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NCB5OR Deficiency in the Cerebellum and Midbrain Leads to Dehydration and Alterations in Thirst Response, Fasted Feeding Behavior, and Voluntary Exercise in Mice

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Abstract

Cytosolic NADHcytochromeb5oxidoreductase (NCB5OR) is ubiquitously expressed in animal tissues. We have previously reported that global ablation of NCB5OR in mice results in early-onset lean diabetes with decreased serum leptin levels and increased metabolic and feeding activities. The conditional deletion of NCB5OR in the mouse cerebellum and midbrain (conditional knock out, CKO mice) results in local iron dyshomeostasis and altered locomotor activity. It has been established that lesion to or removal of the cerebellum leads to changes in nutrient organization, visceral response, feeding behavior, and body weight. This study assessed whether loss of NCB5OR in the cerebellum and midbrain altered feeding or metabolic activity and

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had an effect on serum T3, cortisol, prolactin, and leptin levels. Metabolic cage data revealed that 16 week old male CKO mice had elevated respiratory quotients and decreased respiratory water expulsion, decreased voluntary exercise, and altered feeding and drinking behavior compared to wild-type littermate controls. Most notably, male CKO mice displayed higher consumption of food during refeeding after a 48h fast. Echo MRI revealed normal body composition but decreased total water content and hydration ratios in CKO mice. Increased serum osmolality measurements confirmed the dehydration status of male CKO mice. Serum leptin levels were significantly elevated in male CKO mice while prolactin, T3, and cortisol levels remain unchanged relative to wild-type controls, consistent with elevated transcript levels for leptin receptors (short form) in the male CKO mouse cerebellum. Taken together, these findings suggest altered feeding response post starvation as a result of NCB5OR deficiency in the cerebellum.

Keywords

Ncb5or; Cerebellum; Dehydration; RER; Leptin; Exercise; Mouse

Introduction

Metabolism arguably lies at the core of cellular function, with many pathways contributing to its regulation and virtually all pathways reliant upon sufficient energy production and availability. Thus, changes in pathways influencing metabolism have the potential to severely impact a cell's vitality, function, and survival. Neurodegenerative diseases and diabetes mellitus are two clinically distinct disease classes that share common defects in metabolic influence (for review, see [1]). Changes in bioenergetic homeostasis, influenced largely by mitochondrial and ironrelated pathways, lie at the nexus of these conditions.

NADH-cytochrome-b5-oxidoreductase (NCB5OR) is a ubiquitously expressed, soluble, hemecontaining reductase associated with the endoplasmic reticulum [2, 3]. Recently, naturally occurring non-synonymous mutations that lead to enhanced proteasomal degradation of NCB5OR have been identified in the human population [4]. To date, an exact function and pathway for NCB5OR has yet to be determined; however, it is a potent reductase of iron and heme proteins (e.g., cytochrome c) in vitro, and evidence suggests it plays a role in the maintenance of iron homeostasis and pathways critical to proper metabolic and mitochondrial function [3, 5]. Mice globally deficient for NCB5OR experience early-onset diabetes, growth retardation, decreased serum leptin levels, and increased food consumption despite maintaining a significantly reduced body weight relative to wild-type (WT) littermates [6–9]. We recently reported altered iron homeostasis, decreased iron-positive Purkinje cells, altered locomotor activity, and an increased response to harmaline-induced tremor in mice lacking NCB5OR in the cerebellum and the midbrain (conditional knockout or CKO mice) [10].

There is evidence demonstrating the cerebellum's ability to affect circadian food anticipatory behavior, glucose sensing, satiety, metabolism, nutrient organization, thirst response, and body weight ([11], for review see [12]) through a bi-directional network between the cerebellum and the hypothalamus. Therefore, we hypothesized that selective

loss of NCB5OR in the cerebellum, not the hypothalamus, would alter systemic metabolism, feeding/drinking behavior, and body weight. Through metabolic cage and biochemical analyses, we observed that CKO mice display significant changes in fasted feeding and *ad libitum* drinking behavior, fluctuation of body weight in response to extended fasting, altered metabolic substrate utilization, and decreased voluntary exercise with increases in serum leptin levels. Our findings suggest alterations and dysfunction in the cerebellum's regulation of satiety, feeding and drinking behavior, and exercise behavior as a result of NCB5OR deficiency in the cerebellum and midbrain.

Materials and Methods

Generation of CKO and Animal Husbandry

All mice were treated according to the University of Kansas Medical Center's Institutional Animal Care and Use Committee approval protocol. All mice were of C57BL/6J background. The generation of CKO mice was achieved as previously described [10]. Deletion of NCB5OR in the cerebellum was confirmed through qPCR analysis of functional NCB5OR transcripts, of which the loss of sequences corresponding to exon 3 results in a premature "stop" codon and a highly truncated protein product of 89 residues. All measurements were performed on mice at 16 weeks of age. Mice were fed a standard chow diet from PicoLab (5053). Chow diet total calorie composition is as follows: 62.4% carbohydrates, 24.5% proteins, and 13.1% fat.

Transcript Analysis

Quantitative reverse transcription PCR (qRT-PCR) was performed on RNA extracted from WT and CKO whole mouse cerebellum after mice had been fasted for 4 hours. Total RNA purity was assessed by the A_{260}/A_{280} ratio. One or 2 μg of total RNA underwent reverse transcription using the M-MLV reverse transcriptase and random primers from Life Technologies (Cat no. 28025-013 and 48190-011, respectively). The qPCR reaction was performed on an ABI 7900 HT thermo system using SYBR Green Master Mix from Thermo Fisher (Cat no. 4367659), and all results were normalized to 18S ribosomal RNA (rRNA). Transcript levels were determined by the ΔΔCt method and by defining 18S rRNA as 10^6. Values are presented as the mean ± SEM (standard error of mean). Primer sequences are available upon request.

Serum T3, Cortisol, Prolactin, and Leptin Levels

Blood serum levels of T3, cortisol, prolactin, and leptin were evaluated using enzyme-linked immunosorbent assay (ELISA) kits (T3, Sigma-SE120091; Cortisol, Sigma-SE120082; Prolactin, Sigma-RAB0408; Leptin, Abcam-ab100718). Sixteen (16) week old male CKO mice and WT littermate controls were fasted for 4–6 hours, and whole blood was collected *via* retro-orbital bleeding into a BD serum separator tube (Fisher Cat no. 02-675-188) and centrifuged at 9000 rcf for 10 min at 4 °C for serum collection. Blood serum was then collected and immediately frozen using liquid N_2 and stored at − 80 °C for use at a later date. ELISAs were performed according to the manufacturer's protocol.

Metabolic, Feeding, Drinking, and Locomotion Detection

Sixteen (16) week old CKO mice (n = 12; 6 male, 6 female) and WT littermates (n = 12; 6 male, 6 female) were individually housed and analyzed using the Promethion Indirect Calorimetry System (Sable Systems, Inc., North Las Vegas, NV). All mice were provided access to running wheels. Measurements of feeding, drinking, metabolism, sleeping, and locomotor activity were recorded for 3 days (three light/dark cycles). Data collected from the first day (1st light/dark cycle) were discarded to avoid unintended effects and noise during acclimation. Light-dark cycles were in 12 h intervals with the light cycle commencing from 0700 to 1900 and the dark cycle commencing from 1900 to 0700.

Gait Analysis and Rota-rod Performance

CKO and WT mice underwent gait analysis using a DigiGait apparatus (Mouse Specifics) and were tested on the Rota-rod as described in more detail elsewhere [10]. Briefly, DigiGait analysis was conducted under walking conditions at a speed of 10 cm/s, and Rota-rod measurements were collected at an accelerating speed of 4 to 40 rpm for no longer than 5 min.

Fasted-Feeding Assay

Mice were deprived of food either overnight (~ 14 h) or for 48 h before being allowed *ad libitum* access to food for 24 h. Mice were allowed *ad libitum* access to water at all times and were weighed before fasting began, before addition of food to the cage, and after 24 h of refeeding. To obtain measurement of food and water intake during refeeding, total food and water weight were recorded prior to refeeding and 24 h after refeeding began. Food and water consumption was only measured for the 48 h fasted group.

