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RESEARCH ARTICLE

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CREB coactivator CRTC1 in melanocortin-4 receptor-expressing cells regulate dietary fat intake

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Abstract

Cyclic adenosine monophosphate-response element-binding protein-1-regulated transcription coactivator-1 (CRTC1), a cytoplasmic coactivator that translocates to the nucleus in response to cAMP, is associated with obesity. We previously reported that *CRTC1* deficiency in melanocortin-4 receptor (MC4R)-expressing neurons, which regulate appetite and energy metabolism in the brain, causes hyperphagia and obesity under a high-fat diet (HFD). HFD is preferred for mice, and the dietary fat in HFD is the main factor contributing to its palatability. These findings, along with our previous results, suggest that CRTC1 regulates the appetite for dietary fat. Therefore, in this study, we aimed to investigate the dietary fat intake behavior and energy metabolism of MC4R neuron-specific *CRTC1* knockout mice fed soybean oil or lard. *CRTC1* deficiency increased the intake of soybean oil and significantly increased body weight gain. Furthermore, obesity induced by soybean oil intake was partially due to leptin resistance. No significant changes in soybean oil intake were observed between young *CRTC1*-deficient and wild-type mice; however, soybean oil intake increased with age. Moreover, lard intake did not significantly affect the body weight. Overall, our findings highlighted the crucial role of CRTC1 in the regulation of spontaneous dietary fat intake. Furthermore, the role of CRTC1 becomes increasingly significant with age.

KEYWORDS

aging, appetite, dietary fat, gene expression, metabolism, obesity

Abbreviations: AgRP, Agouti-related protein; ARH, Arcuate nucleus of hypothalamus; BAT, Brown adipose tissue; BDNF, Brain-derived neurotrophic factor; CREB, Cyclic adenosine monophosphate response element-binding protein; CRTC, CREB-regulated transcription coactivator; HFD, High-fat diet; ITT, Insulin tolerance test; MC4R, Melanocortin-4 receptor; MSH, Melanocyte-stimulating hormone; NCD, Normal chow diet; NPY, Neuropeptide Y; OGTT, Oral glucose tolerance test; POMC, Proopiomelanocortin; PTP1B, Protein tyrosine phosphatase-1B; PVH, Paraventricular nucleus of hypothalamus; RER, Respiratory exchange ratio; Rplp0, Ribosomal protein lateral stalk subunit P0; SOCS3, Suppressor of cytokine signaling-3; TCPTP, T cell protein tyrosine phosphatase; UCP1, Uncoupling protein-1; VMH, Ventromedial nucleus of hypothalamus.

1 | INTRODUCTION

Cyclic AMP (cAMP)-response element-binding protein (CREB) is phosphorylated in response to various signals and promotes the transcription of target genes.[1,2] CREB activity is augmented by cytoplasmic coactivators, known as cAMP-regulated transcription coactivators (CRTCs), which are activated via dephosphorylation.[3–6] To date, three CRTC isoforms (CRTC1, CRTC2, and CRTC3) have been identified in rodents and humans, with CRTC1 being the most abundant isoform in neuronal cells.[5,7] Whole-body *CRTC1* knockout (KO) mice are obese, showing hyperphagia and reduced energy expenditure.[8] Therefore, CRTC1 in the brain possibly regulates appetite and energy metabolism by augmenting CREB-induced gene regulation. However, as CRTC1 is expressed in almost all neurons, the specific neuronal populations involved in brain CRTC1 function remain unknown.

Melanocortin systems, comprising melanocortin receptors and their agonists, are critical regulators of appetite and energy expenditure.[9] Specifically, melanocortin-4 receptor (MC4R) plays a crucial role in appetite regulation and its deficiency, similar to that of CRTC1, leads to obesity.[10,11] MC4R, a G protein-coupled receptor, activates adenylate cyclase and elevates intracellular cAMP signaling after binding with the ligand, α-melanocyte-stimulating hormone (αMSH).[12–14] cAMP, via CREB, increases brain-derived neurotrophic factor (BDNF) expression and suppresses appetite.[15] We hypothesized that CRTC1, which is activated by the binding of MC4R to its ligand, functions with CREB to control gene expression and appetite. To validate our hypothesis, we previously generated mice with a specific *CRTC1* deletion in MC4R neurons.[16] *CRTC1* deficiency in MC4R-expessing neurons causes hyperphagia and obesity on high-fat diet (HFD) intake. Under normal chow feeding, MC4R neuron-specific *CRTC1* KO mice do not develop obesity, indicating that CRTC1 regulates the appetite for dietary fat or dietary fat metabolism. However, the mechanisms by which MC4R neuron-specific *CRTC1* KO mice only become obese on HFD intake, but not with normal chow diet (NCD) intake, remain unknown. Although many mouse models with specific gene deficiencies leading to obesity have been developed, only a few animal models have been established that do not become obese on NCD intake but become significantly obese on HFD intake.

Fatty food items, such as donuts, cakes, fried potatoes, and fried chicken, are palatable mainly due to dietary fat.[17,18] We and other research groups have demonstrated that rodents and humans have specific receptors (CD36 and GPR120) for fat molecules in taste bud cells, indicating that dietary fat makes food palatable not only through the olfactory (smell and flavor) and sensory (texture) systems but also through the gustatory system.[19–22] Additionally, we reported that the spontaneous ingestion of dietary fat stimulates the brain reward system, promoting addictive behaviors in mice and rat.[23–25] These reports, along with our findings, suggest that CRTC1 is involved in the preference and craving for dietary fat. Therefore, loss of CRTC1 possibly alters the preference for HFD. Commercial HFDs contain various ingredients, such as soybean oil, in addition to lard. MC4R neuron-specific *CRTC1* KO mice possibly exhibit altered preferences for these components, consequently developing obesity. In this study, we aimed to investigate the dietary fat intake behavior of MC4R neuron-specific *CRTC1* KO mice.

2 | MATERIALS AND METHODS

2.1 | Animals

This study was conducted according to the ethical guidelines of the Osaka Metropolitan University Animal Experimentation Committee and Endocrine Society. All procedures were performed in compliance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals. The Osaka Metropolitan University Animal Care and Use Committee approved all animal procedures (permission number: 23–89). The Animal Research: Reporting of In Vivo Experiments guidelines were adhered to this study. Extensive efforts were made to minimize animal use and limit animal experiments other than those necessary to obtain reliable scientific results. At the end of the experimental period, the mice were euthanized via deep isoflurane anesthesia. For tissue collection, the mice were euthanized via pentobarbital administration (150 mg/kg), followed by cervical dislocation.

MC4R-expressing cell-specific *CRTC1* KO mice were generated by crossing *Crtc1*$^{loxp/loxp}$ mice[26] with *Mc4r-cre* mice (Stock No. 030759; RRID: IMSR_JAX:030759; Jackson Laboratory, ME, USA),[10] as previously described.[16] Briefly, *Crtc1*$^{loxp/loxp}$ and *Mc4r-cre* mice were crossed to obtain *Mc4r-cre*$^{+}$:*Crtc1*$^{loxp/+}$ heterozygote mice, which were intercrossed to obtain *Mc4r-cre*$^{+}$:*Crtc1*$^{loxp/loxp}$ and *Mc4r-cre*$^{-}$:*Crtc1*$^{loxp/loxp}$ mice. *Mc4r-cre*$^{+}$:*Crtc1*$^{loxp/loxp}$ and *Mc4r-cre*$^{-}$:*Crtc1*$^{loxp/loxp}$ (control) mice were used in subsequent experiments. We found that CRTC1 expression was specifically reduced in MC4R-expressing neurons, and no ectopic expression of Cre recombinase or germline recombination was observed in *Mc4r-cre*:*Crtc1*$^{loxp/loxp}$ mice.[16] Then, genomic DNA was extracted from the tail of the mice for polymerase chain reaction (PCR) genotyping. The following genotyping primers were used for the MC4R-Cre allele: F-5′-GGCAAACGGACAGAAGCA-3′, F-5′-TCCGGAGTCAAGAACTGAGG-3′, and R-5′-CAATTCAT

AACGCCCACACT-3′ (MC4R-Cre allele: 300 bp; wild-type [WT] allele: 339 bp).

All mice were housed (4–6 per cage) in a vivarium at 23 ± 2°C under a 12:12 h light/dark cycle (light phase from 0600 to 1800 h). They were individually assessed for food intake and respiratory gas analysis. The animals were fed a normal chow diet (NCD: MF; Oriental Yeast, Tokyo, Japan). The composition and caloric ratios of proteins, fats, and carbohydrates in the NCD are listed in Table 1. Voluntary ingestion of soybean oil (Fujifilm Wako, Japan), lard (Megmilk; Yukijirushi, Sapporo, Japan), and sucrose (Mitsui Sugar, Tokyo, Japan) was initiated at 6 weeks of age.

