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Targeting *Clic1* for the treatment of obesity: A novel therapeutic strategy to reduce food intake and body weight



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ABSTRACT

Objective: Despite great advances in obesity therapeutics in recent years, there is still a need to identify additional therapeutic targets for the treatment of this disease. We previously discovered a signature of genes, including Chloride intracellular channel 1 (*Clic1*), whose expression was associated with drug-induced weight gain, and in these studies, we assess the effect of *Clic1* inhibition on food intake and body weight in mice.

Methods: We studied the impact of *Clic1* inhibition in mouse models of binge-eating, diet-induced obese mice and genetic models of obesity (Magel2 KO mice).

Results: *Clic1* knockout (KO) mice ate significantly less and had a lower body weight than WT littermates when either fed chow or high fat diet. Furthermore, pharmacological inhibition of *Clic1* in diet-induced obese mice resulted in suppression of food intake and promoted highly efficacious weight loss. *Clic1* inhibition also reduced food intake in binge-eating models and hyperphagic Magel2 KO mice. We observed that chronic obesity resulted in a significant change in subcellular localization of *Clic1* with an increased ratio of *Clic1* in the membrane in the obese state. These observations provide a novel therapeutic strategy to block *Clic1* translocation as a potential mechanism to reduce food intake and lower body weight.

Conclusions: These studies attribute a novel role of *Clic1* as a driver of food intake and overconsumption. In summary, we have identified hypothalamic expression of *Clic1* plays a key role in food intake, providing a novel therapeutic target to treat overconsumption that is the root cause of modern obesity.

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Keywords Obesity; Food intake; *Clic1*; Hyperphagia; Body weight regulation

1. INTRODUCTION

Modern-day human eating behavior is characterized by continuous availability and overconsumption of energy-rich food which are key drivers of the obesity epidemic. Over one-third of US adults are obese [1] causing a formidable socioeconomic challenge and widely impacting public health [2]. Despite great advances in efficacious pharmacotherapy for obesity in recent years, there is still a general lack of understanding of the basic biological mechanisms that contribute to obesity and a need to identify additional therapeutic targets [3]. Using a multispecies approach, originating with a screen in *Caenorhabditis elegans*, we previously discovered a signature of genes, including Chloride intracellular channel 1 (*Clic1*), whose expression was associated with drug-induced weight gain [4]. Interestingly, antipsychotic drugs drive a rapid change in food intake, in

both rodents [4–7] and humans [8,9], but the underlying pathways driving this side effect are largely unknown [10–13]. We hypothesized that pathways contributing to hyperphagia and weight gain could be targeted in the reverse direction to stimulate reduction in food intake and drive weight loss. *Clic1*, a 241 amino acid protein, exhibits a dual nature, existing in two distinct forms: a soluble enzymatic form and a membrane-associated ion channel form [14]. The reversible transformation from the soluble, predominant glutathione-S transferase (GST)-like structure [15] to that of an integral membrane protein form [16] is prompted by various stimuli including alterations in cellular pH and oxidative stress [17–19]. This translocation phenomenon has been observed in diverse cell types, such as endothelial cells [20,21] and microglia [22,23]. Importantly, the *Clic1* inhibitor, IAA94, functionally inhibits *Clic1* by preventing translocation to the transmembrane region, and *Clic1* remains predominantly in the cytoplasmic

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compartment [21,24]. Therefore, a crucial aspect of Clic1's function lies in the subcellular localization [25]. Previous studies have identified that whole body Clic1 KO mice, are leaner than WT controls in their early development (age 3–11 weeks of age) [26]. In these studies, we investigate the role of Clic1 in food intake, weight gain and the investigate the impact of Clic1 inhibition as a potential treatment for obesity and metabolic health.

2. METHODS

2.1. In vitro studies in hypothalamic cells

Adult mouse hypothalamic cell line (mHypoA-59, CLU468 cells, Cedarlane) were cultured as previously described. Briefly, cells were grown and maintained in high-glucose, pyruvate-free DMEM supplemented with 10% fetal bovine serum, L-glutamine (Cat. 25030081, Gibco, NY), and 10 u/ml of penicillin and 10 µg/ml of streptomycin (Cat. 15149-122, Gibco) of in a 5% CO₂ environment. Cells were 'fasted' for 8 h using the same growth media but devoid of fetal bovine serum, with or without IAA94 (40 µM).

2.2. Gene expression

RNA was extracted, converted to cDNA and relative expression analyzed by qPCR. Gene expression was calculated after normalization to the housekeeping gene *Hprt1* and *Pgk1* using the $\Delta\Delta C_t$ method. Gene expression was calculated relative to experimental controls. Primer sequences used to measure gene expression are detailed in Supplemental Table 1.

2.3. Clic1 localization studies

Cytoplasmic and membrane-bound hypothalamic proteins from lean and obese mice fed with 60% high-fat diet for 8 weeks were isolated using ProteoExtract Native Protein kit (444810, Calbiochem) as recommended by the manufacturer. Proteins (20 µg) were fractionated in 4–15% Mini PROTEAN TGX acrylamide gels, transferred to PVDF, blocked with 5% BSA, incubated with the primary antibody overnight and secondary antibody for 60 min before detection using ECL. Band intensities were quantified using densitometer in ImageLab. The following antibodies were used: anti-Clic1 (1:100, sc-81873, Santa Cruz Biotechnology), anti-beta actin (1:2000, 3700, Cell Signaling), anti-Na⁺, K⁺-ATPase (1:1000, 3010, Cell Signaling), anti-mouse IgG (1:4000, 115035003, Jackson ImmunoResearch), anti-rabbit IgG (1:4000, NA934V, GE Healthcare).

2.4. Acute fasting and re-feeding studies

All mice studies were approved by UCSD IACUC. WT C57BL/6 mice (stock #000664) were purchased from Jackson labs at 9 weeks of age. At 12 weeks of age, mice were fasted for 23 h and then re-fed for one hour before sacrificed. Hypothalami were dissected, frozen and later analyzed for gene expression studies.

2.5. Clic1 KO mice studies

We obtained the whole body Clic1 knockout (KO) mice, on a CD-1 background, from Prof. John C. Edwards, St Louis University [26]. KO mice and respective WT littermate controls were fed normal chow. The food novelty test was conducted in a similar way as previously described [27]. In brief, mice have a natural phobia to novelty, therefore, when WT mice are exposed to a novel pellet of highly palatable food, they have to overcome this phobia to consume the novel food and this partially models their drive, (or lack of motivation) to consume palatable food. During the 3-day experiment, food was

withdrawn from 9-week chow fed mice 2 h before the onset of the dark period. A pellet of 45% high fat diet, HFD (D12451, Research Diets) was introduced to the food hoppers at the onset of the dark period and were removed and replaced with chow diet after 20 min. Glucose tolerance test was conducted as described previously [28,29]. In brief, mice were fasted for 6 h, injected IP with glucose (1 g/kg, Hospira, Lake Forest, IL) and blood drawn at 0, 10, 30, 60, 90, and 120 min after the injection for blood glucose determination using an Easy Step blood glucose monitor (Home Aid Diagnostics Inc, FL). Plasma insulin was measured in the fasted and 10 min blood sample using 900-MPMI-02 (Alpco, NH).

In a second cohort, male WT and Clic1 KO mice were acclimated for 3 days in metabolic chambers (Promethion, Sable Systems, NV). Food intake, body weight, respiratory gas exchange and activity were measured in the next 7 days. Data was organized using ExpeData macros 1 and 13. Energy expenditure was normalized by body weight. Indanyloxyacetic acid-94 (IAA94, HY-12693, Medchem Express) is a chloride intracellular channel blocker. To assess whether IAA94s hypophagic property is mediated through its inhibitory action against Clic1, we administered IAA94 to chow-fed WT and KO mice at 12 weeks of age by intraperitoneal (IP) injection, at a dose of 10 mg/kg, and food intake measured over the next 24 h.

