There are found some type-errors and misprints. The authors need to polish up throughout the manuscript; ex) (1) blood glucose levels were measured 30, 60, 90, and 120 min after injection ? blood glucose levels were measured 15, 30, 60, 90, and 120 min after injection (P13, L207)., (2) minutes ? min (P13, L212), see above in same page., (3) control mice ($P<0.05$) ? control mice ($P<0.05$) (Fig.2C) (P16, L266)., (4) underlining?underlying (P21, L344).

Author response: In response to the reviewer's comments, typographical errors have been corrected.

2. The authors should standardize the spelling of abbreviations in the Text, the Tables, and the Figures; CRH?CRF. The authors should write original words in front of the abbreviations that appeared for the first time in Text; ex) GLP-1 (L90), GIP (L90) and so on.

Author response:

In accordance with the reviewer's comments, the full term has been included at the first use of each abbreviation throughout the text. In addition, GLP-1 and GIP have been added to the abbreviations listed immediately prior to the abstract. We have standardized the notation to CRF throughout the manuscript.

3. There is no information about genetic background of CRFR1KO mice. Are wild type mice C57BL6J? And, the corticosterone level in CRFR1KO mice was quite low during a day, so the reviewer is afraid that the mice may demand a special drink containing NaCl

for survive and seem to be weaken like addison disease, however, the physical activity of CRFR1KO mice was significantly higher than that of WT mice. What does the authors think this concern?

Author response:

All experiments were performed on the C57BL/6 x 129/Sv hybrid genetic background. A description of the background has been added to the Material and Methods section (P7, lines 113-114). Regarding the reviewer's concern, basal levels of corticosterone were quite low in CRFR1KO mice as shown in Fig.2F. A previous study by Timpl et al. (ref.2) has shown that the plasma corticosterone levels in CRFR1KO mice increased to a measurable level after exposure to stress, such as a forced swim. In addition, there was no difference in basal levels of ACTH between CRFR1KO and WT mice. Consistent with these observations, histological analysis of adrenal cortex did not reveal any apparent morphological differences. Basal ACTH release in CRFR1KO mice might be explained by the influence of other hypothalamic ACTH-stimulating hormones. Collectively, these observations demonstrate that the physiological condition of CRFR1KO mice is clearly different from that of Addison's disease in human. Therefore, replacement of electrolytes such as NaCl is not needed for their survival. The hyperactive phenotype of CRFR1KO mice may be explained by defects in anxiety behavior. CRFR1KO mice exhibited reduced anxiety-related behavior and thus increased exploratory activity.

4. In Figure 1, there is no information about the detail of RT-PCR condition, especially cycles of PCR for three candidate genes. Were the cycles within linear range in RT-PCR analysis? Because of the quantitative PCR, the authors have to explain a quantitative capability carefully.

Author response:

In accordance with this suggestion, details regarding the quantitative RT-PCR have been described in Materials and Methods (P10, lines 156-165). One reference has also been added (18)(P8, line 136).

5. In Figure 1 legends, it is described that CRFR2 mRNA was highly expressed in skeletal muscle and adipose tissues, however, the level of CRFR2 mRNA was low in WAT (Figure 1). Then, the description does not correspond to the Results parts. The authors should rewrite Figure 1 legends. Did the authors investigate the expression of CRFR2 in various

tissues of CRFR1KO mice? CRFR2 expression may be unregulated by compensatory response.

Author response:

In accordance with the reviewer's comment, the legend for Fig.1 has been revised as follows; 'and weakly expressed in brown adipose tissue and liver'.

The expression of CRFR2 in CRFR1KO tissues was not originally analyzed. It has been reported that the expression of other molecules involved in the regulation of the HPA system, including CRFR2, was not altered in the pituitary gland of CRFR1KO mice. However, the question of whether a compensatory mechanism is working between CRFRs in the regulation of energy metabolism, as the reviewer has mentioned, is an interesting one. Therefore, the expression of CRFR2 in islets and livers from CRFR1KO mice has now been analyzed. As shown in reference figure 2A, the expression of CRFR2 was quite low and there was no statistical difference in the expression of CRFR2 between WT and CRFR1KO islets. β-actin was used as an internal control. It is conceivable that CRFR2 functions as a receptor for Ucns, not for CRF. A comment on CRFR2 expression in CRFR1KO islets has been added to the results section (P17, lines 280-283).

6. In Figure 2 and 5, there is no information about the anesthesia when the blood sample was collected from tail vein after intraperitoneal injection with glucose. Did the authors draw blood from total sixteen mice (n=8 per group) without use of an anesthestic every 15 minutes?