2.2 | Respiratory gas analysis

Mice were individually housed in a chamber for 48 h to attain a constant respiratory exchange ratio (RER). Gas analysis was performed using an open-circuit metabolic gas analysis system connected directly to a mass spectrometer (Model Arco2000; ArcoSystem, Chiba, Japan). Room air was pumped into the chambers at a rate of 0.3 L/min. The expired air was directed to an O_2/CO_2 analyzer for mass spectrometry. The motor activity was measured in each chamber using an infrared sensor (NS-AS01; Neuroscience Inc., Tokyo, Japan).

2.3 | Tissue collection

We collected interscapular brown adipose tissue (BAT), epididymal white adipose tissue, and hypothalamus of mice between 9 and 12 am (3–6 h after lights were turned on) and stored them at −70°C until analysis. To collect the hypothalamus, the brain was placed ventral-side-up in the brain matrix (World Precision Instruments, Sarasota, FL, USA), and a 2 mm-thick coronal slice was obtained via an anterior coronal cut in the middle of the optic tract, just rostral to the infundibulum, and a posterior coronal cut at the posterior border of the mammillary bodies. The slices were dissected laterally up to the hypothalamic sulcus and dorsally just above the third ventricle. All procedures were performed on ice to prevent RNA and protein degradation.

TABLE 1 The composition and caloric ratios in NCD.

	Content (g)	Calorie (%)
Water	7.9	
Crude Protein	23.1	25.7
Crude Fat	5.1	12.8
Crude Ash	5.8	
Crude Fiber	2.8	
Nitrogen free extract (Carbohydrate)	55.3	61.5
	100	100.0
Calorie (kcal/100 g)	359.5	

Note: Data from Oriental Yeast Co. Ltd., Tokyo, Japan.

2.4 | Blood analysis

Blood sampling of ad libitum-fed mice was conducted during the light phase (3–6 h after light exposure). Blood was collected via decapitation without anesthesia, and serum was separated using a refrigerated centrifuge. Blood serum samples were analyzed using the appropriate assay kits for glucose (Wako Pure Chemical Industries, Osaka, Japan), nonesterified free fatty acids (Wako Pure Chemical Industries), and triglycerides (Wako Pure Chemical Industries). Insulin and leptin levels were measured using the mouse insulin ELISA (RRID:AB_2783837: Mercodia, Uppsala, Sweden) and leptin ELISA (RRID:AB_2888686: Biovendor Laboratory Medicine, Brno, Czech Republic) kits, respectively.

2.5 | Quantitative PCR analysis

Total RNA from whole tissues was extracted using the TriPure Isolation Reagent (Roche, Mannheim, Germany) and reverse-transcribed to cDNA using the PrimeScript RT Reagent Kit (Takara Bio, Shiga, Japan). The resulting cDNA was amplified on a LightCycler 480 instrument using 2× SYBR Green mix (Roche, Indianapolis, IA, USA). Gene expression levels were determined relative to the levels of the housekeeping gene, ribosomal protein lateral stalk subunit P0 (*Rplp0*). The following primer sequences were used for PCR: *Rplp0*, F-5′-AGATTCGGGATATGCTGTTGGC-3′ and R-5′-TCGGGTCCTAGACCAGTGTTC-3′; Agouti-related protein (*Agrp*), F-5′-CGGCCACGAACCTCTTGTAG-3′ and R-5′-CTCATCCCCTGCCTTTGC-3′; *Bdnf* exon I, F-5′-CCTGCATCTGTTGGGGAGAC-3′ and R-5′-GCCTTGTCCGTGGACGTTTA-3′; neuropeptide Y (*Npy*), F-5′-CTACTCCGCTCTGCGACACT-3′ and R-5′-AGTGTCTCAGGGCTGGATCTC-3′; proopiomelancortin (*Pomc*), F-5′-GAGGCCACTGAACATCTTTGTC-3′ and R-5′-GCAGAGGCAAACAAGATTGG-3′; *Mc4r*, F-5′-CCCGGACGGAGGATGCTAT-3′ and R-5′-TCGCCACGATCACTAGAATGT-3′; *Stat3*, F-5′-AGCTGGACACACGCTACCT-3′ and R-5′-TCCAGTAGAATCCGCTCTCCT-3′; *Socs3*, F-5′-ATGGTCACCCACAGCAAGTTT-3′ and R-5′-TCCAGTAGAATCCGCTCTCCT-3′; *Ucp1*,

F-5′-TGCACCACACTCCTGGCCTCT-3′ and R-5′-GGCC GTCGGTCCTTCCTTGG-3′; *Prdm16*, F-5′-AGGGCA AGAACCATTACACG-3′ and R-5′-GGAGGGTTTTGTC TTGTCCA-3′; *AdipoQ*, F-5′-GGGCTCAGGATGCTA CTGTT-3′ and R-5′-ACCTGCACAAGTTCCCTTGG-3′; *Leptin*, F-5′-TGACACCAAAACCCTCATCA-3′ and R-5′-TGAAGCCCAGGAATGAAGTC-3′; *Adgre1*, F-5′-GTG CCATCATTGCGGGATTC-3′ and R-5′-TGGAAGCCCAT AGCCAAAGG-3′.

2.6 | Immunohistochemistry

Mice were anesthetized with sodium pentobarbital (100 mg/kg), perfused via the aorta with phosphate-buffered saline (PBS), and treated with 4% paraformaldehyde. The brain was removed, fixed with 4% paraformaldehyde, sliced into 30 μm-thick sections, and placed in PBS. After pre-incubation with Block Ace (Yukijirushi, Tokyo, Japan), the sections were incubated with the rabbit anti-SOCS3 antibody (1:1000 dilution; Cat# 14025-1-AP; RRID: AB_ 10,597,854; Proteintech, Rosemont, IL, USA) at room temperature (20–30°C) overnight. After several rinses with PBS, the sections were incubated with the Alexa Fluor 555-conjugated donkey anti-rabbit IgG (1:1000 dilution; Cat# A-31572; RRID:AB_162543; Thermo Fisher Inc., San Jose, CA, USA) for 2 h at 4°C. Then, 4′,6-diamidino-2-phenylindole (Sigma-Aldrich, Burlington, MA, USA) was used for nuclear staining. Immunostained sections were observed using a confocal laser scanning microscope (FV1000; Olympus, Tokyo, Japan) equipped with Olympus FluoView software. Individual optical sections were obtained without further processing.

To quantify SOCS3 immunoreactivity, images of the paraventricular nucleus of the hypothalamus (PVH), ventromedial hypothalamus (VMH), and arcuate nucleus of the hypothalamus (ARH) from approximately the same anatomical location were used for image analysis. Image analysis was performed using ImageJ software (NIH). To measure the intensity of SOCS3 immunoreactivity, the background was determined based on the intensity values in several areas without any detectable SOCS3 immunoreactivity. SOCS3 intensities in the PVH, VMH, and ARH were determined as signals over background intensity.

2.7 | Food intake measurement

In this experiment, mice (young mice: 6–8 weeks old; middle-aged mice: 12–16 weeks old) with no prior intake of soybean oil, lard, and sucrose were used. The mice were housed individually and fed NCD. Each mouse was randomly fed one of the test samples (soybean oil, lard, and sucrose) for at least 3 days to avoid any initial adverse effect. On the test day, the 24-h intake of NCD and test samples was measured. All mice were analyzed for all test samples.

2.8 | Licking behavior test

Mice (young mice: 6–8 weeks old; middle-aged mice: 12–16 weeks old) with no prior intake of soybean oil were used in this test. A two-bottle licking test chamber (LKP2-MC) manufactured by Melquest (Toyama, Japan) was used to investigate the licking behavior. Each lick of a test solution (water or soybean oil) was detected and recorded for 48 h. Initially, for habituation, the mice were placed in a test chamber with a water bottle and NCD. After habituation, each mouse was provided access to water and soybean oil, and the licking counts for each solution were recorded every 10 min. After licking counts for water and soybean oil stabilized, the licking counts were recorded for 48 h.

2.9 | Oral glucose tolerance test (OGTT) and insulin tolerance test (ITT)

After 6 weeks of lard ingestion, OGTT and ITT were performed. Briefly, mice were fasted overnight for 16 h with free access to water. Glucose (1 g/kg, p.o.) was administered, and tail blood glucose levels were measured using the One Touch Ultra glucometer (Johnson & Johnson, Chesterbrook, PA, USA).