2.6. Binge eating studies

The experiment was conducted as previously described [30]. WT mice were divided into one of 4 experimental groups; 'Continuous' (C-Veh or C-IAA94) and 'intermittent' (I-Veh or I-IAA94), (n = 5/group) 'Continuous' groups had *ad libitum* access to high fat diet (HFD) (DS12492, 60% calories from fat) throughout the study. The 'intermittent' groups received an initial 48 h acclimation to HFD after which the HFD was removed for 5 days and only the NC was available *ad libitum*. The HFD was then presented back to the mice for a short 2.5-h period and food intake measured.

2.7. RNA sequencing

RNA from the hypothalamus of WT and KO mice was isolated using RNeasy Lipid tissue kit (Qiagen, 74804) according to the manufacturer's recommended protocol. RNA sequencing was conducted at UCSD Genomic Core. RNA quality and concentration were evaluated using TapeStation and samples with a RIN score of more than 8.0 underwent library preparation and RNA sequencing using Novaseq S4. Quality control of the raw fastq files was performed using the software tool FastQC v0.11.8. [31]. Sequencing reads were trimmed with Trimmomatic v0.38 [32] and aligned to the mouse genome (GRCm38p6 [33]) using the STAR aligner v2.5.1a [34]. Read quantification was performed with RSEM [35] v1.3.0 and the Ensembl release 98 annotation [36]. The R BioConductor packages edgeR [37] and limma [38] were used to implement the limma-voom9 method for differential expression analysis. In brief, lowly expressed genes—those not having counts per million (cpm) ≥ 1 in at least 5 of the samples—were filtered out and then trimmed mean of M-values (TMM) [39] normalization was applied. The experimental design was modeled upon condition ($\sim 0 +$ condition). The voom method was employed to model the mean–variance relationship in the log-cpm values, after which lmFit was used to fit per-gene linear models and empirical Bayes moderation was applied with the eBayes function. Significance was defined by using an adjusted *p*-value cut-off of 0.05 after multiple testing correction [40] using a moderated t-statistic in limma. Functional enrichment of the differentially expressed genes was performed using WebGestalt [41] (including GSEA [42]), GSEA [43], SPIA [44], and fgsea [45].

2.8. Investigation of dose dependent effect of IAA94 and comparison with Liraglutide treatment alone, or combination of Liraglutide and IAA94 on weight loss

Male C57BL6 mice, age 10 weeks, were fed 60% HFD (DS12492, Research Diets) for 8 weeks. At 18 weeks of age, mice were randomly divided into five groups, (1) Vehicle ($n = 9$), (2) Low dose IAA94 ($n = 7$, 10 mg/kg, MedChem Express, HY-12693), (3) High dose IAA94 ($n = 8$, 50 mg/kg), (4) Liraglutide ($n = 6$, 0.4 mg/kg [46,47], SelleckChem, NN2211) and (5) Liraglutide + High dose IAA94 ($n = 5$). All drugs were resuspended in vehicle (Saline + 4% DMSO, 10% Tween 80). At day 8, mice were placed in metabolic chambers (Promethion, Sable Systems, NV) in two staggered cohorts where daily food intake, body weight, oxygen consumption, carbon dioxide production, energy expenditure, activity and respiratory exchange ratio (RQ) were measured at 5 min intervals throughout the daily light and dark cycle, for 7 days. Fat mass and lean mass were determined by Echo MRI analysis at the end of the study. Plasma levels of ALT (EBC-K235, Elabscience) were determined according to manufacturers' instructions.

2.9. Magel2 KO mice studies

Magel2 KO mice recapitulates some aspects of Prader–Willi syndrome. Male Magel2 KO mice and wildtype littermates were provided courtesy of Dr. Marcelo Dietrich, Yale University. After 7 days of acclimation, mice were injected daily with either vehicle or IAA94 (IP) for 9 days. Food intake, weight gain and gonadal adipose tissue weights were measured.

2.10. Psychiatric behavioral analysis

Marble burying is performed as previously described to assess compulsive behavior. Standard polycarbonate rat cages were used with fresh bedding for every mouse. Glass toy marbles were placed on the surface of the bedding in a 4 across and 5 down pattern. The mouse was placed into a corner of the cage and covered with the filter-top lid on the cage. The mouse was allowed to remain undisturbed in the cage for 30 min. The marble is considered buried if two-thirds of its surface is covered with bedding.

Forced swim test was conducted using published protocols to evaluate depression-like behavior. 5L glass beaker was filled with tap water set at room temperature up to the 3.5L mark. The videorecorder was started before the mouse was gently lowered into the water. The mouse was allowed to swim for 6 min. The mouse was then dried and returned to its home cage. A total of 4 mice were studied in each session, with beakers separated by black dividers. Three independent investigators evaluated the time the mice were mobile and immobile during last 4 min of the test. Mobility is defined as any movements other than those necessary to balance the body and keep the head above the water. Increased immobility is an indication of depression-like behavior.

Elevated plus maze was conducted using published protocols to assess anxiety-like behavior. The apparatus used was in a + configuration which comprised of two open arms (25×5 cm) across from each other and perpendicular to two closed arms (25×5 cm) with a center platform (5×5 cm). The closed arm was equipped with 10 cm walls. The apparatus stood 50 cm above the floor. The test was conducted in a room with 100 lux light. The mouse was placed in the center area and was allowed to roam around the maze for 5 min. The experiment was recorded using a video camera which was analyzed by 3 independent investigators for number of exits to and the time spent in the open field.

3. RESULTS

3.1. Fasting induces increased expression of *Clic1* in the arcuate nucleus

We recently identified a gene signature that was specifically associated with antipsychotic-induced hyperphagia which includes *Clic1* [4]. Hypothalamic expression of *Clic1* was significantly elevated in olanzapine-treated mice, and expression reduced by co-treatment with an adjuvant drug, minocycline, that lowered food intake (Figure 1A). To determine if hypothalamic expression of *Clic1* had a broader role in food intake, beyond antipsychotic-induced hyperphagia, we then measured its expression in response to fasting and re-feeding in mice. We found *Clic1* expression was significantly elevated in response to fasting and returned to basal levels one hour after re-feeding (Figure 1B). We then used cell type specific transcriptomics data [48] to determine expression levels of *Clic1* in two key neuronal populations in the arcuate nucleus (ARC) that play a key role in food intake. Notably, the ARC senses and integrates peripheral energy signals [49], such as blood glucose concentration, ghrelin, leptin and insulin [50]. *Clic1* is expressed at a low level in both orexigenic Neuropeptide Y (Npy)/Agouti-related peptide (Agrp) and anorexigenic proopiomelanocortin (Pomc) neurons but is potently and specifically elevated in Npy/Agrp neurons in response to fasting (Figure 1C).

3.2. Obesity results in an increase proportion of hypothalamic *Clic1* localized to the membrane

Clic1 is a particularly unusual protein as it exists in both soluble (enzymatic form) and membrane-associated (ion channel) forms, thus, localization is a key element of *Clic1* function [25]. Importantly, in endothelial cells [21] and cancer models [51], *Clic1* inhibitor, Indanyloxyacetic acid (IAA) has been shown to functionally inhibit *Clic1* by preventing translocation to the transmembrane region, and *Clic1* remains predominantly in the cytoplasmic compartment [21]. To further understand the role of *Clic1* in the hypothalamus, we conducted a series of experiments in an adult mouse hypothalamic cells line (mHypoA). These cells express *Clic1* as well as the pro-feeding neuropeptides Npy and Agrp (Figure 1D). In a similar way observed in mouse hypothalamus, simulation of the fasted state, by nutrient depletion, resulted in increased expression of *Clic1*, as well as Npy and Agrp (Figure 1D). Importantly, IAA94 treatment blocked the fasting-induced increase in expression of *Npy*. In addition, IAA94 did not significantly reduce expression levels of *Clic1* (Figure 1D) as IAA94 functionally inhibits *Clic1* by preventing translocation to the transmembrane region [21,24]. Next, we determined if chronic obesity impacts *Clic1* cellular localization, by studying hypothalamic samples from lean and obese mice. Chronic obesity resulted in an increase proportion of hypothalamic *Clic1* localized to the membrane and lowered proportions in the cytoplasm compared with samples from lean mice (Figure 1E–G). Antibody specificity to *Clic1* is confirmed by western blot (Supplemental Figure 1).