Echo MRI and Serum Osmolality

Animal body composition was assessed using the EchoMRI-1100 system (EchoMRI LCC, Houston TX). Briefly, each mouse was placed in an adjustable plastic cylinder to restrict movement. The cylinder (1.25 inch in diameter) has holes on each end to allow for the animal to breathe normally. The animal (cylinder) was inserted into the Echo MRI for 30–75 s to obtain readings that include lean mass, fat mass, total water, and free water. Serum osmolality measurements were collected using serum from 16 week old male CKO and WT mice and measured using an Advanced Instrument Micro-Osmometer 3300 Osmometer. Reference solution was Clinitrol 290 mOsm, standard for calibration 50 and 850 mOsm.

2-DoG Uptake

Mice were fasted for 4–6 h prior to intra-peritoneal injection of 0.15 μCi per gram body weight of ^{14}C 2-deoxyglucose (Perkin Elmer, Cat no. NEC-945A). Whole cerebellum was collected 2 h later and was placed in 1 mL of lysis buffer containing proteinase K, homogenized, and rotated at 55 °C overnight. Whole homogenate was then mixed with a scintillation fluid (ScintiVerse BD cocktail, Fisher Cat no. SX18-4), and 5 min β-scintillation counts were obtained on a Beckman Coulter LS6500 multi-purpose scintillation counter. The composition of the lysis buffer was as follows (final concentration): 100 mM Tris–HCl (pH 8.8), 5 mM EDTA, 0.2% SDS, 200 mM NaCl, and 10 μg/mL proteinase K.

Experimental Groups

All data for metabolic and wheel running metrics, Echo-MRI results, and qPCR for 2-DoG uptakerelated genes were collected from the same set of mice. Data for ObR (leptin receptor) variant, Agtr1 (Angiotensin II, type 1 receptor), Agtr2 (Angiotensin II, type 2 receptor), and Avpr1a (arginine vasopressin receptor 1A) transcripts was obtained from total RNA derived from the cerebellum of the mice used for the 48 h fast and refeeding groups. All other data, including DigiGait and Rota-rod analyses, were collected from mouse groupings with no overlap.

Statistical Analyses

Two (2)-way ANOVA (sex and genotype) with Student–Newman–Keuls post hoc test analysis was performed for qPCR, Echo MRI, DigiGait analysis, Rota-rod performance, and 2-DoG uptake. Two (2)-way ANOVA (sex and genotype) analysis with Student–Newman–Keuls post hoc test analysis was performed for all metabolic, locomotor, and feeding metrics after values had been split into light and dark cycles and averaged for two consecutive light and dark cycles. One (1)-way ANOVA (genotype) analysis was performed for all ELISA results and for the 48-h fasting, refeeding, and food and water consumption measurements as well as qPCR measurements of Leptin receptor variants, angiotensin I receptor, angiotensin II receptor, and arginine vasopressin receptor type 1a. Two (2)-way ANOVA (genotype and treatment) analysis with Student–Newman–Keuls post hoc test analysis was performed for serum osmolality measurements. Simple linear regression analysis was performed in *R* for correlation between wheel activity and total energy expenditure and correlation between wheel activity and vO_2. Pearson correlation analysis was performed in *R* for correlation between wheel activity and total energy expenditure and correlation between wheel activity and vO_2, and a *t* test was applied to individual correlations. One (1)-way ANOVA results that were found to be significant are presented with *F* and *P* values. Two (2)-way ANOVA results that were found to be significant are presented with *p*, *q*, and *P* values for individual groups found to be significant resulting from Student–Newman–Keuls post hoc tests. Pearson correlation values are presented as Pearson correlation coefficients. All other values are presented as means ± SEM (standard error of mean). *P* values of ≤ 0.05 were considered statistically significant.

Alternative statistical analysis

In order to confirm observations made using the above statistical analysis, metabolic cage data were analyzed using a three-part analysis in *R*. First, interactions between all three independent variables (genotype, sex, and cycle) were analyzed using a linear mixed effects (LME) model. This allowed for interactions between all three independent variables to be analyzed while avoiding an artificially inflated “n” due to measures of light and dark cycles being from the same mice (e.g., 22 measurements for mice during the light cycle and 22 measurements for the mice during the dark cycle should be analyzed using an “n” of 22 instead of “n” of 44). Next, ANOVA analysis was performed using results from the LME analysis. Finally, those interactions that yielded significant *P* values ($P \leq 0.05$) were further analyzed using a correlation matrix approach to delineate which interactions within the groups were significant. For example, if a significant interaction was found between

genotype and sex, further analysis was used to determine if the significant difference between genotypes was found in the male or female populations. This analysis confirmed results and conclusions drawn from the original statistical analysis of metabolic cage data, and the results reported are those from the original statistical analysis.

Definitions

Systemic metabolism: Energy expenditure and substrate utilization measured by O_2 consumption and CO_2 production.

Free water: Contents of the bladder.

Total water: Free water and water content of lean tissue.

Hydration ratio: (Total water – free water) / lean mass.

Pedestrian activity: All non-wheel running locomotion as measured by beam breaks in the metabolic cage system.

Wheel running activity: All wheel running locomotion as measured by the running wheel in the metabolic cage system.

Average vO_2: mean vO_2 in mL/min.

Total energy expenditure: summed energy expenditure for entire cycle in kcal/h.

Resting energy expenditure: mean value for 30 min periods with lowest energy expenditure in kcal/h.

Average respiratory quotient: mean respiratory quotient as calculated by $RQ = CO_{2\ \text{expelled}} / O_{2\ \text{consumed}}$.

Average vH_2O: mean vH_2O in mL/min.

Total retained: total weight gained / (food consumed + water consumed).

Total retained is a metric we use to evaluate how much of the recorded food and water consumption seems to directly apply to the observed weight change (how much of the recorded consumption can be accounted for in weight gain). This is presented due to a tendency for mice to not eat all that they take, resulting in food being left on the cage floor, and water to be wicked away by fur contact or evaporation. Therefore, the measurement of food and water consumption is further evaluated by dividing the total weight gained in the mice by the total food and water consumption in order to give a more accurate representation of how much of the observed feeding and drinking is actually consumed/retained.

Gait symmetry (real number): [Right forelimb step frequency + left forelimb step frequency] /[Right hind limb step frequency + left hind limb step frequency].

Results

CKO Mice Had Normal Body Weight and Composition but had less Total Water, Free Water, and Increased Serum Osmolality (Males Only)

Body weights were collected and averaged for light and dark cycles during individual housing in the metabolic cage system. There were no discernable differences in body weight between CKO (light 28.93 ± 1.04 g, dark 29.04 ± 1.03 g) and WT mice (light 31.62 ± 1.04 g, dark 31.27 ± 1.03 g) in either the light or dark cycles. After metabolic cage experiments were concluded, body composition was assessed by measuring lean and fat mass *via* echo MRI. There were no differences in lean or fat mass between CKO (lean 24.13 ± 0.81 g, fat 2.65 ± 0.37 g) and WT mice (lean 25.77 ± 0.77 g, fat 2.87 ± 0.32 g), but CKO mice had significantly less total water (Fig. 1a) and no changes in free water (Fig. 1b) relative to WT controls. Hydration ratios calculated from Echo MRI data revealed that CKO mice had significantly reduced hydration ratios compared to WT controls (Fig. 1c). However, these results were driven by significantly reduced hydration ratios in male CKO mice while female CKO hydration ratios remained unchanged relative to WT controls. Serum osmolality measurements revealed chow-fed male CKO mice had elevated serum osmolality relative to WT controls, while mice that underwent a 48 h fast and 24 h refeed period demonstrated no changes (Fig. 1d). These changes provide evidence of dehydration in male CKO mice.

Male CKO Mice Had Decreased Voluntary Exercise and Average Energy Expenditure

Mice were allowed access to a running wheel for the entirety of their housing in the metabolic cage system. Both CKO and WT mice predictably traveled significantly greater distances on the running wheel during dark cycles compared to light cycles, indicating no strain differences in diurnal exercise trends (Fig. 2a). However, this pattern was less clear when assessing strictly pedestrian locomotion (Fig. 2b). While there were no apparent changes in diurnal trends, CKO mice exercised significantly less during the dark cycle compared to WT controls, with male CKO mice specifically showing a significant difference (Fig. 2e). This change was not observed in pedestrian locomotion during the light or dark cycle or in exercise during the light cycle (Fig. 2c, d, f).