For ITT, the mice were fasted for 4 h. Insulin (1 U/kg body weight; Humulin-R; Eli Lilly, Indianapolis, IN, USA) was administered, and blood glucose levels were measured ($n = 8$).

2.10 | Statistical analyses

Data are represented as the mean ± standard error of the mean. Statistical analyses were conducted using Welch's *t*-test to compare two groups (Prism 10.0; GraphPad Software Inc., San Diego, CA, USA). Analysis of variance (ANOVA) was used to compare multiple groups. When significant differences were observed, Tukey's post-hoc analysis was performed. Two-way repeated-measures ANOVA was used to compare the group differences in body weight gain and food intake. When significant differences were observed, Sidak's multiple comparison post-hoc test was performed. Differences were considered statistically significant at $p < 0.05$.

3 | RESULTS

3.1 | Body weight gain

Under NCD, *Mc4r-cre:Crtc1*$^{loxp/loxp}$ mice showed no significant body weight gain (Figure 1A). The liver of *Mc4r-cre:Crtc1*$^{loxp/loxp}$ mice was slightly, but significantly, heavier than that of the control mice. Similar to our previous report,[27] voluntary intake of soybean oil did not affect body weight gain in control (*Crtc1*$^{loxp/loxp}$ mice) animals (Figure 1C) compared to that observed in NCD alone. However, soybean oil intake induced significant body weight gain in *Mc4r-cre:Crtc1*$^{loxp/loxp}$ mice (Figure 1C). Moreover, adipose tissue amount and liver size were significantly increased in *Mc4r-cre:Crtc1*$^{loxp/loxp}$ mice at 14 weeks of age (Figure 1D).

Lard intake did not cause a significant difference in the body weight gain between *Mc4r-cre:Crtc1*$^{loxp/loxp}$ and control mice (Figure 1E,F). Sucrose intake significantly increased body weight gain in *Mc4r-cre:Crtc1*$^{loxp/loxp}$ mice (Figure 1G,H). However, compared with soybean oil intake, the difference in body weight between *Mc4r-cre:Crtc1*$^{loxp/loxp}$ and control mice was modest.

3.2 | Blood analysis

To clarify the impact of soybean oil intake on metabolism, blood samples of mice were collected after 8 weeks of soybean oil intake, and the energy substrates in the blood were measured. Additionally, blood samples were obtained from mice consuming lard to compare with the results of mice consuming soybean oil.

Blood glucose levels were significantly lower in *Mc4r-cre:Crtc1*$^{loxp/loxp}$ mice fed NCD (Figure 2A). Conversely, blood glucose levels were significantly increased in

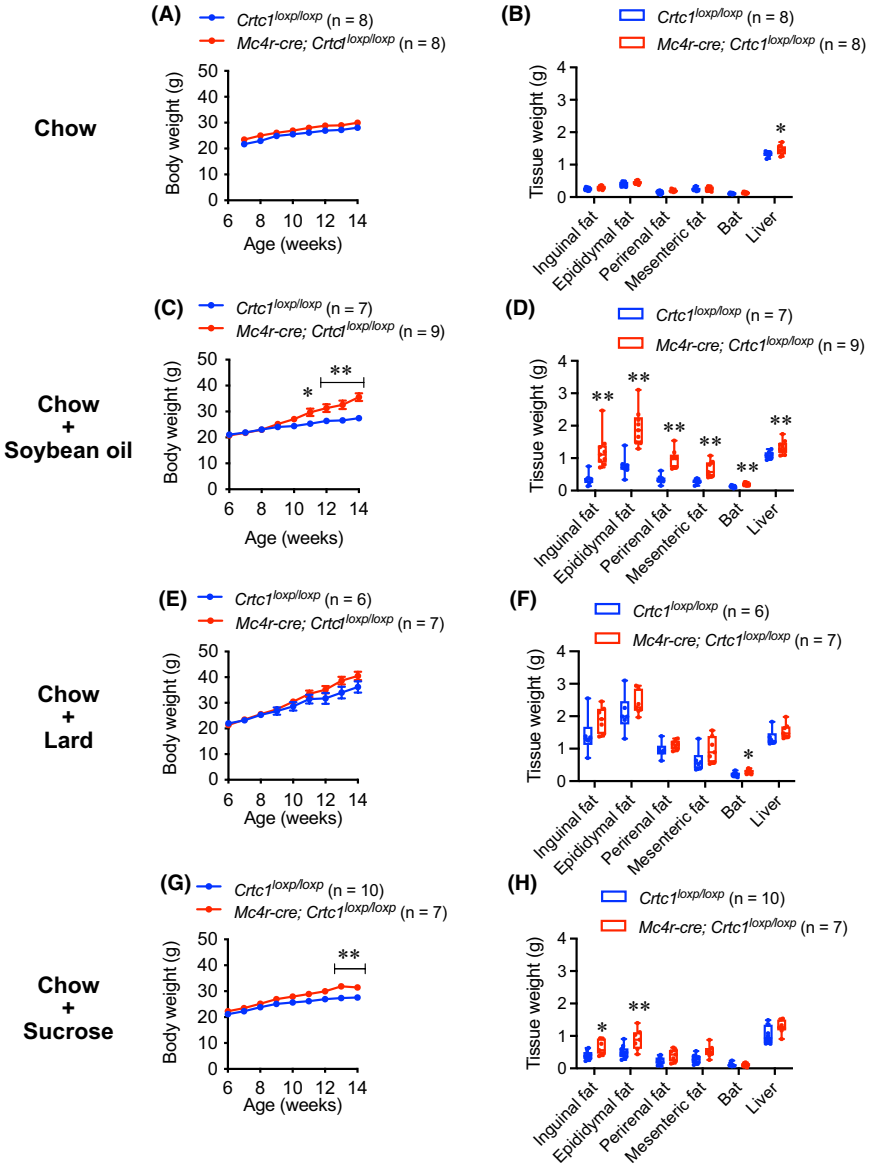

FIGURE 1 (A) Body weight change and (B) tissue weight at 14 weeks old in *Crtc1*$^{loxp/loxp}$ and *Mc4r-cre:Crtc1*$^{loxp/loxp}$ mice fed a normal chow diet (Chow; $n = 8$). (C) Body weight change and (D) tissue weight at 14 weeks old fed a normal chow diet with soybean oil (Chow + soybean oil; $n = 7$–9). (E) Body weight change and (F) tissue weight at 14 weeks old fed a normal chow diet with lard (Chow + lard; $n = 6$–7). (G) Body weight change and (H) tissue weight at 14 weeks old fed normal chow diet with sucrose (Chow + sucrose; $n = 7$–10). Data are represented as the mean ± standard error of the mean (SEM). $^{**}p < 0.01$ and $^{*}p < 0.05$ via two-way repeated-measures analysis of variance (ANOVA), followed by Bonferroni's multiple comparisons post-hoc test. Right panel: Tissue weight at 14 weeks of age. $^{**}p < 0.01$ and $^{*}p < 0.05$ (right panel). In the box and whisker plots, the median value is indicated by the horizontal line, with the top and bottom of the box indicating the 75th and 25th percentiles, respectively, and whiskers indicating the maximum and minimum points.