3.3. *Clic1* KO mice eat less and weight less than WT littermates

We next studied the effect whole body *Clic1* deletion on food intake, body weight and glucose homeostasis. *Clic1* KO mice fed normal chow ate significantly less (Figure 2A) and weighed less (Figure 2B) than their WT littermates. In addition, when given access to highly palatable 45% HFD for 20 min per day, *Clic1* KO mice showed less 'motivation' to overcome their innate fear of novelty and ate less HFD during this limited exposure time compared with their WT littermates (Figure 2C)

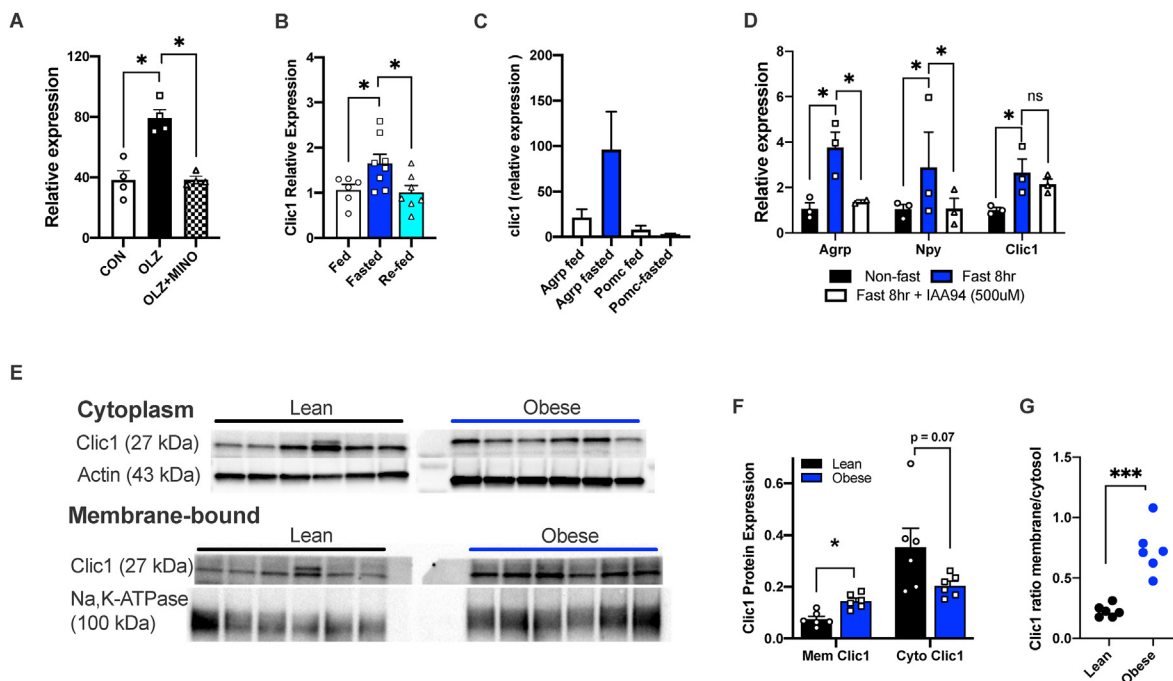


Figure 1: Hypothalamic Expression of *Clic1*. **A.** Hypothalamic expression of *clic1* in control, OLZ and OLZ + minocycline (MINO) treated mice ($n = 4$ /group) determined from RNA seq studies (GSE119772) [4]. **B.** Hypothalamic expression of *Clic1* in *ad libitum* fed, 24 h fasted and 23 h fasted mice and then re-fed for 1 h ($n = 6-8$ per group) determined by quantitative PCR. **C.** Expression of *Clic1* in *Agrp* and *Pomc* neurons, using RNA seq data from Henry et al., 2015, (GSE93374) [48]. **D.** *Clic1*, *Npy* and *Agrp* expression in non-fasted, 8-h fasted, and 8-h fasted + IAA94 treated hypothalamic cells (HypoA-59, $n = 3$ /group). **E–F.** Immunoblots and densitometric quantification of *Clic1* membrane (Mem) and cytosolic (Cyto) localization in the hypothalamus of lean and obese mice ($n = 6$ /group) and **G.** ratio of membrane and cytosolic *Clic1*. A, B, D, * $p < 0.05$ One-way ANOVA followed by Two-stage linear step-up procedure of Benjamini, Krieger and Yekutieli with 0.05 FDR. F. * $p < 0.05$ Two-way ANOVA followed by Two-stage linear step-up procedure of Benjamini, Krieger and Yekutieli with 0.05 FDR. G. *** $p < 0.01$ Student's t-test.

suggesting *Clic1* KO resulted in less motivation to consume calorically dense food. *Clic1* KO mice were then placed in metabolic chambers to determine the impact of *Clic1* ablation on energy homeostasis (Figure 2D and E). *Clic1* KO mice displayed similar levels of energy expenditure (Figure 2D) and had no differences in respiratory quotient (Figure 2E) compared with WT littermates, suggesting the lower body weight was attributable to lower food intake. *Clic1* KO mice also had increased lean mass and decreased fat mass, relative to their body weights (Figure 2F). Glucose tolerance tests in normal chow fed mice revealed similar levels of glucose tolerance (Figure 2G), with lower levels of insulin in KO mice, indicative of improved insulin sensitivity in the *Clic1* KO compared with WT (Figure 2H).

3.4. Inhibition of food intake by IAA94 is specifically mediated through *Clic1*

To determine if pharmacological inhibition of *Clic1* could affect food intake, we fasted mice and then treated with IAA94 before re-feeding. IAA94 has been widely used a potent inhibitor of *Clic1* in many pre-clinical studies [51–53]. In fasted WT mice, IAA94 treatment significantly blunted 24hr food intake when mice were re-fed compared with vehicle treated controls. Importantly, when IAA94 was administered to *Clic1* KO mice, there was no effect on fasting-induced re-feeding, proving that IAA94's effects on food intake are specifically mediated through *Clic1* rather than other members of the *Clic* protein family (Figure 3A and B). To further explore the role of *Clic1* in food intake in broader translation models of eating disorders, we tested whether IAA94 treatment impacted binge-eating in mice [30]. When mice were given intermittent access to HFD, they consumed significantly more of the palatable HFD than when it was continuously available. However,

when IAA94 is administered prior to intermittent HFD exposure, this significantly lowered the amount of food that was consumed during this 'binge eating' period (Figure 3C).