The rate of oxygen consumption (vO_2) was used as an indicator to measure metabolic rate and total energy expenditure in CKO and WT mice (Fig. 3). Although vO_2 was unchanged between CKO and WT mice during the light cycle (Fig. 3a, c, e), a period when there is reduced activity and wheel running, we observed a marked decrease in vO_2 and energy expenditure in male CKO mice compared to WT controls during the dark cycle (Fig. 3b, d), a period of time when mice are more active and perform more wheel running. However, resting energy expenditure was not different between male CKO and WT mice (Fig. 3f), suggesting that vO_2/energy expenditure differences were due to lower activity/wheel running in the CKO mice. Pearson correlation analysis revealed strong correlations between wheel activity and energy expenditure ($R = 0.77$, $P = 0.006$) and wheel activity and vO_2 ($R = 0.79$, $P = 0.004$). In addition, CKO mice did not present with abnormalities in gait symmetry (Fig. S1A) or Rota-rod performance (Fig. S1B), indicating no overt ataxia.

Male CKO Mice Exhibited Altered Metabolic Substrate Preference, Respiratory Water Expulsion, and Water Consumption Behavior

Using indirect calorimetry, we explored a number of metrics that are indicative of the metabolic state of the mice being observed. Average respiratory quotients (RQ) (Fig. 4a) of male CKO mice were significantly elevated during both the light (Fig. 4b, top) and dark cycles (Fig. 4c, top), indicating a greater reliance on carbohydrate utilization compared to WT controls. Elevated resting RQ values in male CKO mice during both the light (Fig. 4b, bottom) and dark (Fig. 4c, bottom) cycles confirmed the conditional independence of this observation.

An increased RQ is indicative of a metabolic preference for glucose utilization. Since the brain uses glucose as its primary metabolic substrate, we asked whether the cerebellum in CKO mice was hypermetabolic, resulting in an increase in uptake and utilization of glucose. In order to test this hypothesis in a simple manner, we assessed whether there were changes in glucose uptake and key messenger RNA (mRNA) transcripts associated with brain glucose uptake and metabolism in the CKO cerebellum. We did not observe increased 2-deoxyglucose (2-DoG) uptake in CKO mice relative to WT controls (Fig. S2A). In addition, qPCR analysis confirmed that mRNA transcripts for glucose transporters, lactate dehydrogenase, insulin, and insulin receptor were not increased in CKO mice compared to WT controls (Fig. S2B). Notably, no functional NCB5OR transcripts were detected in the cerebellum of both male and female CKO mice (Fig. S2B).

Interestingly, male CKO mice also exhibited a lowered rate of respiratory water expulsion (vH_2O) over WT controls during both the light (Fig. 5a) and dark (Fig. 5d) cycles. This corresponded with decreased total water consumption that neared significance ($P = 0.06$. $q = 2.77$, $P = 2$), (Fig. 5e) and a significantly increased number of drinking bouts (Fig. 5f) in CKO male mice during the dark cycle. It is important to note that female CKO mice maintained a normal vH_2O (Fig. 5d) when compared to controls but had an increased number of drinking bouts during the dark cycle (Fig. 5f). Additionally, qPCR revealed elevated angiotensin 2 receptor transcripts in the cerebellum of male CKO mice compared to WT controls (Agtr2; WT 0.087 ± 0.008, CKO 0.139 ± 0.020, CKO/WT 1.59, $F = 5.23$, $P = 0.04$) but no elevation in transcripts of angiotensin I receptor (Agtr1; WT 0.089 ± 0.017, CKO 0.100 ± 0.020, CKO/WT 1.13, $P = 0.62$) or arginine vasopressin receptor 1A (Avpr-1a; WT 0.012 ± 0.002, CKO 0.016 ± 0.003, CKO/WT 1.31, $P = 0.34$). These observations are consistent with a decreased hydration state in male, not female, CKO mice (Fig. 1c).

Male CKO Mice Had Increased Weight Gain as a Result of Refeeding After an Overnight or 48-h Fast

We observed no changes in normal feeding behavior between CKO and WT control mice (Fig. S3). While there were no overt changes in *ad libitum* feeding in CKO mice, we observed differences in feeding behavior under fasted and refeeding conditions (Fig.6a–c). Upon resumption of *ad libitum* feeding after an overnight fast, male CKO mice gained approximately 11.5% of their fasted body weight in the 24 h after feeding had resumed compared to the 5% gained by male WT mice. Conversely, female CKO gained 2.5% of their fasted body weight compared to the 5% gained by female WT mice.

Food and water consumption was assessed in male mice after a 48h fast and 24-h refeeding period. Again, we observed that male CKO mice gained significantly more weight during the refeeding period compared to WT controls (Fig. 6d). This was found to be associated with increased food consumption (Fig. 6e), total food, and water consumption, and a significant retention of the food and water consumed during the refeeding period (Fig. 6f). All CKO and WT mice were allowed *ad libitum* access to water at all times.

Male CKO Mice Had Normal Serum T3, Cortisol, and Prolactin Levels but Elevated Leptin Levels

Increased weight gain during refeeding after fasting, reduced voluntary exercise, and elevated RQ with no apparent explanation led us to investigate whether male CKO mice had alterations in hypothalamic–pituitary–adrenal (HPA) axis function, hypothalamic–pituitary–thyroid (HPT) axis function, or hypothalamic response to satiety cues. Therefore, we evaluated serum T3, cortisol, prolactin, and leptin levels. Corticosterone and cortisol are both present in rodents and have both been used as markers of stress in previous studies [13, 14]; however, cortisol has been found to respond quicker as a result of more acute, rather than chronic, stress [15] and was therefore evaluated. When compared to WT controls, male CKO mice had normal serum T3 (Fig. 7a), cortisol (Fig. 7b), and prolactin levels (Fig. 7c), but their leptin levels were significantly increased (Fig. 7d). Pearson correlation statistics revealed that while male WT mice maintained an expectedly strong inverse correlation between prolactin and leptin levels ($R = -0.85$, $P = 0.03$), this trend was lost in male CKO mice ($R = 0.135$, $P = 0.77$) (Fig. S4). Additionally, we observed significantly elevated transcript levels of short-form, but not long-form, leptin receptors in the cerebellum of male CKO mice in comparison to WT counterparts upon *ad libitum* refeeding following 48 h fasting (Fig. 7e). Similar findings were observed in CKO mice under *ad libitum* feeding (data not shown).

Discussion and Conclusions

The cerebellum was once thought to influence only motor coordination, planning, and sensorimotor integration. This view has since changed with a growing body of evidence indicating an integral role for the cerebellum in non-somatic and visceral functions including feeding, micturition, immune function, and emotional and higher-order cognate processes (for reviews, see [16–19]). This study provides evidence that absence of a reductase, NCB5OR, in the cerebellum and midbrain of mice results in changes consistent with altered cerebellar-hypothalamic pathway function.

Previous studies revealed an increased basal metabolic rate (vO_2) and lower body weight in mice globally deficient for NCB5OR [6]. In the current study, initial observation indicated that deleting NCB5OR in the cerebellum and midbrain resulted in normal body weight accompanied by a lowered vO_2 and total energy expenditure in male CKO mice. Generally, such results would indicate a lower basal metabolic rate with decreased appetite and food intake. Theoretically, this combination would balance the lowered caloric energy expenditure with a lowered caloric intake, resulting in normal body weight. However, we did not observe changes in *ad libitum* feeding behavior in CKO mice compared to WT. Further

investigation revealed that male CKO mice had normal resting energy expenditure and participated significantly less in voluntary exercise, in the absence of ataxia, during those times in which we observed lowered vO_2 and energy expenditure. Therefore, we concluded that CKO mice do not have changes in basal metabolic rate or resting energy expenditure. Rather, male CKO mice have decreased locomotor activity during times in which peak activity is expected (dark cycle). This observation is consistent with our previous study detailing altered locomotor behavior in CKO mice [10]. However, the finding is novel in that these data indicate male CKO mice might possess an atypical disposition to voluntary wheel running: an activity normally reveled by mice.