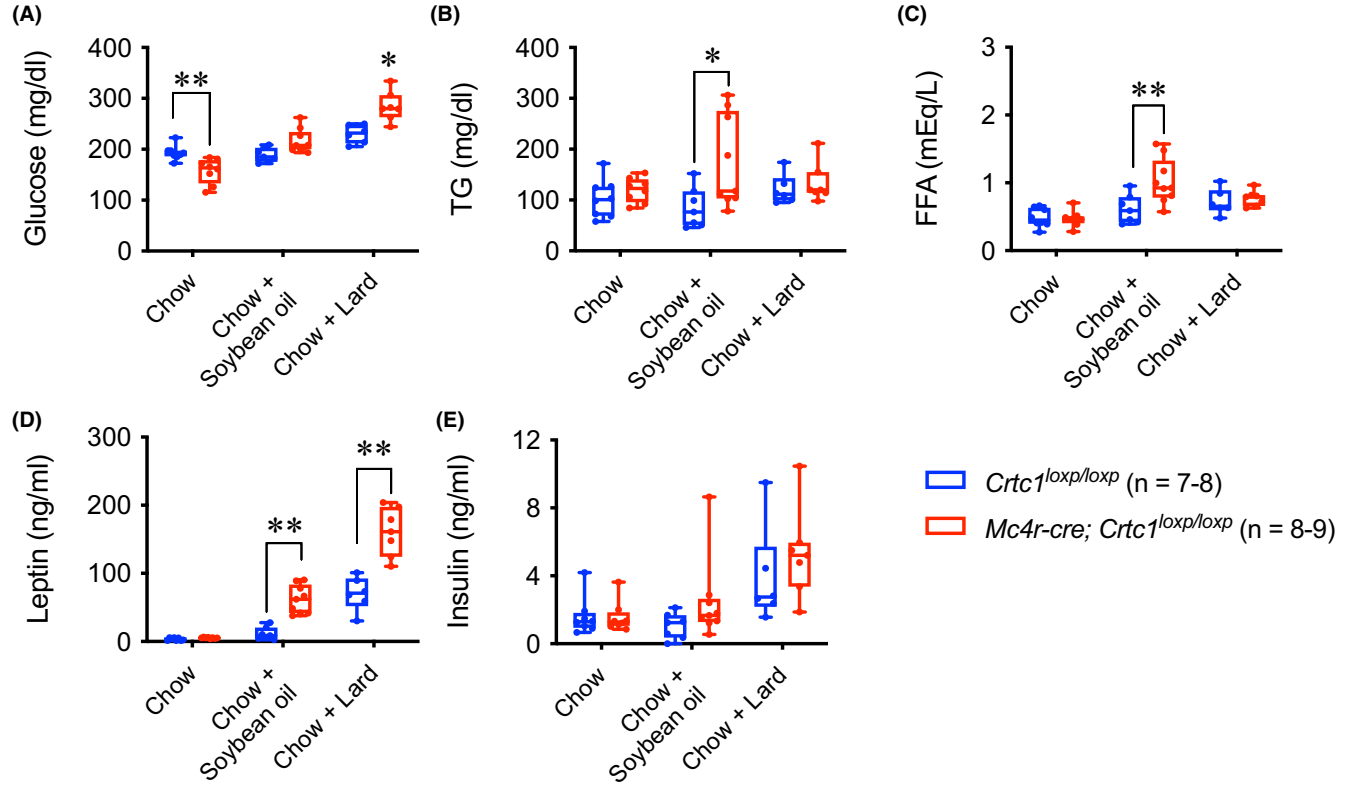

FIGURE 2 (A) Levels of serum glucose, (B) triglycerides (TGs), (C) free fatty acids (FFAs), (D) leptin, and (E) insulin in *Crtc1*$^{loxp/loxp}$ and *Mc4r-cre:Crtc1*$^{loxp/loxp}$ mice after 8 weeks of soybean oil or lard intake ($n = 7–9$). $^{**}p < 0.01$ and $^{*}p < 0.05$. In the box and whisker plots, the median value is indicated by the horizontal line, with the top and bottom of the box indicating the 75th and 25th percentiles, respectively, and the whiskers indicating the maximum and minimum points.

Mc4r-cre:Crtc1$^{loxp/loxp}$ mice compared to those in the control mice fed lard. Soybean oil intake significant increased the serum triglyceride and free fatty acid levels in *Mc4r-cre:Crtc1*$^{loxp/loxp}$ mice (Figure 2B,C). In contrast, lard intake did not affect the triglyceride and free fatty acid levels in *Crtc1*$^{loxp/loxp}$ and *Mc4r-cre:Crtc1*$^{loxp/loxp}$ mice compared to those in NCD-fed mice, and no significant differences were observed between the groups.

Next, we measured the levels of leptin and insulin, which are hormones related to glucose metabolism and energy regulation. Soybean oil intake did not affect, whereas lard intake increased the leptin levels in *Crtc1*$^{loxp/loxp}$ (control) mice (Figure 2D). In *Mc4r-cre:Crtc1*$^{loxp/loxp}$ mice, both soybean oil and lard intake significantly increased the leptin levels. Notably, both soybean oil and lard intake did not affect the insulin levels in mice (Figure 2E).

3.3 | Energy expenditure, food intake, gastric emptying

As significant differences in body weight were observed following soybean oil intake (6 weeks), we further conducted respiratory gas analysis. No significant differences were observed in the RER, oxygen consumption, and activity of *Mc4r-cre:Crtc1*$^{loxp/loxp}$ and control mice following soybean oil intake (Figure 3A–F).

As no changes in oxygen consumption were observed, we examined the calorie intake. NCD and soybean oil intake was measured in individually housed cages. Soybean oil intake was initiated at 6 weeks of age, consistent with the experiment shown in Figure 1. NCD intake was significantly high in *Mc4r-cre:Crtc1*$^{loxp/loxp}$ mice at 11 weeks of age (Figure 3G). Similar to the body weight change graph shown in Figure 1, no differences were observed in the soybean oil intake of *Mc4r-cre:Crtc1*$^{loxp/loxp}$ and control mice up to 3 weeks after the initation of soybean oil intake. Soybean oil and total caloric intake (calories of NCD plus calories of soybean oil) were significantly high in *Mc4r-cre:Crtc1*$^{loxp/loxp}$ mice after 9 weeks of age (Figure 3H,I).

Next, we measured the gastric emptying rate to determine whether increased soybean oil intake correlates with increased gastric emptying speed. We administered soybean oil containing ^{13}C-labeled octanoic acid to mice and measured the concentration of $^{13}CO_2$ expelled in the mouse breath. Administration of ^{13}C-labeled octanoic

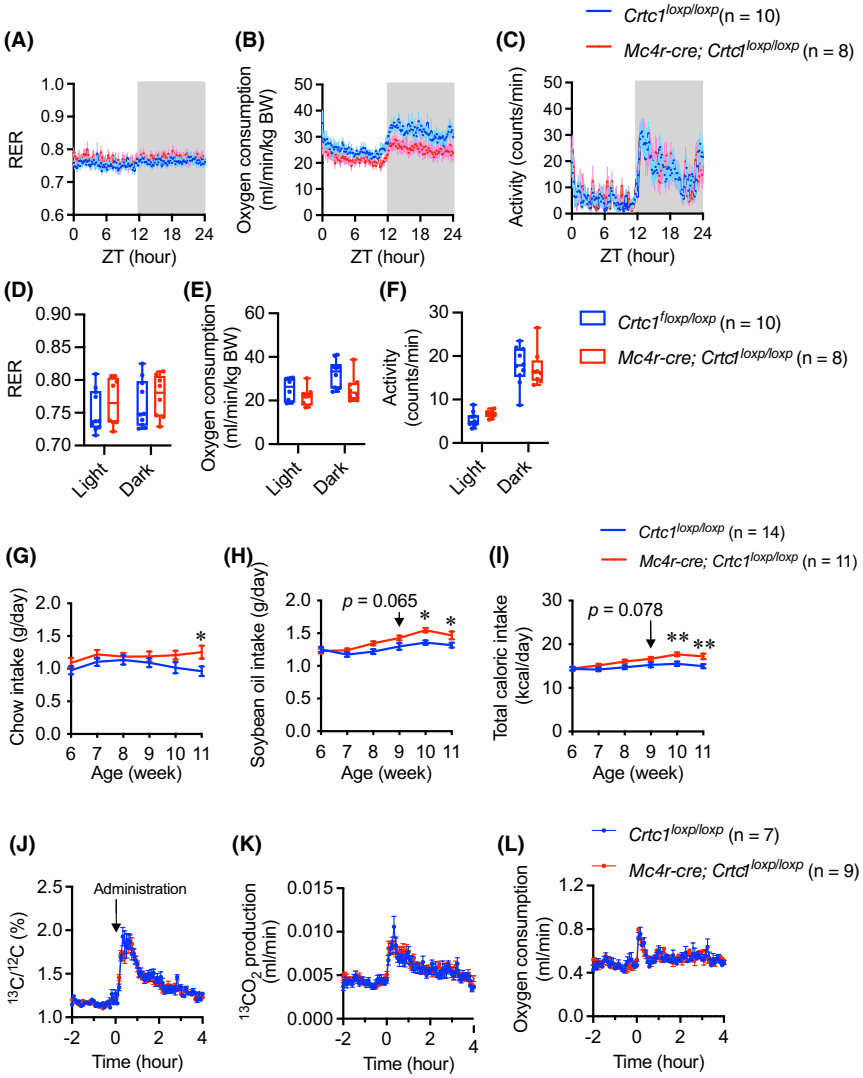

FIGURE 3 (A) Respiratory exchange ratio (RER), (B) oxygen consumption, and (C) spontaneous motor activity in *Crtc1*$^{loxp/loxp}$ and *Mc4r-cre:Crtc1*$^{loxp/loxp}$ mice after 8 weeks of voluntary soybean oil intake ($n = 7–8$). Data are represented as the mean ± SEM. (D) Average RER, (E) oxygen consumption, and (F) motor activity after 12 h (light and dark phases). In the box and whisker plots, the median value is indicated by the horizontal line, with the top and bottom of the box indicating the 75th and 25th percentiles, respectively, and the whiskers indicating the maximum and minimum points. (G) Daily chow intake, (H) soybean oil intake, and (I) total caloric intake in *Crtc1*$^{loxp/loxp}$ and *Mc4r-cre:Crtc1*$^{loxp/loxp}$ mice fed a normal chow diet with soybean oil. Data are represented as the mean ± SEM. $^{**}p < 0.01$ and $^{*}p < 0.05$ via two-way repeated-measures ANOVA, followed by Bonferroni's multiple comparisons post-hoc test. (J) Changes in the $^{13}C/^{12}C$ ratio in respiratory gas, (K) $^{13}CO_2$ production, and (L) oxygen consumption in mice after ^{13}C-octanoic acid administration. Data are represented as the mean ± SEM.

acid increased the $^{13}C/^{12}C$ ratio (Figure 3J) and $^{13}CO_2$ production (Figure 3K). However, no significant differences were observed in the $^{13}C/^{12}C$ ratio, $^{13}CO_2$ production, and oxygen consumption (Figure 3L) of *Mc4r-cre:Crtc1*$^{loxp/loxp}$ and control mice.