3.5. *Clic1* inhibition has a dose-dependent effect on reducing body weight and has an additional effect on weight loss when given in combination with GLP-1 agonist, Liraglutide

To test whether *Clic1* inhibition could reduce food intake and induce weight loss in the context of obesity, we administered IAA94 to diet-induced obese mice. Chronic treatment with IAA94 resulted in a dose-dependent reduction in food intake (Figure 4A) and body weight (Figure 4B and C). IAA94-induced reduction in body weight was attributed to a significant reduction in fat mass with no significant changes in lean mass in all groups, compared with vehicle treatment (Supplemental Figure 2A and B). IAA94 treatment at a dose of 50 mg/kg resulted in slightly greater weight loss than liraglutide, and importantly combination treatment of Liraglutide and IAA94 (50 mg/kg) induced greater weight loss than either drug alone (Figure 4B and C). No significant differences in energy expenditure (Figure 4D) or activity (Figure 4E) were observed, further suggesting *Clic1* inhibition drives weight loss due by reducing food intake. IAA94 treatment did not impact respiratory quotient while liraglutide treatment resulted in slightly lower respiratory quotient levels indicative of a mild increase in fat oxidation rather than utilization of carbohydrate as a fuel source. IAA94 (50 mg/kg) alone or in combination with Liraglutide also significantly improved glucose tolerance compared with vehicle (Figure 1G and H) as a likely secondary effect to the lower body weight (Figure 1B and C). Importantly, IAA94 did not change Alanine transaminase (ALT) enzymatic activity, which is a marker of hepatocyte

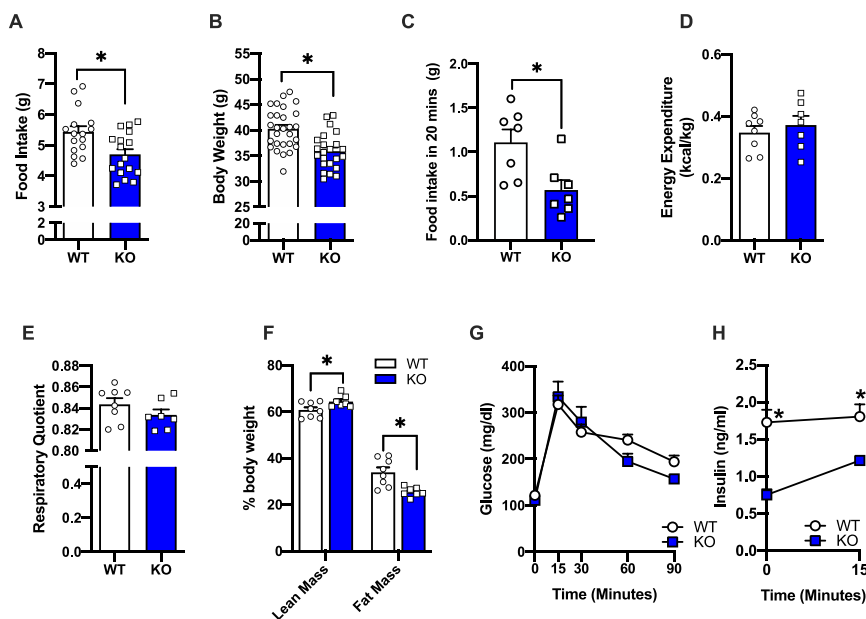


Figure 2: Whole body knockout of *Clic1* is associated with lower food intake and body weight. A. daily food intake (n = 7–8/group) B. Body weight (n = 7–8/group), C. Food novelty test, average daily consumption of 45% HFD when administered for 20 min per day (n = 7–8/group). D. Energy expenditure (n = 7–8/group), E. Respiratory quotient (n = 7–8/group), F. Average lean mass and fat mass (n = 7–8/group), G. Glucose tolerance (n = 7/group), H. Glucose stimulated insulin levels (n = 7/group). A–F * $p < 0.05$ Students t-test, G–H * $p < 0.05$, repeated measures ANOVA followed by Two-stage linear step-up procedure of Benjamini, Krieger and Yekutieli with 0.05 FDR.

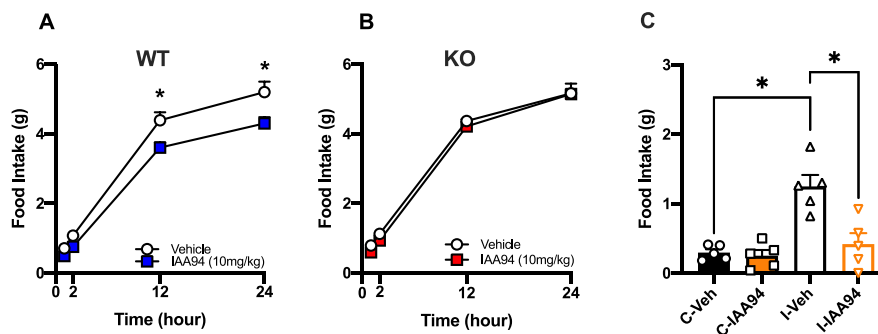


Figure 3: IAA94 mediated reduction in food intake is mediated via *Clic1*. Male (A) WT mice and (B) *Clic1* KO were fasted for 23 h and then injected once with IAA94 (10 mg/kg) or vehicle (VEH) before being re-fed with normal chow and then food intake measured 24 h after re-feeding (n = 9–10/group). C. Food intake measured in WT mice during binge eating study (n = 5/group). WT mice were divided into one of 4 experimental groups; 'Continuous' (C-Veh or C-IAA94) and 'intermittent' (I-Veh or I-IAA94), 'Continuous' groups had *ad libitum* access to HFD throughout the study. The 'intermittent' groups received an initial 48-h acclimation to HFD followed by 5 days of access to NC only, before the HFD was then presented back to the mice for a short 2.5-h period and food intake measured. A–B * $p < 0.05$, repeated measures ANOVA, C. * $p < 0.05$, two-way ANOVA followed by Two-stage linear step-up procedure of Benjamini, Krieger and Yekutieli with 0.05 FDR.

injury and toxicity, and in fact, IAA94 resulted in a trend in lower levels of ALT (Supplemental Figure 2C).

3.6. *Clic1* inhibition reduces hyperphagia and weight gain in mouse model of Prader–Willi syndrome

To explore the broader potential of *Clic1* inhibition in genetic models of obesity, we tested the effect of IAA94 in the *Magel2* KO mouse model of Prader–Willi syndrome [54]. As previously described, HFD feeding of *Magel2* KO mice resulted in hyperphagia and weight gain [54] (Figure 5A and B). *Magel2* KO mice were fed HFD at 18 weeks of age and treated with IAA94 (10 mg/kg) or vehicle. Treatment with IAA94 significantly reduced food intake (Figure 5A) and prevented body weight gain in *Magel2* KO mice (Figure 5B, Supplemental

Figure 3). In addition, IAA94 treatment resulted in lower epididymal fat mass compared with vehicle treated control mice (Figure 5C).

3.7. *Clic1* ablation had no effect on behavioral tests associated with depression or anxiety

Many previous efforts to develop centrally acting anti-obesity therapeutics have failed due to neuropsychiatric adverse effects including anxiety and depression [55]. Therefore, we tested whether ablation of *Clic1* had any overt effects on depression or anxiety-like behavior in mice. *Clic1* KO mice performed in a similar way to WT mice in all three behavioral assays, elevated plus maze (Figure 6A and B), marble burying assays (Figure 6C) and forced-swim tests (Figure 6D), suggesting *Clic1* ablation had no overt effect on depression, compulsive