Respiratory quotients (RQ) provide information about the status ofmetabolic substrate utilization in an animal by dividing the amount of CO_2 eliminated by the amount of O_2 consumed during respiration. Normal RQ values generally range from 0.7, indicating pure fat oxidation, to 1.0, indicating pure carbohydrate oxidation. These values are helpful in assessing metabolic substrate preference and have been helpful in identifying metabolic deficiencies and trends in disease [20, 21]. During rest (e.g., sleep or prolonged periods of immobility) or in a fasted state, RQ values are expected to be close to 0.70 due to a reliance on fatty acid utilization. This preference is apparent in male WT mice during rest as indicated by resting RQ values of ~0.70. However, male mice lacking NCB5OR in the cerebellum and midbrain have elevated RQ values, even during rest, suggesting a state of mixed carbohydrate and fatty acid utilization. Since RQ values in male CKO mice indicated an increase in glucose utilization, it was within reason to consider the possibility that the neural tissue devoid of NCB5OR might have an increased basal metabolic rate comparative to the rest of the animal, causing increased glucose metabolism and the resulting elevated RQ values. However, transcript data for mRNA from CKO and WT mice failed to reveal increases in transcripts responsible for or attributed to changes in glucose uptake or demand in the brain. Transcript data would be unlikely to detect small changes in metabolic rate; however, considering the limited region in which NCB5OR was deleted combined with the size of effect on RQ value, we posited that the change in metabolic activity would need to be sizeable, requiring significant changes in glucose uptake. In addition, a detectable increase in RQ value due to glucose preference derived from neural tissue would result in an increased basal metabolic rate (vO_2) since glucose is the primary source of energy for neural tissue. However, we did not observe any changes in resting vO_2 or energy expenditure in CKO mice. This data supports the observation that CKO mice do not have increased uptake of ^{14}C-labeled 2-deoxyglucose in the cerebellum.

Hydration and drinking behavior are controlled by highly complex neuroendocrine mechanisms that have yet to be fully understood (for review, see [22]). Angiotensin II is a peptide hormone that plays a central role in the regulation of thirst and drinking behavior in the central nervous system ([23], for reviews, see [24, 25]). The expression of angiotensin II receptors is generally restricted in the brain but has been confirmed in the cerebellum [26, 27]. Direct projections from the deep cerebellar nuclei to osmoresponsive neurons in the paraventricular hypothalamus have been identified, and neuroimaging evidence supports the theory that the cerebellum contributes to thirst [28, 29]. In our model, we observed significantly decreased levels of free and total water in CKO mice compared to WT controls as well as increases in angiotensin II receptor transcript levels in the male CKO cerebellum.

Changes indicative of altered thirst sensing were further observed in male CKO mice who had decreased water expulsion during exhalation (vH_2O) accompanied by indications of decreased water intake under normal conditions. We did not see this trend in female CKO mice, which could be explained by the effects of estrogen on drinking behavior and fluid intake in females [30, 31] as well as sexual dimorphism in angiotensin II mediated processes [32]. Although further investigation is needed, our data indicate that male CKO mice have an altered thirst response that leads to a state of dehydration, supported by decreased hydration ratios and increased serum osmolality measurements. Interestingly, hyperosmolality leads to a shift in metabolic substrate utilization similar to what is seen in male CKO mice: increased glucose utilization and decreased lipid oxidation leading to elevated RQ values [33]. In fact, it has been demonstrated that dehydration in scorpions elevates RER (RQ) levels in order to generate more water and aid in prevention of further dehydration [34]. Additionally, dehydration increases glycogen and glucose oxidation in male humans during exercise [35]. Thus, we suspect that the increased RER observed in male CKO mice likely manifests as a result of increased carbohydrate metabolism due to dehydration. We are currently conducting experiments to investigate the peripheral systems affected by these observed phenotypes.

A role for the cerebellum in the modulation of appetite and feeding behavior has been proposed based on mounting evidence that dysfunction or lesion in the cerebellum can result in changes in feeding behavior. Evidence for a circadian oscillator involved in food anticipatory behavior has been found in mice with the hotfoot mutation ($Grid2^{ho/ho}$), which results in altered cerebellar function and an ataxic phenotype [11]. Interestingly, food-restricted hotfoot mutant mice lacked food anticipatory behavior in the presence of normal corticosterone levels. HPA-regulated corticosterone levels play an integral role in food anticipatory behavior and change according to food anticipatory trends [36], suggesting that cerebellar modulation might involve the integration of peripherally expressed signals. However, we did not observe alterations in *ad libitum* feeding in the presence of normal cortisol levels in CKO mice, indicating that cortisol-mediated regulation of *ad libitum* food intake is intact in CKO mice.

Another peripherally expressed modulator of feeding behavior is leptin. Leptin is expressed by adipose tissue and plays a central role in mediating satiety during feeding [37]. Avast majority of studies on leptin action in the brain have focused around the hypothalamus [38]; however, leptin receptor expression in the brain appears to be widespread, suggesting that leptin may play more complex roles than previously thought [39]. Studies have confirmed the presence of leptin receptors in the cerebellum, with the active isoform found to be most densely expressed in the cerebellum [40, 41]. Leptin has also been shown to promote cell survival in cerebellar Purkinje neurons as well as modulate posterior cerebellar morphology in response to food cues in adults who are genetically deficient for leptin [42, 43]. In our study, male mice deficient for NCB5OR in the cerebellum and midbrain were found to gain significantly more weight as a result of *ad libitum* feeding after an overnight fast even in the presence of significantly elevated leptin levels as well as increased transcript levels for the short forms (ObRa and ObRc), but not long form (ObRb), of leptin receptors. Interestingly, female CKO mice had an opposite response to the fasted and refeeding conditions compared to their male counterparts with results demonstrating significant reductions in weight gained

after resumption of feeding compared to WT controls. The reason for the difference in this response is not immediately evident, although previous studies show sexually dimorphic responses to fasting as it pertains to corticosterone and hypothalamic activation [44, 45]. These changes are suggestive of an abnormal peripheral response to fasting conditions between the two genotypes and an abnormal response to satiety cues in the central nervous system (CNS).

The regulation of leptin secretion from adipocytes has been linked to a number of different factors, including prolactin-mediated inhibition of leptin secretion [46]. Previous studies demonstrating interaction and reciprocal regulation of prolactin and leptin lead us to hypothesize that lowered prolactin levels might explain increased leptin levels in male CKO mice. There were no significant differences in serum prolactin levels between CKO and WT mice; however, the negative correlation between prolactin and leptin levels was lost in CKO mice. Prolactin release is negatively regulated by hypothalamic dopaminergic stimulation of pituitary dopamine D2 receptors (D2R) and has been shown to be stimulated by chronic elevation of leptin levels [47–49]. Therefore, the lack of increased prolactin levels in the presence of increased basal leptin levels might indicate a decreased response to leptin. It is important to note that this response may not be an indication of changes in leptin sensitivity specifically, but rather increased hypothalamic dopaminergic tone leading to subsequent increased inhibition of prolactin release. We have previously observed changes in CKO mice indicative of altered dopaminergic function [10] as well as significantly elevated dopamine levels in the brains of mice globally deficient for NCB5OR (unpublished data). Dopaminergic signaling in leptin-mediated processes has been explored, and leptin modulation of dopaminergic tone in the ventral tegmental area (VTA) has been shown to reduce the reward response to running, leading to decreased voluntary exercise [50,51]. Direct dopaminergic projections from the VTA to the cerebellum have also been described [52]. In addition, the actions of leptin-mediated satiety cues have been shown to be increased in the absence of hypothalamic D2R [53], and stimulation of adipocyte D2R has been shown to increase leptin secretion [54]. While CKO mice display characteristic changes of altered hypothalamic function, the effects of leptin, prolactin, and dopamine on food intake in the CNS are considerably complex processes involving many factors. In addition, the experiments conducted in this study did not directly test effects on cerebellar–hypothalamic tract function or integrity. Therefore, the exact effect of NCB5OR deficiency on hypothalamic function and leptin response in the CNS cannot be determined from the present findings and warrants future study.