3.4 | Quantitative PCR analysis

To investigate the effect of soybean oil intake on gene expression, we administered soybean oil to mice for 8 weeks and collected the hypothalamus, BAT, and epididymal white adipose tissue for quantitative PCR analysis.

mRNA levels of appetite-stimulating NPY were significantly decreased in mice fed soybean oil compared to those in mice not fed soybean oil (Figure 4A, top panel). However, no effects of *CRTC1* deficiency were observed. However, no differences were observed in the appetite-stimulating AgRP, appetite-suppressing POMC, and MC4R levels between the groups with and without soybean oil intake. mRNA levels of *Socs3*, *Ptpn1*, and *Ptpn2*, which inhibit pathways downstream of leptin, were increased in soybean oil-fed *Mc4r-cre:Crtc1*$^{loxp/loxp}$ mice

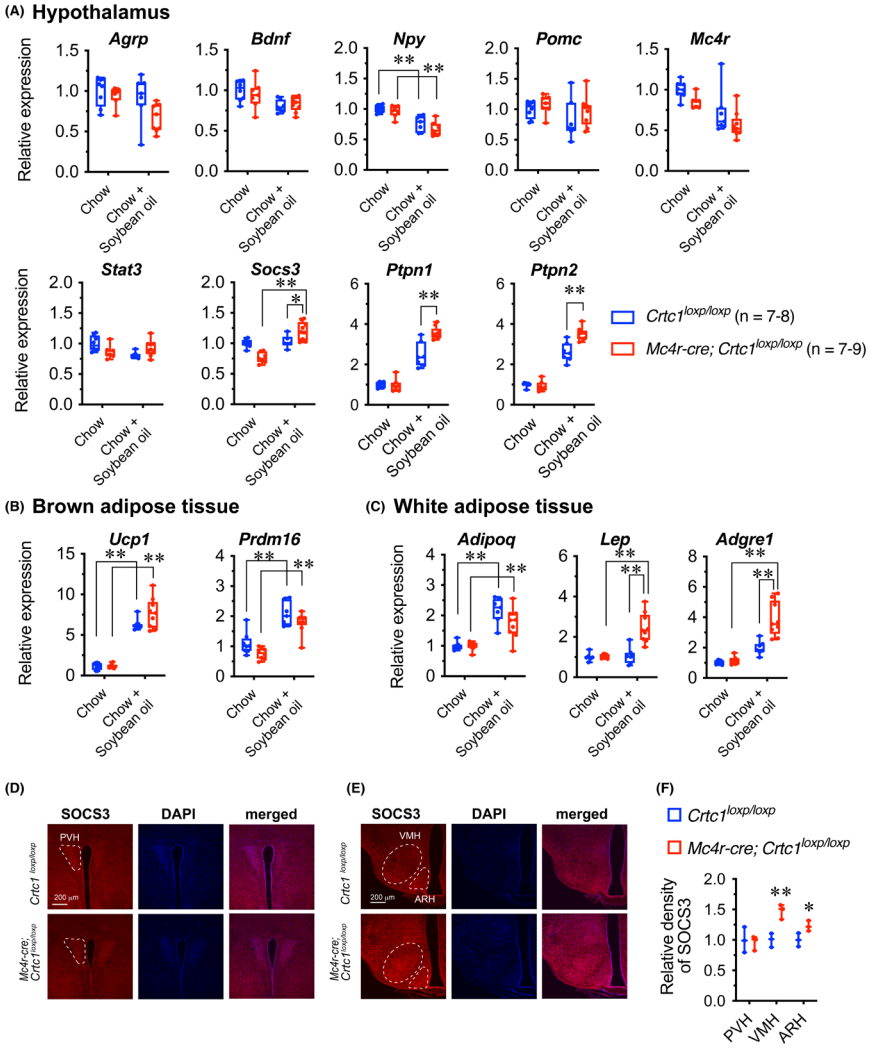

FIGURE 4 (A) mRNA levels in the hypothalamus, (B) epididymal adipose tissue, and (C) interscapular brown adipose tissue ($n = 7$–9). $^{**}p < 0.01$ and $^{*}p < 0.05$ via one-way ANOVA, followed by Tukey's post-hoc test. In the box and whisker plots, the median value is indicated by the horizontal line, with the top and bottom of the box indicated the 75th and 25th percentiles, respectively, and the whiskers indicating the maximum and minimum points. (D and E) Immunohistochemical analysis of the suppressor of cytokine signaling-3 (SOCS3) expression in the hypothalamus. Brain slices were stained with the anti-SOCS3 antibody (red) and 4′,6-diamidino-2-phenylindole (DAPI; blue). (F) Density of SOCS3 immunoreactivity in PVH, VMH, and ARH ($n = 3$). In the box and whisker plots, the median value is indicated by the horizontal line, with the top and bottom of the box indicated the 75th and 25th percentiles, respectively, and the whiskers indicating the maximum and minimum points.

compared to those in the control group (Figure 4A, bottom panel). Notably, Stat3, which plays an important role in leptin signaling, was not affected by *CRTC1* deficiency or soybean oil intake.

Next, we examined thermogenesis-related gene levels in BAT and observed increased mRNA levels of *Ucp1* and *Prdm16* in mice fed soybean oil compared to those in mice not fed soybean oil (Figure 4B). However, no differences in gene expression levels were observed due to *CRTC1* deficiency. In the epididymal white adipose tissue, *AdipoQ* levels were increased with soybean oil intake (Figure 4C). Soybean oil intake also increased the mRNA levels of leptin and inflammation marker *Adgre1* (encoding F4/80) in *Mc4r-cre:Crtc1*$^{loxp/loxp}$ mice compared to those in control mice.

3.5 | Immunohistochemistry

We examined SOCS3 protein expression via immunohistochemistry using the anti-SOCS3 antibody. In the paraventricular nucleus of hypothalamus (PVH), with abundant MC4R neurons and CRTC1,[9,10,16] SOCS3 immunoreactivity was weak in both *Mc4r-cre:Crtc1*$^{loxp/loxp}$ and control mice (Figure 4D). In the ventromedial nucleus of the hypothalamus (VMH) and arcuate nucleus of the hypothalamus (ARC), with abundant leptin receptors and leptin signaling components, stronger SOCS3 immunoreactivity was observedin *Mc4r-cre:Crtc1*$^{loxp/loxp}$ mice than in the control mice (Figure 4E,F).

3.6 | Food intake in young and middle-aged mice

As indicated by the weight change (Figure 1B), *Mc4r-cre:Crtc1*$^{loxp/loxp}$ mice did not exhibit weight gain after initiation soybean oil intake at 6 weeks of age until 10 weeks of age; however, a significant increase in weight gain began at approximately 11 weeks of age. To investigate whether age influences the dietary preferences of mice, we measured the intake of NCD, soybean oil, lard, and sucrose in young (6–8 weeks old) and middle-aged (12–16 weeks old) mice and calculated the total calorie intake. All diets were

freely available, as shown in Figure 1, and intake was assessed over a 24-h period.