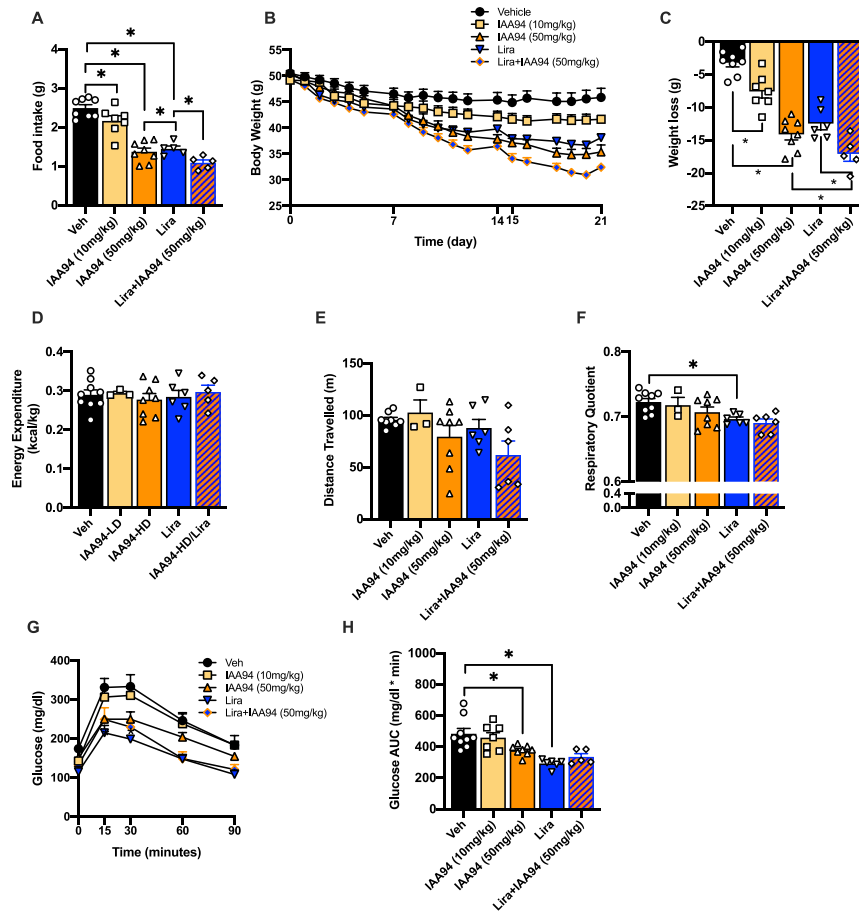


Figure 4: *Clic1* inhibition has a dose dependent effect on reducing body weight and has an additional effect on weight loss when given in combination with GLP-1 agonist, Liraglutide. **A.** average daily food intake, **B.** weight loss, **C.** % weight loss, **D.** Energy expenditure, **E.** Activity, **F.** Respiratory Quotient, **G.** Glucose tolerance test, **H.** Area under the curve of GTT of HFD-fed obese mice treated with Vehicle (n = 9), IAA94 50 mg/kg (n = 8), IAA94 10 mg/kg (n = 7), Lira (n = 6), combo [IAA94 50 mg/kg + Lira] (n = 6) for 21 days. Metabolic chamber analysis was conducted during days 8–15 of treatment. A, C–F, H **p* < 0.05 One-way ANOVA followed by Two-stage linear step-up procedure of Benjamini, Krieger and Yekutieli with 0.05 FDR. B, G **p* < 0.05, repeated measures ANOVA.

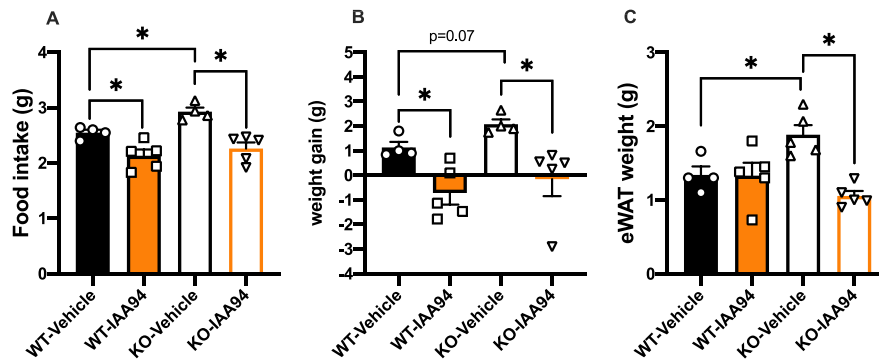


Figure 5: *Magel2* KO mice. **A.** Average daily food intake, **B.** weight change and **C.** epididymal white adipose tissue weight of *Magel2* WT and null mice treated with either Vehicle or IAA94 (10 mg/kg), n = 4–5 per group. **p* < 0.05 Two-way ANOVA followed by Two-stage linear step-up procedure of Benjamini, Krieger and Yekutieli with 0.05 FDR.

behavior and anxiety. In addition, we treated a subgroup of mice with IAA94 and further confirmed that *Clic1* inhibition had no impact on the forced swim test (Figure 6E), confirming that both genetic ablation and pharmacological inhibition of *Clic1* had no measurable impact on behavioral tests associated with depression and anxiety in mice.

3.8. *Clic1* ablation in the hypothalamus

To explore potential hypothalamic molecular mechanisms underlying *Clic1* regulation of energy balance, we conducted RNA sequencing from the mouse hypothalamus of normal chow fed WT and *Clic1* KO mice (GSE229799). Differential gene expression analysis revealed 38

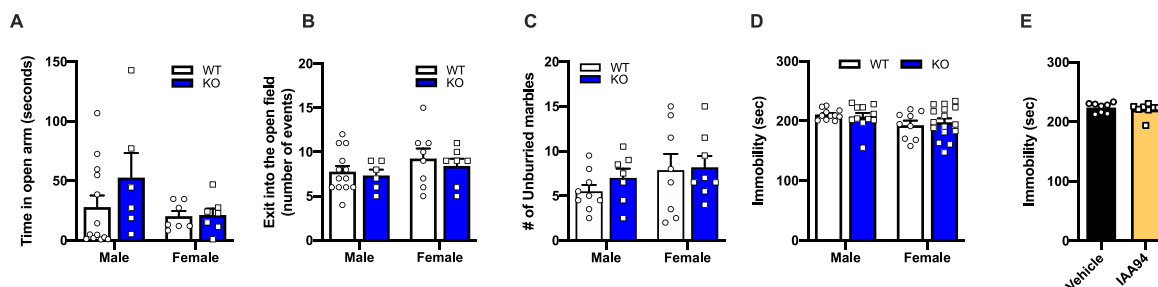


Figure 6: *Clic1* ablation had no effect on behavioral tests associated with depression or anxiety. **A.** Time spent in open arm in elevated plus maze, **B.** Number of entries to closed arms during elevated plus maze test, (n = 6–13/group) **C.** Number of unburred marbles in marble burying anxiety test (n = 8–9/group). **D.** Time spent immobile in forced swim test in KO and WT mice (n = 9–16/group). **E.** Time spent immobile in forced Swim test in IAA94 (10 mg/kg) and Vehicle treated WT mice, (n = 7–9 per group). * $p < 0.05$ two-way ANOVA followed by Two-stage linear step-up procedure of Benjamini, Krieger and Yekutieli with 0.05 FDR.

significantly differentially expressed genes (Adjusted $P < 0.05$), notably including *Clic1* (serving as an internal control) (Figure 7A and B, and Supplemental Tables 1 and 2). These differentially expressed genes represent potential molecular targets downstream of *Clic1* that could also be therapeutic targets for modulation of food intake in obesity.

The RNA seq gene signature include Neurensin 1 (*Nrsn1*) and Leucine rich repeat neuronal 1 (*Lrrn1*) in which polymorphisms in these gene have been linked to changes in food intake [56] and obesity [57]. In addition, we noted significantly higher expression of key appetite regulating hormone Arginine vasopressin (*Avp*) (Figure 7B). *Avp* is robustly expressed in regions of the hypothalamus that regulated food intake, including the paraventricular hypothalamus (PVH) [58,59] and ARC [60]. Activation of *Avp* neurons in rats results in reduction in food intake [58] and injection of AVP dose dependently decreases food intake [61]. This association of lower *Clic1* and higher *Avp* suggests *Avp* may be a key component of *Clic1* mediated lowering of food intake.

To determine which hypothalamic neurons express *Clic1*, we analyzed single-cell transcriptomic data (GSE93374) [60]. *Clic1* is broadly expressed in many cell types but of particular interest is found in a neuronal population (a.13.Neurons.1) that also co-express *Npy* and *Avp* (Figure 7C). In addition, *Clic1* is broadly expressed in many non-neuronal cell types (Figure 7C) including perivascular macrophages and microglia (PVMMicro), endothelial cells, mural cells (form part of the blood brain barrier), ependymocytes (glial cell), tanycytes (glial cells that are part of the blood–brain barrier), and vascular and leptomeningeal cells (VLMCS).