Our study presents an original characterization of effects of NCB5OR deficiency on genes, circuits, and behaviors as they apply to cerebellar and midbrain influence on metabolism, feeding behavior, and physiology. The data presented suggests or infers cerebellar influence over hypothalamic function in NCB5OR deficiency, but does not directly test or prove this hypothesis. The exact cause of these effects is a matter of current investigation, and experiments are being conducted to test hypotheses pertaining to the specific and individual observations presented here.

In summary, this study provided evidence that loss of NCB5OR in the cerebellum and midbrain likely affects pathways and processes integral to the regulation of feeding and

drinking behavior, possibly through dysfunctional peripheral signal response. Lack of NCB5OR in the cerebellum and midbrain altered neuroendocrine thirst regulation in a manner that resulted in dehydration and subsequently elevated RQ values due to a shift to glucose utilization. In addition, NCB5OR deficiency in the cerebellum and midbrain reduced energy expenditure due to lowered voluntary wheel running, which may be an indication of altered leptin-mediated reward response to exercise. Although further experimentation is needed to investigate the mechanism, our current data demonstrate a role for NCB5OR in maintaining the integrity of cerebellar regulation of satiety cues and voluntary exercise.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

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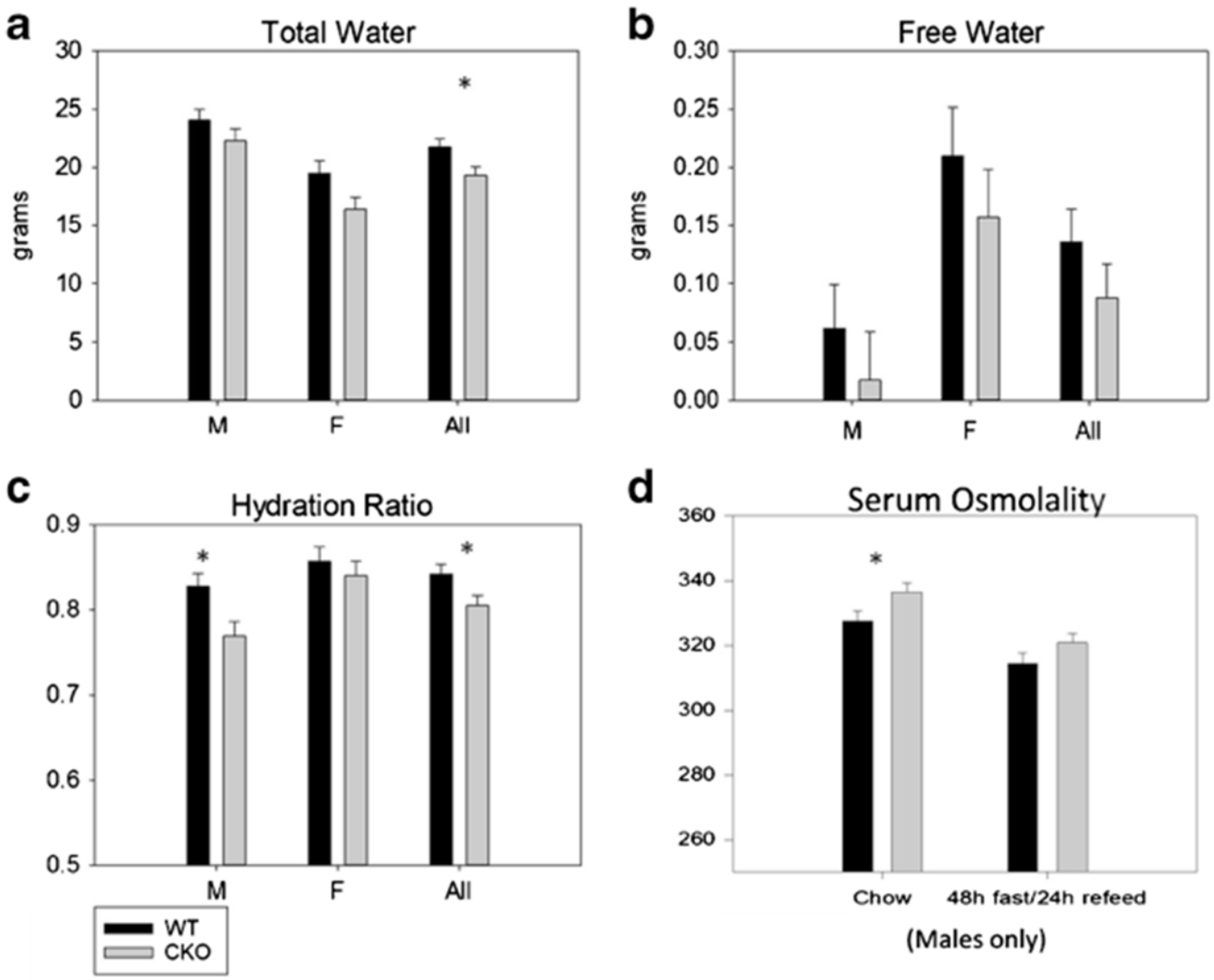


Fig. 1.

Hydration status of CKO and WT mice. **a** Total water was significantly reduced in CKO mice compared to WT mice. **b** CKO mice had comparable free water content relative to WT controls. **c** Hydration ratios in male CKO mice were significantly lower than those of WT controls, while female CKO hydration ratios remained unchanged. **d** Serum osmolality was significantly elevated in male CKO mice relative to controls when chow fed, but not after a 48 h fast and 24 h refeed period. See the “Materials and Methods” section for definitions and calculations. Values are presented as means ± SEM. Water measurements: $n = 8$ for CKO (4 male, 4 female) and $n = 9$ for WT (5 male, 4 female). Osmolality measurements: All male. Chow fed: $n = 9$ for CKO and $n = 7$ for WT. Forty-eight hour fast with 24 h refeed: $n = 10$ for CKO and $n = 8$ for WT. *: $P \leq 0.05$