In young mice, no significant differences were observed in the NCD and soybean oil intake of *Mc4r-cre:Crtc1*$^{loxp/loxp}$ and control mice (Figure 5A,B). Under conditions where lard and NCD were available for intake in the cage, no differences were observed in the lard intake of *Mc4r-cre:Crtc1*$^{loxp/loxp}$ and control mice; however, NCD and total calorie intake were higher in *Mc4r-cre:Crtc1*$^{loxp/loxp}$ mice than in the control mice (Figure 5E). When sucrose and NCD were available for intake, sucrose intake was higher in *Mc4r-cre:Crtc1*$^{loxp/loxp}$ mice than in the control mice (Figure 5C), but no differences were observed in the total calorie intake (Figure 5F). In middle-aged mice, soybean oil and total calorie intake were higher in *Mc4r-cre:Crtc1*$^{loxp/loxp}$ mice than in the control mice (Figure 5G,J). Moreover, under conditions where

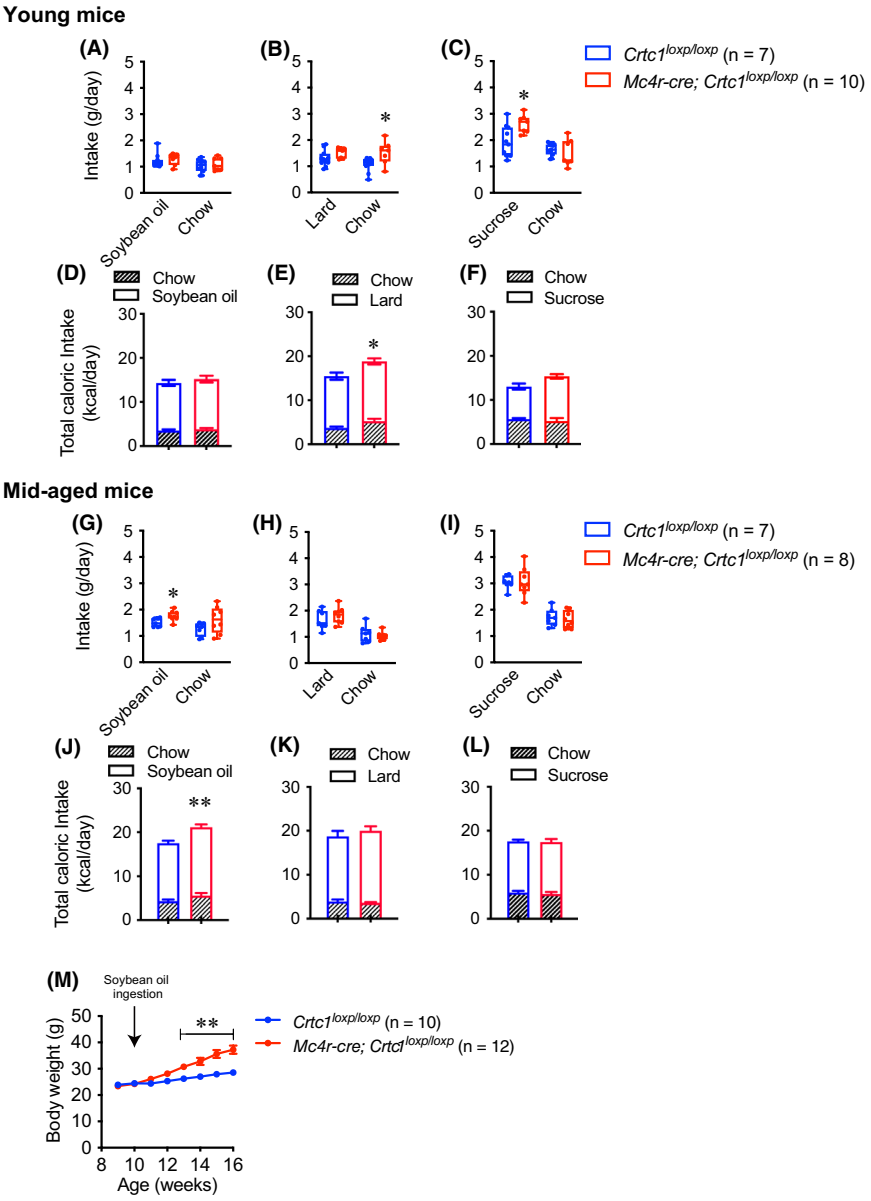

FIGURE 5 Comparison of food intake between young (6–8 weeks old) and middle-aged (12–16 weeks old) mice. (A) Daily intake of chow and soybean oil, (B) chow and lard, and (C) chow and sucrose in *Crtc1*$^{loxp/loxp}$ and *Mc4r-cre:Crtc1*$^{loxp/loxp}$ mice (young mice) ($n = 7$–10). (D–F) Total caloric intake for 24 h in each experimental condition. (G) Daily intake of chow and soybean oil, (H) chow and Lard, (I) chow and sucrose in *Crtc1*$^{loxp/loxp}$ and *Mc4r-cre:Crtc1*$^{loxp/loxp}$ mice (middle-aged mice) ($n = 7$–8). (J–L) Total caloric intake for 24 h in each experimental condition. $^{**}p < 0.01$ and $^{*}p < 0.05$ via one-way ANOVA, followed by Tukey's post-hoc test. In the box and whisker plots, the median value is indicated by the horizontal dividing line, with the top and bottom of the box indicating the 75th and 25th percentiles, respectively, and the whiskers indicating the maximum and minimum points. (M) Body weight changes in *Crtc1*$^{loxp/loxp}$ and *Mc4r-cre:Crtc1*$^{loxp/loxp}$ mice fed normal chow diet with soybean oil ($n = 10$–12). Soybean oil intake in mice began at 10 weeks of age. Data are represented as the mean ± SEM. $^{**}p < 0.01$ and $^{*}p < 0.05$ via two-way repeated-measures ANOVA, followed by Bonferroni's multiple comparisons post-hoc test.

lard or sucrose was available for intake, no significant differences were observed in the NCD, lard, or sucrose intake of *Mc4r-cre:Crtc1*$^{loxp/loxp}$ and control mice (Figure 5H,I).

As only middle-aged *Mc4r-cre:Crtc1*$^{loxp/loxp}$ mice showed a significant increase in soybean oil intake, we administered soybean oil at 10 weeks of age and observed body weight gain. As shown in Figure 5M, *Mc4r-cre:Crtc1*$^{loxp/loxp}$ mice showed significant body weight gain soon after soybean oil ingestion.

3.7 | Licking behavior

To determine whether the weight gain caused by soybean oil intake in *Mc4r-cre:Crtc1*$^{loxp/loxp}$ mice is due to meal rhythm disruption, the frequency of soybean oil licking was measured for 2 days. In young mice, no significant differences were observed in the licking frequency of water (Figure 6A,C) and soybean oil (Figure 6B,D) between *Mc4r-cre:Crtc1*$^{loxp/loxp}$ and control mice during both the light and dark phases over the two-day period.

In middle-aged mice, no significant differences were observed in the water-licking frequency of *Mc4r-cre:Crtc1*$^{loxp/loxp}$ and control mice over the two-day period (Figure 6E,G). However, soybean oil licking frequency was significantly increased in *Mc4r-cre:Crtc1*$^{loxp/loxp}$ mice during the dark phase (Figure 6F,H).

3.8 | Energy expenditure and glucose metabolism in mice fed lard

Elevated blood glucose levels were observed in *Mc4r-cre:Crtc1*$^{loxp/loxp}$ mice fed lard for 8 weeks (Figure 2A). To