We then examined the co-expression of *Clic1*, *Agrp*, *Npy* and *Avp* across 34 clusters of molecularly distinct neuronal subtypes (Figure 7D). This analysis confirmed that *Clic1* is found in *Agrp/Npy* expressing neurons (n13.*Agrp/Gm8773* as well as n.12 *Agrp/Sst*). Additionally, *Clic1* is highly expressed in Oxytocin neurons (n.06.Oxt) that also express high levels of *Avp*. These hypothalamic single cell studies confirm that *Clic1* is expressed in neuronal populations that co-express neuropeptides with key roles in appetite regulation, including *Agrp*, *Npy* and *Avp*.

4. DISCUSSION

In these studies, we describe the novel role of hypothalamic expression of *Clic1* in food intake regulation, specifically, overconsumption. Fasting drives an increase in *Clic1* expression in *Agrp/Npy* neurons. Genetic ablation of *Clic1* in mice results in a significant reduction in

food intake and lower body weight compared with WT littermates. Furthermore, pharmacological inhibition of *Clic1* results in suppression of food intake and promotes highly efficacious weight loss in obese mice. While IAA94 resulted in 28% reduction in body weight, combination treatment with both liraglutide and IAA94 resulted in an average of 35% weight loss, suggesting improved potential efficacy than many currently available anti-obesity drugs [62]. *Clic1* inhibition was also highly efficacious in broader models of food intake including a mouse model of binge eating. This binge eating assay revealed that pre-treatment with *Clic1* inhibitor prevents the overconsumption of HFD when presented with this highly palatable food for a short period of time. Further detailed studies are needed to investigate the impact of *Clic1* inhibition of food motivation and reward. IAA94 treatment also reduced food intake and weight gain in a *Magel2* KO mouse model of the genetic condition Prader–Willi syndrome, suggesting additional areas of future clinical benefits of *Clic1* inhibition. Many previous efforts to develop centrally acting anti-obesity therapeutics have failed due to neuropsychiatric adverse effects including anxiety and depression [55]. With a view to future translational potential, we studied the effect of *Clic1* ablation on behavioral assays and confirmed that *Clic1* ablation has no effect on depression or anxiety-like phenotypes in mice. Many of the previously described anti-obesity drugs modulate a number of neurochemical systems (dopamine, serotonin, noradrenaline, endocannabinoid) involved in regulation of mood, cognition and sleep and thus have an elevated risk for widespread neuropsychiatric adverse effects. Therefore, targeting specific regulators of overconsumption, and not targeting fundamental neurochemical systems is likely to reduce the risk of unwanted side effects. Obesity often develops in response to small changes in food intake over time, and thus, is hard to delineate the underlying mechanisms. The neural circuits that regulate food overconsumption are highly complex and many molecular components of these circuits remain unknown [63]. Utilizing a model of drug-induced hyperphagia, that is applicable to both rodents and humans, enabled us to identify a novel role for *Clic1* in the regulation of food intake. *Clic1* is a particularly unusual protein as it exists in two forms: a soluble enzymatic form and a membrane-associated ion channel form with a single transmembrane region [14]. Transformation from the predominant glutathione-S transferase (GST)-like structure soluble form [15] to that of an integral membrane protein [16] that is triggered by changes in cellular pH and oxidative stress [17]. *Clic1* inhibitor IAA94 prevents the translocation of *Clic1* to the membrane-associated ion channel form [21,24]. Chloride ions play an important role in controlling neuronal excitability whereby accumulation of intracellular chloride drives

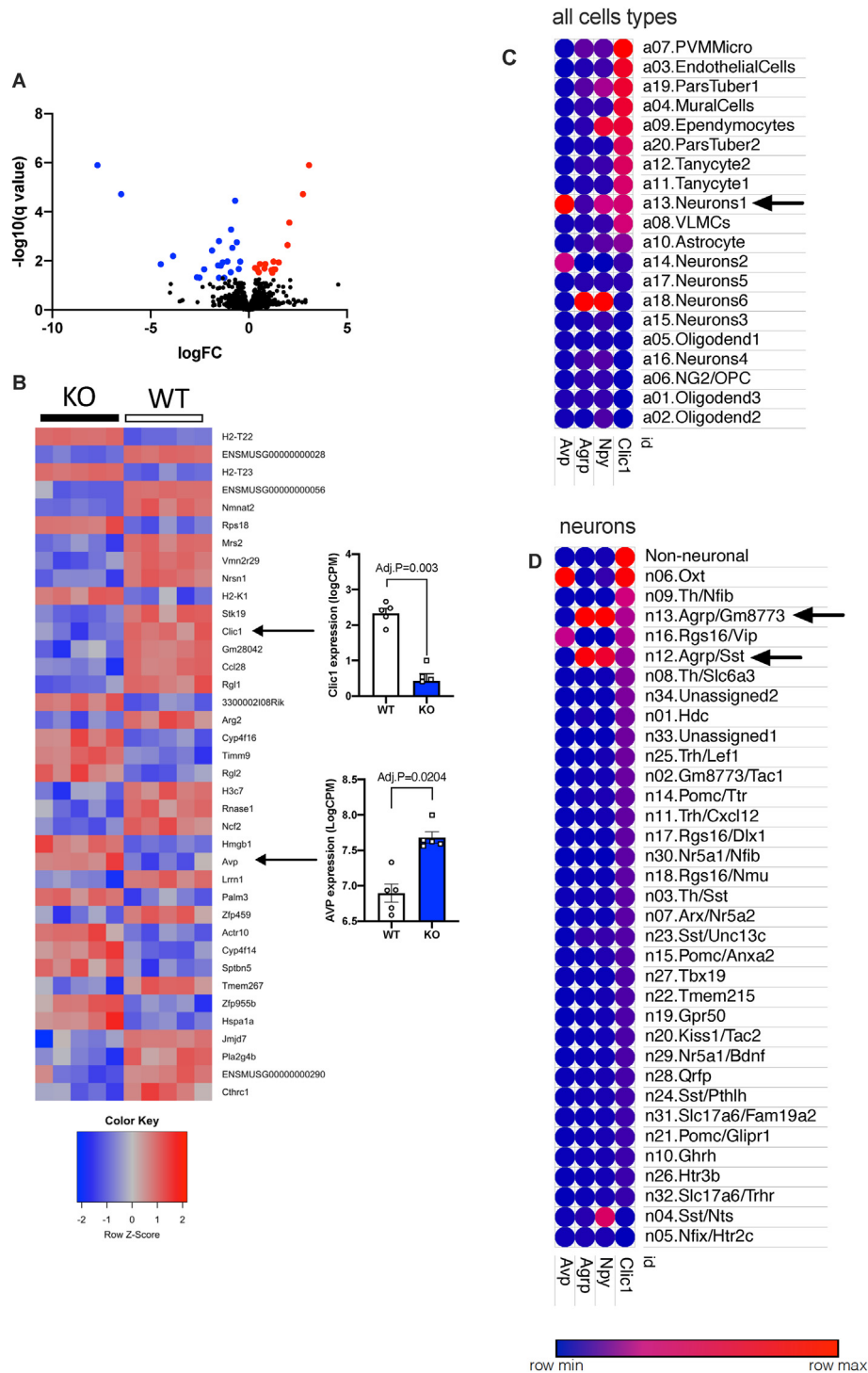


Figure 7: Hypothalamic RNA seq in *Clic1* KO and WT mice. **A.** Volcano plot and **B.** Heat map of differentially expressed genes between the hypothalamus of *Clic1* WT and KO mice. **C.** All cell-types and **D.** Specific neuronal population co-expression of *Clic1* hypothalamic DEGs.

hyperpolarization which leads to neuronal inactivation [64]. However, the strength of inhibition depends on the chloride ion gradient across the membrane. Therefore, increasing intracellular chloride could also decrease the concentration-driven entry of extracellular chloride which can lead to reduced inhibitory postsynaptic potential generated by chloride-permeable GABA receptors [64,65]. *Agrp/Npy* neurons receive inhibitory GABA inputs from leptin-responsive neurons [66]. Thus, it is

possible that in the obese state, elevated levels of the membrane form of *Clic1* results in increased concentration of intracellular chloride leading to reduced-inhibition of *Agrp/Npy* neurons which then drives feeding. Further studies are warranted to investigate the role of *Clic1* specifically in *Npy/Agrp* expressing neurons. Moreover, chronic obesity results in a significant change in subcellular localization of *Clic1* with an increased ratio of *Clic1* in the membrane in the obese state [67]. It is

also likely that the chronic inflammatory state associated with obesity plays a role in stimulating the persistent increase in membranal localization of *Clc1*. These observations provide a novel therapeutic strategy to block *Clc1* translocation as a potential mechanism to reduce food intake and lower body weight.