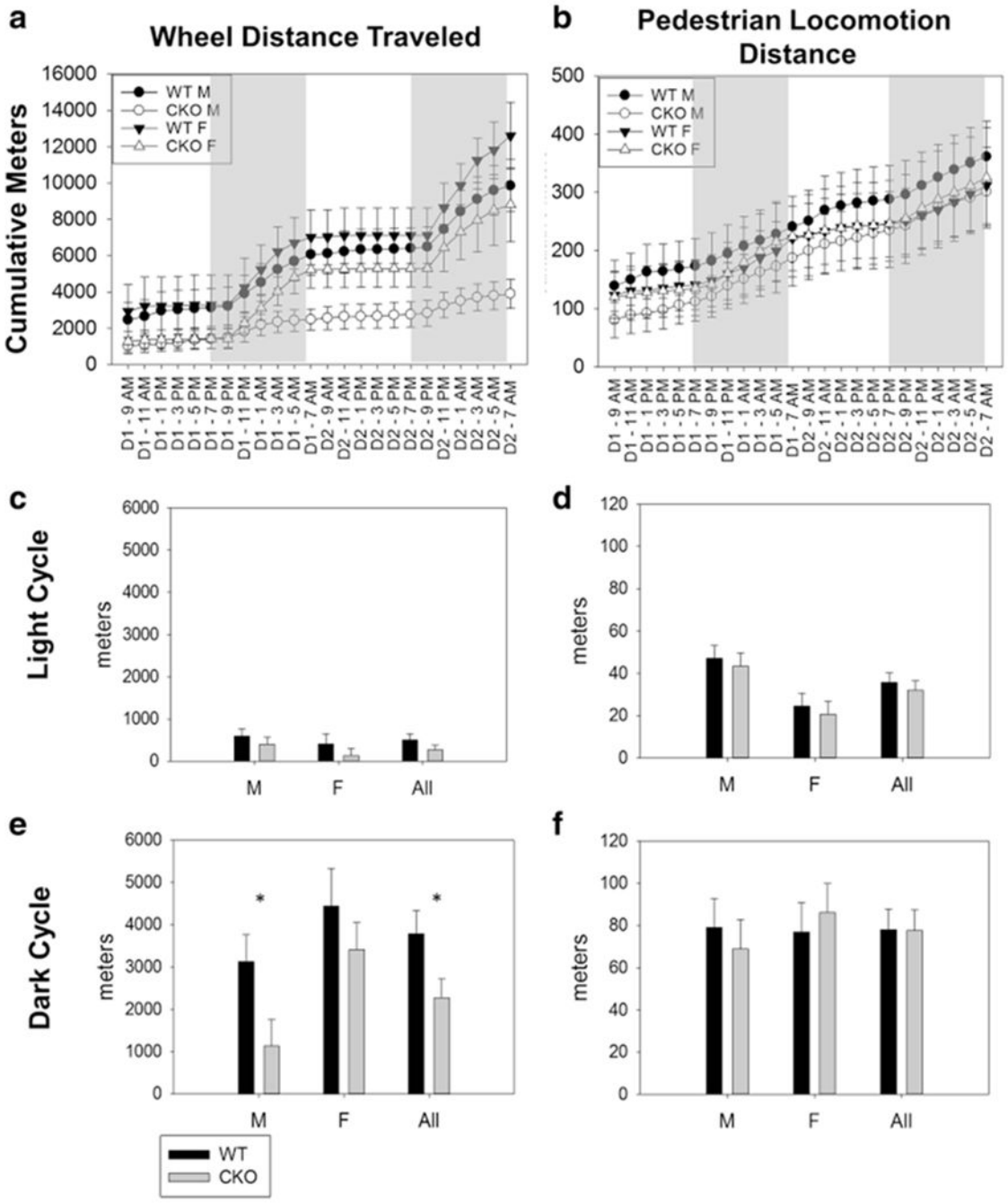


Fig. 2.

Exercise trends in CKO and WT mice. **a**, **b** Longitudinal representations of cumulative meters during two consecutive light and dark cycles. Note the lack of distance accumulative during the light cycle in **a**. There were no differences between CKO and WT mice during the light cycle for (**c**) average wheel distance traveled per cycle and (**d**) average pedestrian locomotion distance per cycle. Significantly lowered average wheel distance traveled in male CKO mice (*: $P = 0.03$, $q = 3.18$, $p = 2$) and all CKO mice combined (*: $P = 0.03$, $q = 3.04$, $p = 2$) compared to WT during the dark cycle is shown in **e**, while there were no differences

in pedestrian locomotion (**f**). See the “Materials and Methods” section for definitions. Values are presented as means ± SEM. Light and shaded areas in (**a**) and (**d**) represent light and dark cycles, respectively. $n = 12$ for CKO (6 male, 6 female) and $n = 12$ for WT (6 male, 6 female)

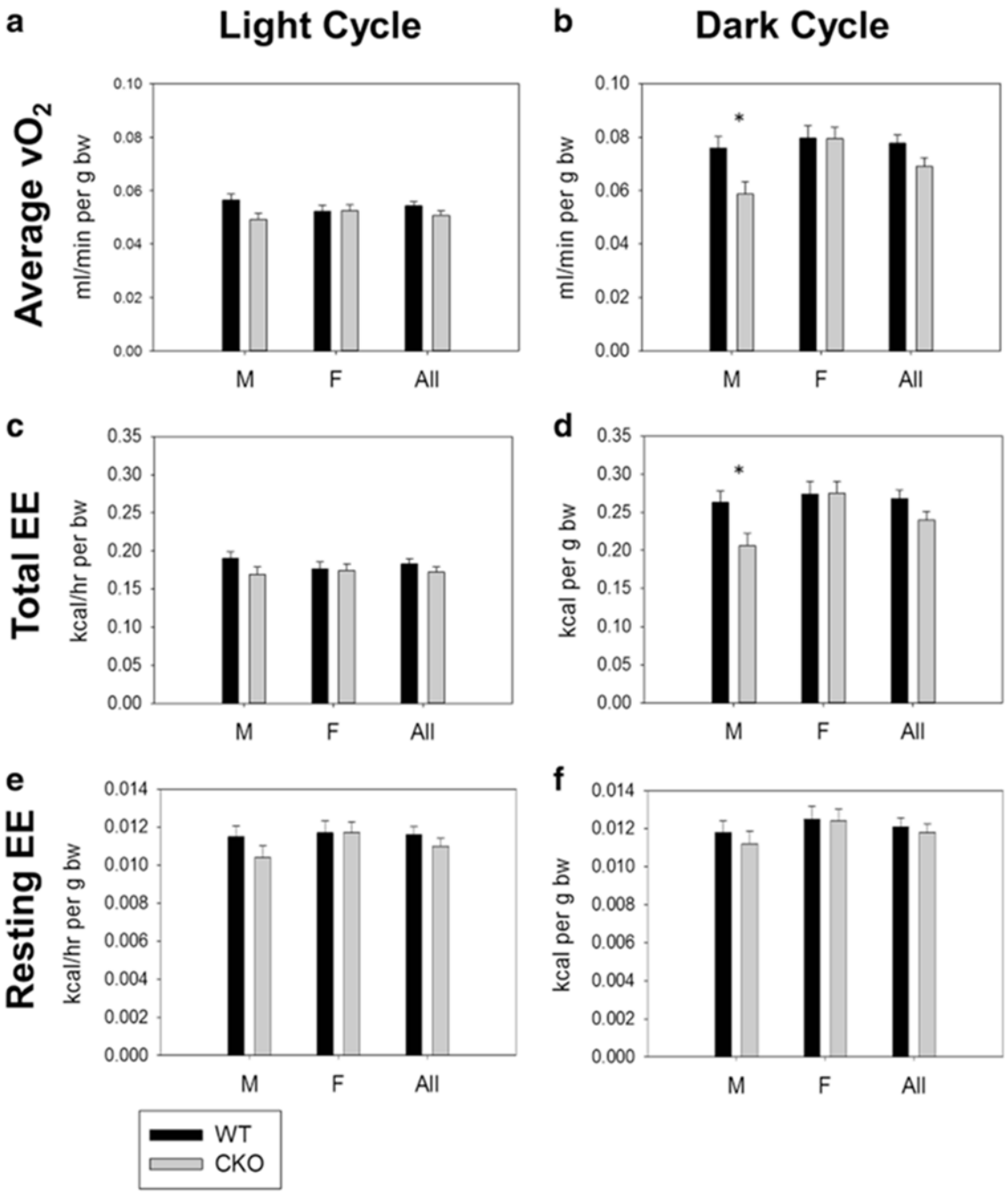


Fig. 3.

Energy expenditure and oxygen consumption in CKO and WT mice. **a** Average rate of oxygen consumption (vO_2) was comparable between WT and CKO mice during the light cycle, but (**b**) was lower in male CKO mice during the dark cycle (***: $P = 0.002$, $p = 5.15$, $q = 2$). **c** Total energy expenditure (EE) was unchanged in CKO mice during the light cycle, but (**d**) was lower in male CKO mice during the dark cycle (*: $P = 0.02$, $p = 3.59$, $q = 2$). **e**, **f** Resting EE was unchanged between WT and CKO mice during both light and dark cycles. See the "Materials and Methods" section for definitions. Values are presented as means ±

SEM and were normalized to body weight (per g bw) $n = 12$ for CKO (6 male, 6 female) and $n = 12$ for WT (6 male, 6 female)