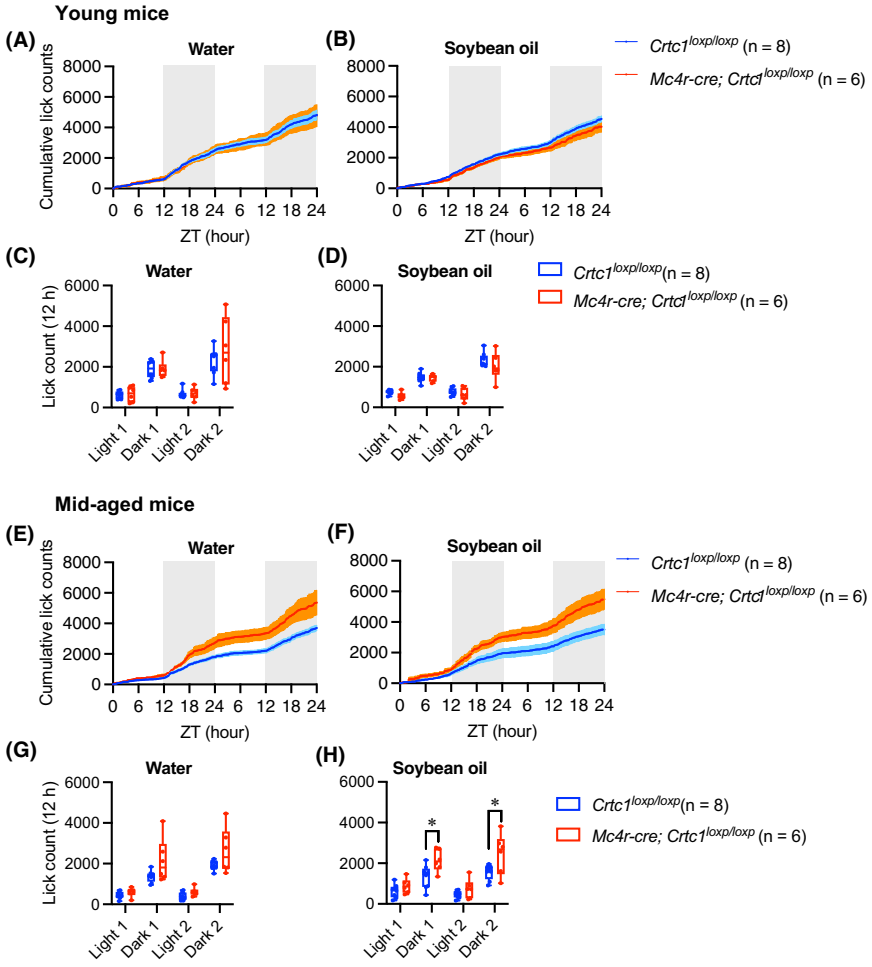

FIGURE 6 (A) Cumulative lick counts for water and (B) soybean oil in *Crtc1*$^{loxp/loxp}$ and *Mc4r-cre:Crtc1*$^{loxp/loxp}$ mice (young mice). $n = 6–8$. Data are represented as the mean ± SEM. (C) Twelve-hour lick counts for water and (D) soybean oil. In the box and whisker plots, the median value is indicated by the horizontal line, with the top and bottom of the box indicating the 75th and 25th percentiles, respectively, and the whiskers indicating the maximum and minimum points. (E) Cumulative lick counts for water and (F) soybean oil (right panel) in *Crtc1*$^{loxp/loxp}$ and *Mc4r-cre:Crtc1*$^{loxp/loxp}$ mice (middle-aged mice). $n = 6–8$. Data are represented as the mean ± SEM. (G) Twelve-hour lick counts for water and (H) soybean oil. *$p < 0.05$. In the box and whisker plots, the median value is indicated by the horizontal line, with the top and bottom of the box indicating the 75th and 25th percentiles, respectively, and the whiskers indicating the maximum and minimum points.

determine whether any metabolic abnormalities or impairment of glucose metabolism occur in *Mc4r-cre:Crtc1*$^{loxp/loxp}$ mice due to lard intake, we conducted respiratory gas analysis, OGTT, and ITT. Respiratory gas analysis showed no significant differences in the RER, oxygen consumption, and locomotor activity of *Mc4r-cre:Crtc1*$^{loxp/loxp}$ and control mice after 8 weeks of lard intake (Figure 7A–F). Additionally, OGTT and ITT revealed no significant differences between *Mc4r-cre:Crtc1*$^{loxp/loxp}$ and control mice (Figure 7G,H).

4 | DISCUSSION

Fat-rich food is highly desirable for animals. Dietary fat enhances the food quality. These properties of dietary fat make high-energy diets favorable for wild animals. However, in modern society, the selective intake of fatty food is a significant problem that leads to obesity. Although fatty food items are palatable, their intake must be strictly controlled to maintain good health.

In this study, we examined the effects of MC4R deficiency on dietary fat intake and subsequent body weight gain. Four experimental groups were established: NCD alone, NCD with soybean oil, NCD with lard, and NCD with sucrose. Free access was provided to each diet to examine their effects on weight gain. *Mc4r-cre:Crtc1*$^{loxp/loxp}$ mice showed the most significant weight gain when soybean oil and NCD were provided ad libitum. Moreover, under conditions with freely available soybean oil and NCD, mice showed higher daily intake of soybean oil than the control animals. These findings suggest that CRTC1 in MC4R-expressing cells regulates dietary fat intake.

Mc4r-cre:Crtc1$^{loxp/loxp}$ mice exhibited significant weight gain with soybean oil intake, but not with lard intake, despite both being dietary fats. The precise mechanisms underlying this phenomenon remain unclear. Mice prefer unsaturated fatty acids over saturated fatty acids.[28,29] We previously reported that interactions between fatty acid receptors CD36[20,21] and GPR120 expressed in taste cells[22] and dietary fat molecule influence fat palatability, with physically solid fats, such as lard, exerting less impact on mouse taste cells, resulting in decreased palatability. Solid fats generally have slower gastric emptying rates and longer digestion periods than liquid fats. These differences may have contributed to the increased soybean oil intake in *Mc4r-cre:Crtc1*$^{loxp/loxp}$ mice observed in this study.

Direct measurement of the gastric expulsion rate of soybean oil did not reveal any significant differences between *Mc4r-cre:Crtc1*$^{loxp/loxp}$ and control mice. This suggests that CRTC1 in MC4R-expressing cells does not influence the gastric emptying rate. Therefore, *Mc4r-cre:Crtc1*$^{loxp/loxp}$ mice may fail to sense satiety despite excessive gastric distension due to overconsumption.

Obesity primarily results from not only overeating but also reduced energy expenditure. Therefore, we conducted respiratory gas analysis in environments in which mice freely consumed NCD with either soybean oil or lard. Although *Mc4r-cre:Crtc1*$^{loxp/loxp}$ mice were heavier than control mice with soybean oil intake, no significant differences were observed in oxygen consumption compared to

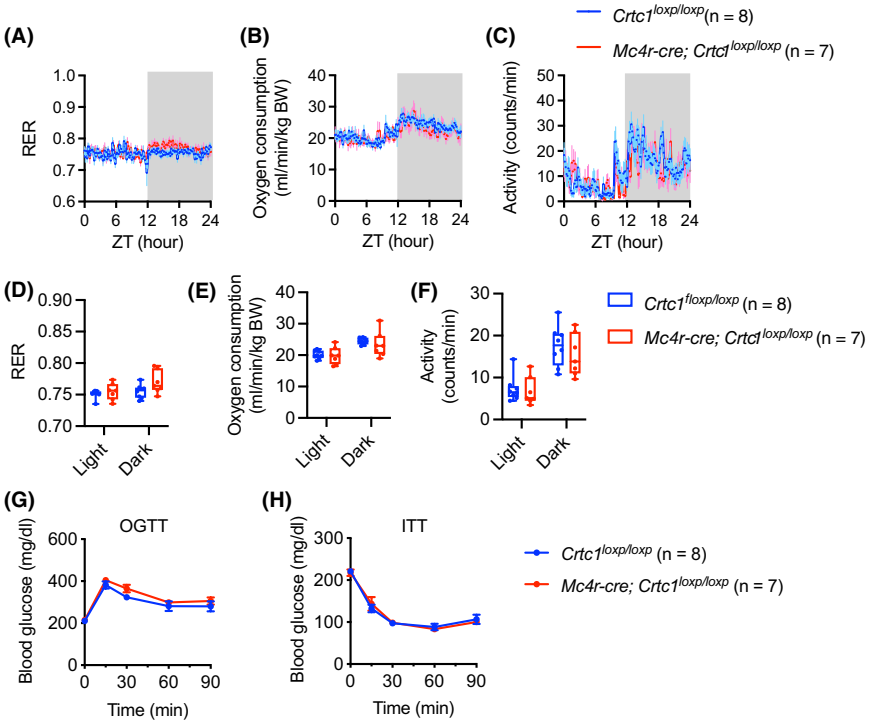

FIGURE 7 (A) RER, (B) oxygen consumption, and (C) spontaneous motor activity in *Crtc1*$^{loxp/loxp}$ and *Mc4r-cre:Crtc1*$^{loxp/loxp}$ mice after 8 weeks of voluntary lard intake ($n = 7–8$). Data are represented as the mean ± SEM. (D) Average RER, (E) oxygen consumption, and (F) motor activity after 12 h (light and dark phases). In the box and whisker plots, the median value is indicated by the horizontal line, with the top and bottom of the box indicating the 75th and 25th percentiles, respectively, and the whiskers indicating the maximum and minimum points. (G) Oral glucose tolerance and (H) insulin tolerance tests ($n = 7–8$). Data are represented as the mean ± SEM.

control mice. Similarly, there were no changes in oxygen consumption under lard intake conditions. These results suggest that CRTC1 expression in MC4R-expressing cells is not crucial for energy expenditure.