Author response:

Because anesthesia has effects on heart rate and blood flow and induces hyperglycemia, tests of glucose metabolism on anesthetized mice are not recommended. Therefore, blood samples after IP injection were collected without anesthesia in our study. However, to minimize stress, we applied a small amount of topical anesthetic cream to the tail of the mice, and scored the tip of the tail using a sterilized scalpel blade. A small drop of blood was placed on the test strip of the blood glucose meter. This was the baseline glucose level and was recorded in the experiment record sheet. The mouse was then injected intraperitoneally with the appropriate amount of glucose solution, as previously determined, and the time of injection noted. The blood glucose levels were then measured at 15, 30, 60, 90 and 120 min after glucose injection, by placing a small drop of blood on a new test strip and recording the measurements. The clot from the first incision was gently removed, and the tail gently massaged if the blood flow was inadequate, for each subsequent blood sample. To ensure that further blood loss from the incision was minimal,

pressure was briefly applied to the incision after each measurement. At the end of the experiment food was added to the cage and an adequate supply of water was made available to the animals. We thus follow the standard operating procedures for performing metabolic tests in mice, which have been proposed by The Mouse Metabolic Phenotyping Center Consortium (Ref. doi:10.1242/dmm.006239).

7. There is no information about the age in week of mice in which the expression of CRF receptors was examined (Figure 1), the corticosterone levels were measured (Figure 2) and the immunostaining was performed (Figure 3). The authors should add the information to Figure legends and/or Method parts. Then, the ages in week of mice used for each experiment were too discrete. The physical activity was measured at 20 weeks of age, the histologic and morphometric analyses of pancreatic islets were performed at 40 weeks of ages, and GSIS in vitro was examined using the islets from mice (4-6 months old). These seem to be no plan. Why did not the authors examine in close age-groups?

Author response:

In accordance with the reviewer's comment, information regarding the age of the mice has been added to the Figure legends. The same mice were used for several experiments. For instance, OGTT and ITT were performed at 1-week intervals. For the measurement of metabolic parameters such as oxygen consumption and physical activity, at least 3 days were required for one measurement. Because of limits in the availability of equipment, the age of the mice used in the study varied slightly. However, we believe that these variations of age do not affect the conclusions.

There was no statistical difference in average size of islets between CRFR1KO and WT mice at 5 and 8 weeks of age. Therefore, the histological analyses of the islets were performed on relatively old mice (40 weeks of age) and islet hypoplasia was found in CRFR1KO mice, indicating that hypoplasia may be secondary to the reduced insulin demand in KO mice. The description of islet histology of 8-week-old mice has been added to the results (P20, lines 331-333). For islet isolation, relatively old mice are preferentially used due to technical issues; diameter of bile duct and size of pancreas are large enough to manipulate after approximately 20 weeks of age. Collectively, we believe this demonstrates that all mice experiments were carried out according to well-laid plans.

8. In Figure 3B, the reviewer cannot realize the quantification analysis of islets. How many islets did the authors check for calculation of the sizes? How about microscopic

field? There is no information about the pancreas weights and how to measure the protein contents of pancreas (Figure 3D). The authors should add the information to Figure legends and/or Method parts.

Author response:

The reviewer has asked about the number of islets analyzed. The authors feel that this comment resulted from our mistake in the organization of figures. To make this more easily understood, figures 3B and 3C have been combined and the corresponding legend has been changed. For WT, *n*=219; CRFR1KO, *n*=189.

In the case of figure 3D, the incorrect value for the Y-axis was accidentally provided. This has therefore been replaced by a correct figure 3C. Insulin content was expressed as ng/mg pancreas weight.

9. The description about treatment period of high fat diet in Figure 5 legend does not correspond to the Figure 5 and Results parts (P19). There is no information about the method of histological analysis of liver sections and epididymal WAT (Figure 5G). The authors should add the information to Method parts.

Author response:

As pointed out by the reviewer, the high fat diet started at 6 weeks of age. Therefore, a typing error in Figure 5 has been corrected. In accordance with the reviewer's comment, the method of histological analysis of the liver and WAT has been added to section 2.3. in the Materials and Methods (P12, lines 190-194).

10. There is no information about the measurements of locomotive activity, oxygen consumption and so on (Figure 6). The authors should add the information to Method parts.

Author response:

In accordance with the reviewer's comment, methodological information regarding metabolic measurements including oxygen consumption has been added to section 2.5. in the Materials and Methods (P13, line 219 to P14, line 226).

11. In Statistics, In vivo data are described as the mean +- SEM". How about another data of the experiments excepting in vivo?

Author response:

In all cases, data are described as the mean ± SEM. Therefore, 'In vivo data are described|-' has been changed to 'Data are described-' (P16, line 262).

12. One third of Introduction is described the results in the present study. The author should eliminate the redundant description in Introduction parts.

Author response:

In accordance with the reviewer's comment, the introduction has been revised.

13. Immunostaining for CRFR1 revealed CRFR1 expresses in whole islets containing <alpha> cell in WT mice. This suggests that CRFR1 may also effect glucagon secretion. There is no data about glucagon contents in between CRFR1KO and WT mice. What does the author think that insulin secretion and sensitivity in CRFR1KO mice may be effected by glucagon level? Is the low corticosterone level enough to explain improved insulin sensitivity despite less insulin secretion during IPGTT, especially in HFD study (Fig. 5)?