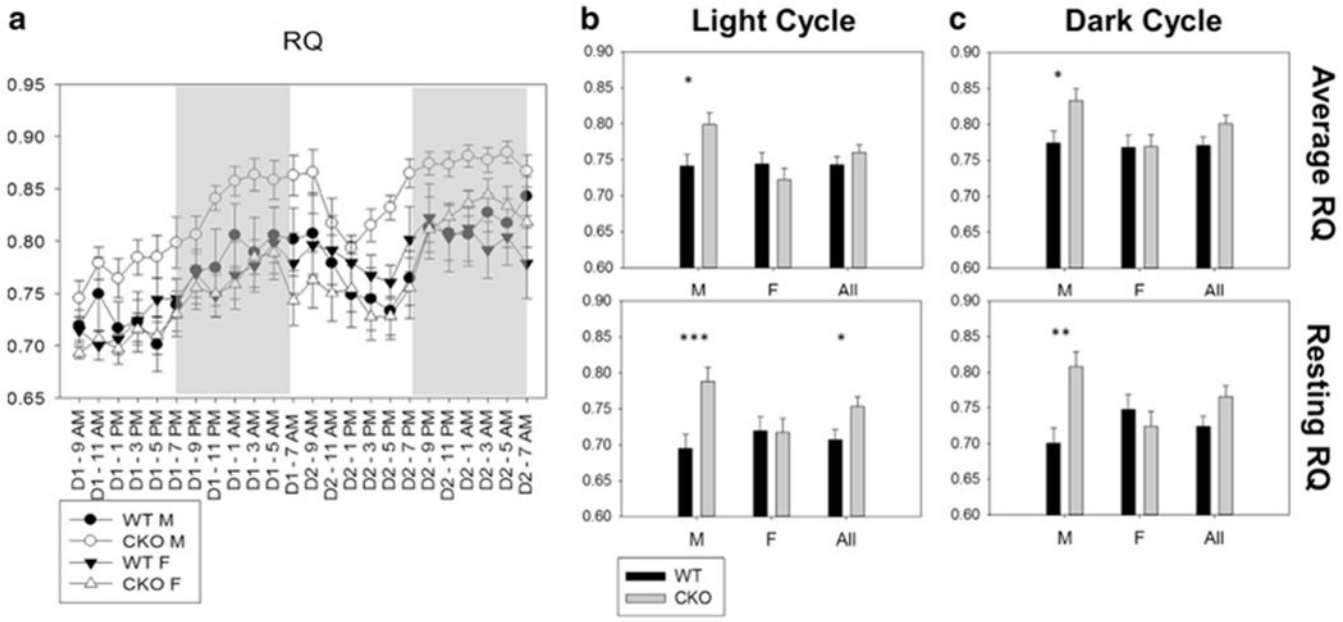


Fig. 4.

Metabolic substrate utilization in CKO and WT mice. **a** A longitudinal representation of average respiratory quotients during two consecutive light and dark cycles. Note that male CKO RQ values were consistently higher. **b** During the light cycle, male CKO mice had significantly elevated average RQ values (top-*: $P = 0.02$, q = 3.57, $p = 2$) and resting RQ values (bottom- ***: $P = 0.003$, q = 4.76, $p = 2$). **c** The same trend was seen in the dark cycle (top- *: $P = 0.02$, q = 3.91, $p = 2$; bottom- ***: $P = 0.002$, q = 5.16. $p = 2$) See the "Materials and Methods" section for definitions and calculations. Values are presented as means ± SEM. Light and shaded areas in **a** represent light and dark cycles, respectively. $n = 12$ for CKO (6 male, 6 female) and $n = 12$ for WT (6 male, 6 female)

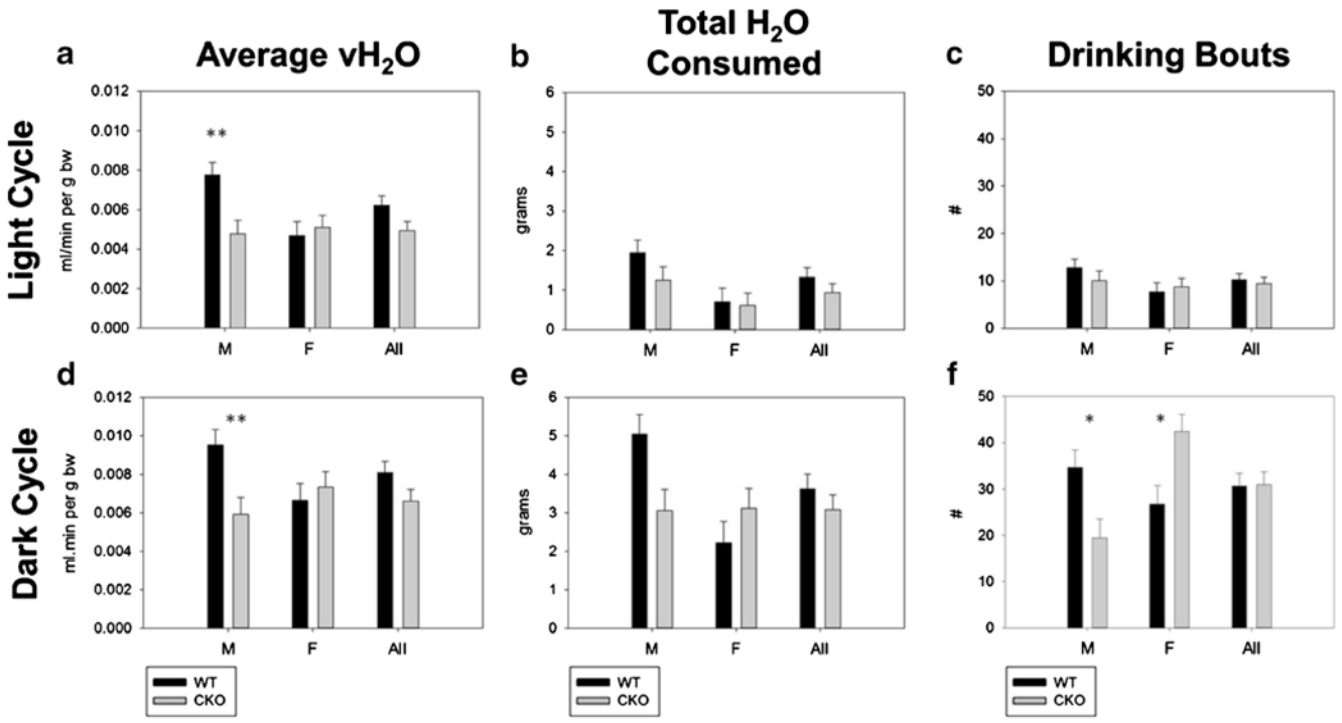


Fig. 5.

Respiratory water expulsion and water consumption behavior. During the light cycle, male CKO mice had (**a**) lowered average respiratory water consumption (vH_2O) (**: $P = 0.006$, $q = 4.46$, $p = 2$) but consume comparable amounts of water (**b**) and had a comparable average number of bouts (**c**) compared to WT mice. During the dark cycle, male CKO mice had (**d**) lowered average vH_2O (**: $P = 0.008$, $q = 4.27$, $p = 2$) along with decreased average water consumption that neared significance ($P = 0.06$, $q = 2.77$, $p = 2$) (**e**) and average number of drinking bouts (*:$P = 0.05$, q = 2.83, $p = 2$) (**f**). Note that in **f**, female CKO mice had an elevated number of drinking bouts converse that of their male counterparts (*:$P = 0.01$, $q = 3.89$, $p = 2$). See the "Materials and MethodsS5" section for definitions. Values are presented as means ± SEM and were normalized to body weight (per g bw) in **a**, **d**. $n = 12$ for CKO (6 male, 6 female) and $n = 12$ for WT (6 male, 6 female)