5. CONCLUSION

These studies identify a novel role for *Clc1* in the regulation of food intake and preclinical studies support the further development of *Clc1* inhibitors for the treatment of obesity.

AUTHOR CONTRIBUTIONS

OO, RCZ and MP conceptualized the studies. RCZ, DZ, AL, OO, BSC and MLV performed experiments and analyzed data. DP and HS provided the *Clc1* KO mouse model. CAN and DRCF performed RNAseq analysis. OO and RCZ wrote and edited the manuscript.

DATA AVAILABILITY

Data will be made available on request.

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DECLARATION OF COMPETING INTEREST

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

O.O is a consultant to *ClcBio*. The remaining authors have no conflicts of interest to declare.

APPENDIX A. SUPPLEMENTARY DATA

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.molmet.2023.101794>.

REFERENCES

- [1] Flegal KM, Kruszon-Moran D, Carroll MD, Fryar CD, Ogden CL. Trends in obesity among adults in the United States, 2005 to 2014. *JAMA* 2016;315(21):2284–91.
- [2] Ng M, Fleming T, Robinson M, Thomson B, Graetz N, Margono C, et al. Global, regional, and national prevalence of overweight and obesity in children and adults during 1980–2013: a systematic analysis for the Global Burden of Disease Study 2013. *Lancet* 2014;384(9945):766–81.
- [3] Muller TD, Bluher M, Tschop MH, DiMarchi RD. Anti-obesity drug discovery: advances and challenges. *Nat Rev Drug Discov* 2022;21(3):201–23.
- [4] Perez-Gomez A, Carretero M, Weber N, Peterka V, To A, Titova V, et al. A phenotypic *Caenorhabditis elegans* screen identifies a selective suppressor of antipsychotic-induced hyperphagia. *Nat Commun* 2018;9(1):5272.
- [5] Davoodi N, Kalinichev M, Korneev SA, Clifton PG. Hyperphagia and increased meal size are responsible for weight gain in rats treated sub-chronically with olanzapine. *Psychopharmacology (Berl)* 2009;203(4):693–702.
- [6] Ferno J, Varela L, Skrede S, Vazquez MJ, Nogueiras R, Dieguez C, et al. Olanzapine-induced hyperphagia and weight gain associate with orexigenic hypothalamic neuropeptide signaling without concomitant AMPK phosphorylation. *PLoS One* 2011;6(6):e20571.
- [7] Morgan AP, Crowley JJ, Nonneman RJ, Quackenbush CR, Miller CN, Ryan AK, et al. The antipsychotic olanzapine interacts with the gut microbiome to cause weight gain in mouse. *PLoS One* 2014;9(12):e115225.
- [8] Fountaine RJ, Taylor AE, Mancuso JP, Greenway FL, Byerley LO, Smith SR, et al. Increased food intake and energy expenditure following administration of olanzapine to healthy men. *Obesity (Silver Spring)* 2010;18(8):1646–51.
- [9] Ratzoni G, Gothelf D, Brand-Gothelf A, Reidman J, Kikinon L, Gal G, et al. Weight gain associated with olanzapine and risperidone in adolescent patients: a comparative prospective study. *J Am Acad Child Adolesc Psychiatry* 2002;41(3):337–43.
- [10] Bak M, Fransen A, Janssen J, van Os J, Drukker M. Almost all antipsychotics result in weight gain: a meta-analysis. *PLoS One* 2014;9(4):e94112.
- [11] Goncalves P, Araujo JR, Martel F. Antipsychotics-induced metabolic alterations: focus on adipose tissue and molecular mechanisms. *Eur Neuropharmacol* 2015;25(1):1–16.
- [12] Musil R, Obermeier M, Russ P, Hamerle M. Weight gain and antipsychotics: a drug safety review. *Expert Opin Drug Saf* 2015;14(1):73–96.
- [13] Newcomer JW. Second-generation (atypical) antipsychotics and metabolic effects: a comprehensive literature review. *CNS Drugs* 2005;19(Suppl 1):1–93.
- [14] Ulmasov B, Bruno J, Woost PG, Edwards JC. Tissue and subcellular distribution of *CLIC1*. *BMC Cell Biol* 2007;8:8.
- [15] Al Khamici H, Brown LJ, Hossain KR, Hudson AL, Sinclair-Burton AA, Ng JP, et al. Members of the chloride intracellular ion channel protein family demonstrate glutaredoxin-like enzymatic activity. *PLoS One* 2015;10(1):e115699.
- [16] Goodchild SC, Angstmann CN, Breit SN, Curmi PM, Brown LJ. Transmembrane extension and oligomerization of the *CLIC1* chloride intracellular channel protein upon membrane interaction. *Biochemistry* 2011;50(50):10887–97.
- [17] Averaimo S, Milton RH, Duchon MR, Mazzanti M. Chloride intracellular channel 1 (*CLIC1*): sensor and effector during oxidative stress. *FEBS Lett* 2010;584(10):2076–84.
- [18] Domingo-Fernandez R, Coll RC, Kearney J, Breit S, O'Neill LAJ. The intracellular chloride channel proteins *CLIC1* and *CLIC4* induce IL-1 β transcription and activate the NLRP3 inflammasome. *J Biol Chem* 2017;292(29):12077–87.
- [19] Littler DR, Harrop SJ, Fairlie WD, Brown LJ, Pankhurst GJ, Pankhurst S, et al. The intracellular chloride ion channel protein *CLIC1* undergoes a redox-controlled structural transition. *J Biol Chem* 2004;279(10):9298–305.
- [20] Mao Y, Kleinjan ML, Jilishitz I, Swaminathan B, Obinata H, Komarova YA, et al. *CLIC1* and *CLIC4* mediate endothelial S1P receptor signaling to facilitate Rac1 and RhoA activity and function. *Sci Signal* 2021;14(679).
- [21] Xu Y, Zhu J, Hu X, Wang C, Lu D, Gong C, et al. *CLIC1* inhibition attenuates vascular inflammation, oxidative stress, and endothelial injury. *PLoS One* 2016;11(11):e0166790.
- [22] Milton RH, Abeti R, Averaimo S, DeBiasi S, Vitellaro L, Jiang L, et al. *CLIC1* function is required for beta-amyloid-induced generation of reactive oxygen species by microglia. *J Neurosci* 2008;28(45):11488–99.
- [23] Novarino G, Fabrizi C, Tonini R, Denti MA, Malchiodi-Albedi F, Lauro GM, et al. Involvement of the intracellular ion channel *CLIC1* in microglia-mediated beta-amyloid-induced neurotoxicity. *J Neurosci* 2004;24(23):5322–30.
- [24] Varela L, Hendry AC, Cassar J, Martin-Escolano R, Cantoni D, Ossa F, et al. A Zn²⁺-triggered two-step mechanism of *CLIC1* membrane insertion and activation into chloride channels. *J Cell Sci* 2022;135(15).
- [25] Singh H. Two decades with dimorphic Chloride Intracellular Channels (*CLICs*). *FEBS Lett* 2010;584(10):2112–21.
- [26] Ulmasov B, Bruno J, Oshima K, Cheng YW, Holly SP, Parise LV, et al. *CLIC1* null mice demonstrate a role for *CLIC1* in macrophage superoxide production and tissue injury. *Physiol Rep* 2017;5(5).