Author response:

In accordance with the reviewer's comment on the expression of CRFR1 in islets, we have performed double IHC with insulin and CRFR1. As shown in the reference figure 2B, CRFR1 signal can be seen at the periphery of the islet where the insulin signal is negative, suggesting that α cells may express CRFR1, as the reviewer mentioned. The problem with the CRFR1 antibody is that it only can give a weak signal when applied to the staining of islets. As shown in the reference figure 2C (upper panel), weak but positive signals can be seen in whole islet cells, similar to the present figure 3A. However, positive staining also can be seen in the periphery of the islet in CRFR1KO mice (figure 2C, lower panel, arrowheads), indicating the limits of the specificity of the CRFR1 antibody that is commercially available. Based on these results, we are yet not confident that we have demonstrated CRFR1 expression in α cells. Nonetheless, CRFR1 positive signal observed in WT β cells was missing in CRFR1KO beta cells. We know that it is very important to address the issue of CRFR1 expression in α, δ, and PP cells. Therefore, the comment regarding the cell types within the islet has been added in the result section (P19, lines 315-317).

14. The reviewer cannot realize the sentence at L330-L333, P20 (Although CRFR1KO showed insulin resistance on the high fat diet, insulin secretion during glucose challenge was significantly lower compared with the level observed with normal diet (Fig. 2A), indicating defective insulin secretion in response to high glucose). First of all, the cited

figure is likely to be wrong. Fig. 2E is correct? Even so, insulin secretion in CRFR1KO mice treated with high fat diet (Fig. 5E) was not lower compared with the level observed with the normal diet (Fig. 2E). Moreover, the authors cannot actually compare the results of Fig. 5 to those of Fig. 2 because these experiments were not performed at the same time. It is only allowed to compare CRFR1KO with WT in Figure 2 or 5. There is same problematic point in description of ITT study (Results and Discussion parts).

Author response:

To make it easier to understand, the sentence pointed out by the reviewer has been revised as follows. As the reviewer suggested, the reference to Fig.2A has been corrected to Fig.2E (P22, line 362). The part of the original sentence ' –significant lower compared with the level observed with the normal diet, indicating-' has been changed to '-equivalent to the level observed with the normal diet, suggesting- '(P22, lines 360-362).

The authors fully agree with the reviewer's comment that data from different settings cannot be directly compared. However, in this study, we performed ipGTT and ITT on the mice using the same protocols, namely 2 g glucose/kg BW and 0.75 U insulin/kg BW, respectively. Blood glucose levels in CRFR1KO mice on both GTT and ITT increased after HF diet, indicating that insulin resistance was induced in CRFR1KO mice as well as WT mice. Insulin levels during GTT increased in WT mice, reflecting increased insulin demand due to insulin resistance. However, insulin levels during GTT did not increase in CRFR1KO mice (or less than we expected). Therefore, the interpretation of defective insulin secretion in CRFR1KO mice is likely to be justified.

15. The marks for statistical difference were not shown in Figure 6A. There was a significant disruption of the circadian distribution of food intake. CRFR1KO mice consumed more food during the light period. It is reasonable that oxygen consumption during the light period was significantly increased. On the other hand, locomotive activity and oxygen consumption during the dark period was also significantly increased but food intake was reduced. What does the author explain the mechanism? If total food intake is same and energy expenditure is significantly high, CRFR1KO mice should be lean phenotype on normal chaw feeding. What does the author think this comment?

Author response:

The marks for statistical difference have been added to Figure 6A. Increased locomoter activity does not solely reflect activity for seeking food. CRFR1KO mice exhibit

significantly reduced anxiety-related behavior, which may explain hyperactivity of the mutant mice.

In the present study, CRFR1KO mice did not show any significant difference in body weight when fed on normal chow until 18 weeks of age, as shown in Figure 2A. Consistent with this finding, the total food intake over a 24-h period did not differ between the different genotypes. However, the circadian pattern of food intake was markedly disrupted in CRFR1KO mice. Disruption of circadian rhythmicity is known to lead to energy imbalance and obesity. This may explain, in part, how energy expenditure excess may be compensated for on the animals consuming normal chow. However, under the energy excess condition of the high fat diet, the body weight gain in WT mice was exaggerated and the body weight difference became apparent. Furthermore, locomoter activity was measured in an open field box, while body weight was measured in a small cage in which free movement is limited. Therefore, energy expenditure may be less accurately estimated during the body weight measurements.

Reviewer #3: The paper by Sakammoto et al. investigates the role of CRH-R1 in metabolism and energy homeoastasis. The deeply characterized the KO mice for this receptor. The paper is clearly written and experiments are very clealrly presented and are of great interest. The authors suggest that CRH-R1 plays important roles in whole body energy homeostasis, providing compelling evidence of the close relationship between energy homeostasis and the function of the hypothalamus-pituitary-adrenal axis.

Author response:

The authors thank the reviewer for taking the time to review our manuscript, and appreciate their positive assessment.

Reference figure 1. Sakamoto et al.

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Reference figure 2. Sakamoto et al.

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