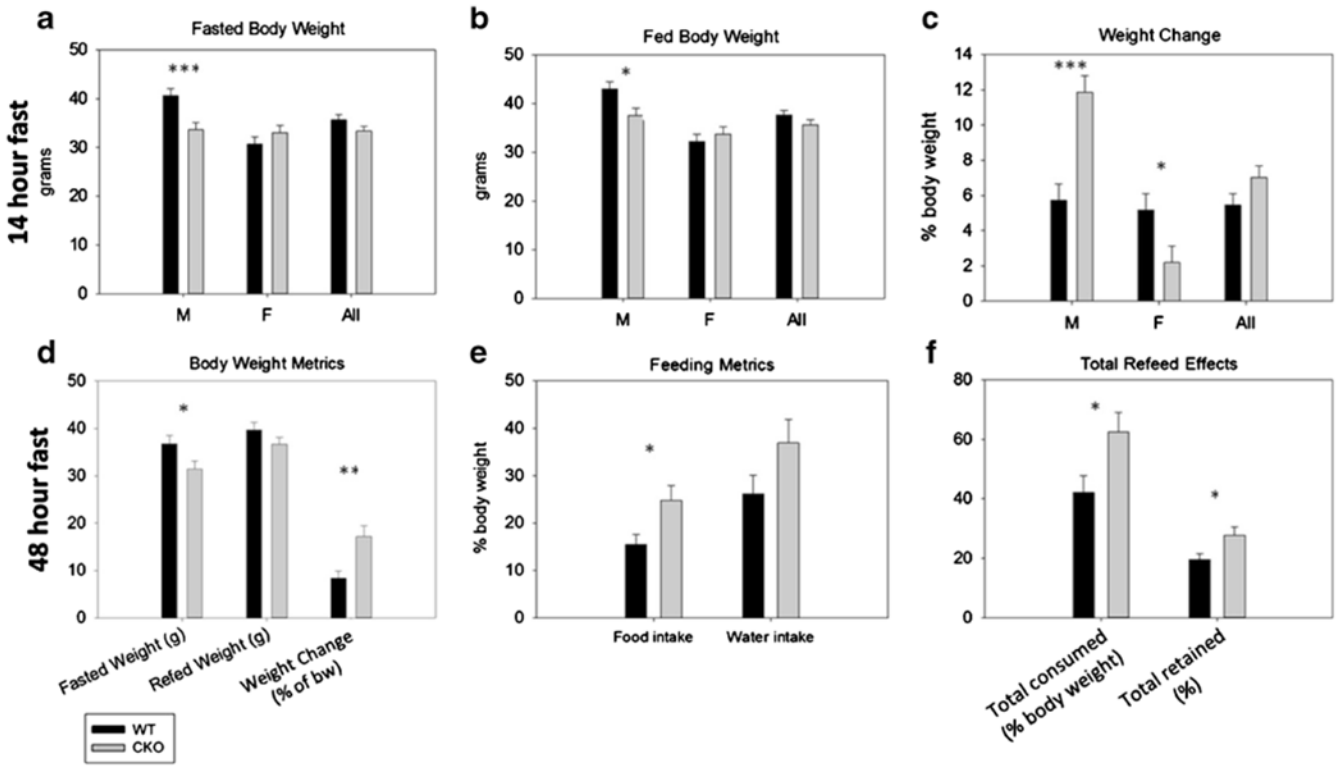
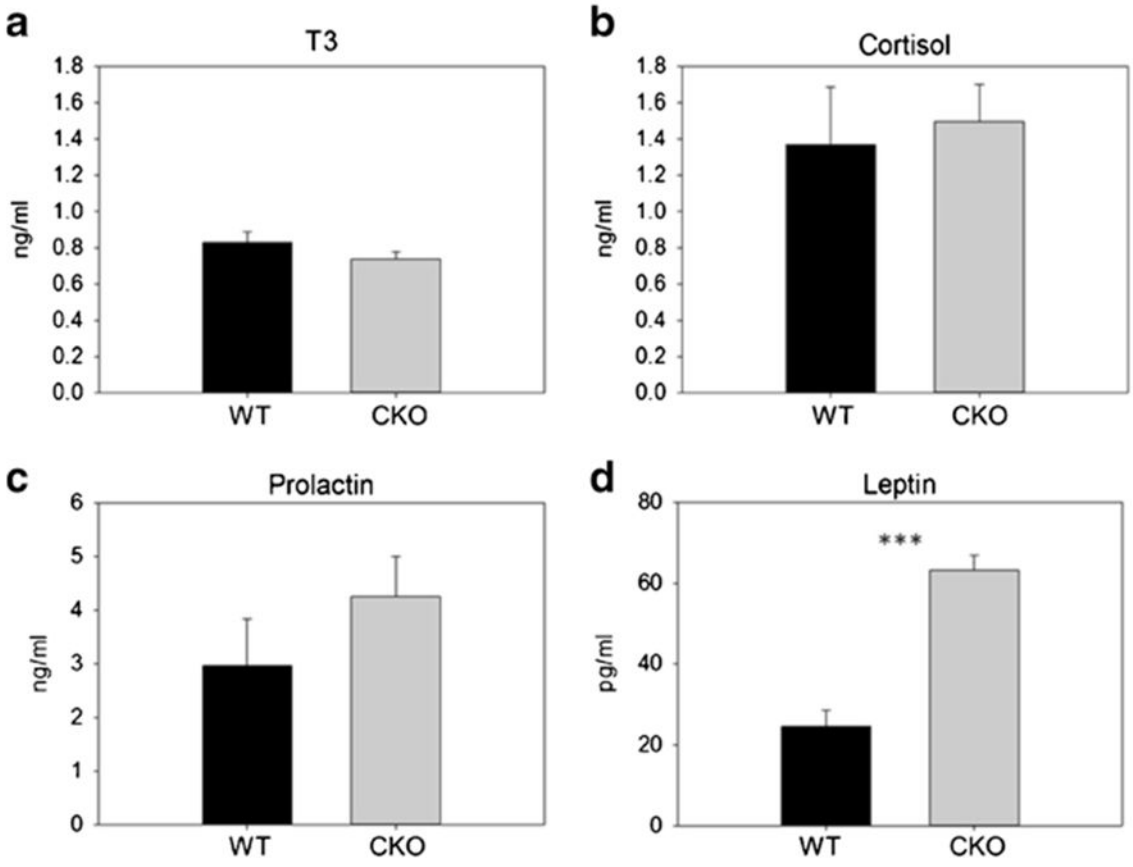


Fig. 6.

Fasted-refeeding weight change (**a–d**) and food and water consumption (**e**, **f**) in CKO mice. **a** Male CKO mice displayed decreased body weight after an overnight fast (~ 14 h) (**: $P = 0.004$, $q = 4.78$, $p = 2$) and **b** after resuming *ad libitum* feeding for 24 h (*: $P = 0.02$, $q = 3.65$, $p = 2$). **c** Male CKO mice gained significantly more body weight after refeeding (***: $P \leq 0.001$, $q = 6.64$, $p = 2$) while female CKO gained less compared to WT controls (*: $P = 0.04$, $q = 3.24$, $p = 2$). **d** A 48h fast of only male CKO and WT mice reveals significantly lower weight (*: $P = 0.05$, $F = 4.7$) and gain of weight in CKO mice after 24 h of refeeding (**: $P = 0.006$, $F = 11.3$). **e** Male CKO mice have significantly elevated food intake compared to WT ($P = 0.03$, $F = 6.34$). **f** The total consumed (food and water) of CKO mice is significantly elevated (*: $P = 0.04$, $F = 5.51$) as well as the total retained compared to WT controls (*: $P = 0.03$, $F = 6.38$). See the "Materials and Methods" section for definition and calculation of total retained values are presented as means ± SEM (standard error of mean). Fourteenhour fast and refeed: $n = 12$ for CKO (6 male, 6 female) and $n = 12$ for WT (6 male, 6 female). Forty-eight hour fast and refeed: $n = 6$ for CKO and $n = 8$ for WT, all male



e

Gene	16 weeks			Statistics	
	CKO	WT	CKO/WT	F	p
Ncb5or	0.165±0.03	3.68±0.43	0.04	76.4	<0.001
ObR All – 5′	1.49±0.152	1.14±0.101	1.32	3.69	0.077
ObR All - Mid	0.774±0.097	0.487±0.057	1.59	6.06	**0.029**
ObR All – 3′	0.849±0.069	0.579±0.075	1.46	6.99	**0.020**
ObRa	0.387±0.038	0.266±0.020	1.45	7.68	**0.016**
ObRb	0.024±0.003	0.030±0.009	0.79	0.551	0.472
ObRc	0.109±0.012	0.072±0.012	1.51	4.92	**0.045**

Fig. 7.

Serum T3, cortisol, proclactin, and leptin levels as well as cerebellar transcript levels of leptin receptor variants in male CKO mice. There were no differences in serum T3 (**a**), cortisol (**b**), or prolactin (**c**) levels between male CKO and WT mice. However, male CKO mice had significantly elevated serum levels of leptin (**d**) relative to WT controls (***: $P \leq 0.001$, $F = 47.6$). **e** Transcript levels of leptin receptors are elevated in the male CKO cerebellum relative to WT controls. ObRa and ObRc: short-form lectin receptors. ObRb:

long-form leptin receptors. Values are presented as means ± SEM. Serum ELISA: $n = 13$ (7 CKO, 6 WT), all male. qPCR of leptin receptor transcripts: $n = 15$ (8 CKO, 7 WT), all male