Obesity can also occur because of leptin resistance, in which leptin is less effective.[30] In the current study, *Mc4r-cre:Crtc1*$^{loxp/loxp}$ mice, under conditions where they freely consumed NCD and soybean oil, exhibited significant weight gain despite increased leptin mRNA expression in the adipose tissue and higher blood leptin levels. Therefore, these mice may develop leptin resistance upon free consumption of soybean oil.

Leptin activates POMC neurons in the ARH, suppress appetite.[31] POMC is the precursor of αMSH, an agonist of MC4R. Activated POMC neurons release αMSH into PVH, thereby inhibiting feeding behavior.[32,33] As MC4R neurons are abundant in the PVH, CRTC1 may be involved in leptin-mediated feeding suppression through MC4R neurons.

Leptin resistance involves various factors, such as the suppressor of cytokine signaling-3 (SOCS3), protein tyrosine phosphatase-1B (PTP1B, encoded by *Ptpn1*), and T-cell protein tyrosine phosphatase (TCPTP, encoded by *Ptpn2*).[34,35] SOCS3 expressed in POMC neurons in the ARH affects weight regulation and leptin resistance under HFD conditions.[36] PTP1B in POMC neurons negatively affects leptin signaling.[37,38] Overexpression of TCPTP induces leptin resistance due to obesity.[39]

In this study, mRNA expression levels of *Socs3*, *Ptpn1*, and *Ptpn2*, which inhibit leptin signaling, were higher in *Mc4r-cre:Crtc1*$^{loxp/loxp}$ mice than in the control mice after soybean oil intake. These changes in gene expression may have been caused by an increase in body weight following increased leptin levels.

Immunostaining showed increased expression of SOCS3 in VMH and ARH, but not in the PVH, of *Mc4r-cre:Crtc1*$^{loxp/loxp}$ mice after soybean oil intake. Both PTP1B and SOCS3, which act as inhibitors of leptin signaling, are regulated by their phosphorylation status.[40,41] In the current study, we did not assess the phosphorylation status of these leptin signaling inhibitors; therefore, it is unclear whether PTP1B and SOCS3 inhibit leptin signaling in *Mc4r-cre:Crtc1*$^{loxp/loxp}$ mice fed soybean oil. Therefore, further studies are required to clarify this.

Leptin stimulates POMC neurons, promoting the release of αMSH, an MC4R agonist, into the PVH, leading to appetite suppression.[31,33] CRTC1 is downstream of MC4R signaling,[16] implying that CRTC1 in MC4R neurons is indirectly activated via leptin-induced αMSH release, which mediates appetite suppression. Therefore, lack of CRTC1 in MC4R neurons diminishes the appetite-suppressing effects of leptin, potentially inducing high leptinemia and increasing the factors related to leptin resistance in *Mc4r-cre:Crtc1*$^{loxp/loxp}$ mice. However, whether leptin resistance in these mice is due to obesity resulting from the overconsumption of soybean oil or is a direct consequence of CRTC1 deficiency remains uncertain. Further studies are required to investigate the effect of CRTC1 deficiency in MC4R-expressing cells on the leptin pathway.

We observed heavier BAT in *Mc4r-cre: Crtc1*$^{loxp/loxp}$ mice following soybean oil intake. However, soybean oil intake did not result in significant differences in the expression of *Ucp1* and *Prdm16*. Therefore, it is possible that soybean oil intake promotes triglyceride accumulation in BAT rather than enhancing brown adipocyte differentiation and proliferation.

We demonstrated that the effects of CRTC1 deficiency on feeding behavior varied with age. As shown in Figure 1, despite feeding soybean oil from 6 weeks of age, there was no observable change in body weight until 10 weeks of age, after which a weight difference emerged between the control mice and *Mc4r-cre:Crtc1*$^{loxp/loxp}$ mice. Similarly, increased soybean oil intake in *Mc4r-cre:Crtc1*$^{loxp/loxp}$ mice was observed from 10 weeks of age onwards (Figure 2C). Initially, we hypothesized that continuous soybean oil consumption would influence these outcomes. However, even middle-aged *Mc4r-cre:Crtc1*$^{loxp/loxp}$ mice without prior exposure to soybean oil showed an increased intake compared to control mice (Figure 5B). Furthermore, *Mc4r-cre:Crtc1*$^{loxp/loxp}$ mice displayed more pronounced weight gain soon after starting soybean oil intake at 10 weeks of age (Figure 5M). Collectively, these findings suggested that the contribution of CRTC1 to weight regulation differed with age. Therefore, in young mice, CRTC1 in MC4R neurons may not significantly contribute to feeding regulation, implying that other factors may regulate feeding. As the mice aged, the contribution of CRTC1 became more significant. MC4R sensitivity decreases due to aging or HFD intake.[42] MC4R localizes to primary cilia on neuronal bodies, which shorten with aging or a HFD, potentially reducing MC4R signaling and impairing appetite suppression. However, the influence of CRTC1 and CREB on primary cilia remains unclear and requires further research.

HFD increases feeding frequency during the inactive phase (light phase) in mice, thus contributing to obesity.[43,44] Therefore, the increase in soybean oil intake observed in *Mc4r-cre:Crtc1*$^{loxp/loxp}$ mice is expected to occur during the light phase. To investigate this, we utilized a lick analysis-based preference test system to analyze licking behavior toward soybean oil and water over a 24-h period. During the light phase, there was no difference in the number of licks for soybean oil between *Mc4r-cre:Crtc1*$^{loxp/loxp}$ mice and control mice in either the young or middle-aged groups. However, during the dark phase, middle-aged *Mc4r-cre:Crtc1*$^{loxp/loxp}$ mice exhibited

an increased number of licks for soybean oil compared to controls. Therefore, it became evident that the deficiency of CRTC1 does not increase the intake of soybean oil during the light phase by disrupting feeding rhythms, but rather enhances the craving for soybean oil during the dark phase.

We observed lower glucose levels in *Mc4r-cre:Crtc1*$^{loxp/loxp}$ mice fed chow alone without changes in insulin or leptin levels. Another research group has demonstrated that MC4R-deficient mice exhibit improved glucose tolerance due to elevated glucosuria rather than enhanced β-cell function.[45] The deficiency of CRTC1 in MC4R-cells may partially mimic the effects of MC4R deficiency, potentially leading to more efficient glucose metabolism.

In environments with freely available NCD and lard, mice with MC4R-expressing cell-specific *CRTC1* deficiency exhibited higher blood glucose levels than the control mice. However, no changes were observed in the OGTT and ITT. Despite the elevated blood glucose levels in *Mc4r-cre:Crtc1*$^{loxp/loxp}$ mice, no significant alterations were observed in glucose metabolism, suggesting that impaired glucose tolerance and insulin sensitivity do not cause hyperglycemia. However, further research is necessary to understand the combined effects of diet and *CRTC1* deficiency on glucose metabolism. We also observed no significant differences in RER, oxygen consumption, or motor activity between *Mc4r-cre:Crtc1*$^{loxp/loxp}$ and control mice, although lard intake promoted body weight gain in both groups compared with the chow-fed group, as shown in Figure 1A,E. In a previous study, we observed significant body weight gain, decreased oxygen consumption, and hyperglycemia induced by HFD, in that the main source of fat was lard, in *Mc4r-cre:Crtc1*$^{loxp/loxp}$ mice.[16] The difference between the current study and our previous study was the method of feeding lards to mice. In the current study, the mice had the option of choosing between chow and lard, allowing for spontaneous lard ingestion. In contrast, a previous study involved HFD feeding, in which the mice had no choice but to eat an HFD.[16] The difference in the method of lard administration may have influenced the metabolism, resulting in the observed differences in outcomes.

In conclusion, our study identified a role for CRTC1 in MC4R-expressing cells in regulating dietary fat intake in mice. Moreover, the regulatory effects of CRTC1 increase with age. Both excessive dietary fat intake and aging are major factors in obesity; therefore, further studies to understand the precise mechanism of obesity caused by CRTC1 deficiency will help develop new therapeutic strategies against obesity.

AUTHOR CONTRIBUTIONS

S. Matsumura designed the study; M. Fujisawa, M. Fujiwara, and H. Okayama analyzed the data; S. Matsumura, M. Fujisawa, M. Fujiwara, H. Okayama, E. Nousou, T. Sasaki, and N. Harada performed the experiments; S. Matsumura and M. Fujisawa wrote the original manuscript; and T. Sasaki and N. Harada revised and edited the manuscript.

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflicts of interest. All co-authors have read the manuscript and approved the content. The authors have no financial interests to declare.

DATA AVAILABILITY STATEMENT

Data supporting the findings of this study are available upon request from the corresponding author, S. Matsumura.

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