- [27] Campos CA, Bowen AJ, Roman CW, Palmiter RD. Encoding of danger by parabrachial CGRP neurons. *Nature* 2018;555(7698):617–22.
- [28] Lackey DE, Reis FCG, Isaac R, Zapata RC, El Ouarrat D, Lee YS, et al. Adipocyte PU.1 knockout promotes insulin sensitivity in HFD-fed obese mice. *Sci Rep* 2019;9(1):14779.
- [29] Hernandez-Carretero A, Weber N, LaBarge SA, Peterka V, Doan NYT, Schenk S, et al. Cysteine- and glycine-rich protein 3 regulates glucose homeostasis in skeletal muscle. *Am J Physiol Endocrinol Metab* 2018;315(2):E267–78.
- [30] Czyzyk TA, Sahr AE, Statnick MA. A model of binge-like eating behavior in mice that does not require food deprivation or stress. *Obesity (Silver Spring)* 2010;18(9):1710–7.
- [31] F AS. FastQC: a quality control tool for high throughput sequence data. 2010.
- [32] Bolger AM, Lohse M, Usadel B. Trimmomatic: a flexible trimmer for Illumina sequence data. *Bioinformatics* 2014;30(15):2114–20.
- [33] The Genome Reference Consortium. https://www.ncbi.nlm.nih.gov/datasets/genome/GCF_000001635.26/.
- [34] Dobin A, Davis CA, Schlesinger F, Drenkow J, Zaleski C, Jha S, et al. STAR: ultrafast universal RNA-seq aligner. *Bioinformatics* 2013;29(1):15–21.
- [35] Li B, Dewey CN. RSEM: accurate transcript quantification from RNA-Seq data with or without a reference genome. *BMC Bioinformatics* 2011;12:323.
- [36] Frankish A, Diekhans M, Ferreira AM, Johnson R, Jungreis I, Loveland J, et al. GENCODE reference annotation for the human and mouse genomes. *Nucleic Acids Res* 2019;47(D1):D766–73.
- [37] Robinson MD, McCarthy DJ, Smyth GK. edgeR: a Bioconductor package for differential expression analysis of digital gene expression data. *Bioinformatics* 2010;26(1):139–40.
- [38] Ritchie ME, Phipson B, Wu D, Hu Y, Law CW, Shi W, et al. limma powers differential expression analyses for RNA-sequencing and microarray studies. *Nucleic Acids Res* 2015;43(7):e47.
- [39] Law CW, Chen Y, Shi W, Smyth GK. voom: precision weights unlock linear model analysis tools for RNA-seq read counts. *Genome Biol* 2014;15(2):R29.
- [40] Benjamini YH, Y. Controlling the false discovery rate: a practical and powerful approach to multiple testing. *J R Stat Soc Series B Methodol* 1995;57(1):289–300.
- [41] Zhang B, Kirov S, Snoddy J. WebGestalt: an integrated system for exploring gene sets in various biological contexts. *Nucleic Acids Res* 2005;33(Web Server issue):W741–8.
- [42] Subramanian A, Tamayo P, Mootha VK, Mukherjee S, Ebert BL, Gillette MA, et al. Gene set enrichment analysis: a knowledge-based approach for interpreting genome-wide expression profiles. *Proc Natl Acad Sci U S A* 2005;102(43):15545–50.
- [43] Hanzelmann S, Castelo R, Guinney J. GSEA: gene set variation analysis for microarray and RNA-seq data. *BMC Bioinformatics* 2013;14:7.
- [44] Tarca AL, Draghici S, Khatri P, Hassan SS, Mittal P, Kim JS, et al. A novel signaling pathway impact analysis. *Bioinformatics* 2009;25(1):75–82.
- [45] Korotkevich G, Sukhov V, Sergushichev A. Fast gene set enrichment analysis. *BioRxiv* 2019.
- [46] Adams JM, Pei H, Sandoval DA, Seeley RJ, Chang RB, Liberles SD, et al. Liraglutide modulates appetite and body weight through glucagon-like peptide 1 receptor-expressing glutamatergic neurons. *Diabetes* 2018;67(8):1538–48.
- [47] Buganova M, Pelantova H, Holubova M, Sediva B, Maletinska L, Zelezna B, et al. The effects of liraglutide in mice with diet-induced obesity studied by metabolomics. *J Endocrinol* 2017;233(1):93–104.
- [48] Henry FE, Sugino K, Tozer A, Branco T, Sternson SM. Cell type-specific transcriptomics of hypothalamic energy-sensing neuron responses to weight-loss. *Elife* 2015;4.
- [49] Broberger C, Johansen J, Johansson C, Schalling M, Hokfelt T. The neuropeptide Y/agouti gene-related protein (AGRP) brain circuitry in normal, anorectic, and monosodium glutamate-treated mice. *Proc Natl Acad Sci U S A* 1998;95(25):15043–8.
- [50] Kohno D, Yada T. Arcuate NPY neurons sense and integrate peripheral metabolic signals to control feeding. *Neuropeptides* 2012;46(6):315–9.
- [51] Gritti M, Wurth R, Angelini M, Barbieri F, Peretti M, Pizzi E, et al. Metformin repositioning as antitumoral agent: selective antiproliferative effects in human glioblastoma stem cells, via inhibition of CLIC1-mediated ion current. *Oncotarget* 2014;5(22):11252–68.
- [52] Jiang L, Salao K, Li H, Rybicka JM, Yates RM, Luo XW, et al. Intracellular chloride channel protein CLIC1 regulates macrophage function through modulation of phagosomal acidification. *J Cell Sci* 2012;125(Pt 22):5479–88.
- [53] Valenzuela SM, Mazzanti M, Tonini R, Qiu MR, Warton K, Musgrove EA, et al. The nuclear chloride ion channel NCC27 is involved in regulation of the cell cycle. *J Physiol* 2000;529 Pt 3:541–52.
- [54] Bischof JM, Stewart CL, Wevrick R. Inactivation of the mouse *Mage2* gene results in growth abnormalities similar to Prader-Willi syndrome. *Hum Mol Genet* 2007;16(22):2713–9.
- [55] Nathan PJ, O'Neill BV, Napolitano A, Bullmore ET. Neuropsychiatric adverse effects of centrally acting antiobesity drugs. *CNS Neurosci Ther* 2011;17(5):490–505.
- [56] Ding R, Yang M, Wang X, Quan J, Zhuang Z, Zhou S, et al. Genetic architecture of feeding behavior and feed efficiency in a Duroc pig population. *Front Genet* 2018;9:220.
- [57] Kraja AT, Lawson HA, Arnett DK, Borecki IB, Broeckel U, de las Fuentes L, et al. Obesity-insulin targeted genes in the 3p26-25 region in human studies and LG/J and SM/J mice. *Metabolism* 2012;61(8):1129–41.
- [58] Pei H, Sutton AK, Burnett KH, Fuller PM, Olson DP. AVP neurons in the paraventricular nucleus of the hypothalamus regulate feeding. *Mol Metab* 2014;3(2):209–15.
- [59] Kim A, Madara JC, Wu C, Andermann ML, Lowell BB. Neural basis for regulation of vasopressin secretion by anticipated disturbances in osmolality. *Elife* 2021;10.
- [60] Campbell JN, Macosko EZ, Fenselau H, Pers TH, Lyubetskaya A, Tenen D, et al. A molecular census of arcuate hypothalamus and median eminence cell types. *Nat Neurosci* 2017;20(3):484–96.
- [61] Meyer AH, Langhans W, Scharrer E. Vasopressin reduces food intake in goats. *Q J Exp Physiol* 1989;74(4):465–73.
- [62] Srivastava G, Apovian CM. Current pharmacotherapy for obesity. *Nat Rev Endocrinol* 2018;14(1):12–24.
- [63] Sternson SM, Atasoy D, Betley JN, Henry FE, Xu S. An emerging technology framework for the neurobiology of appetite. *Cell Metab* 2016;23(2):234–53.
- [64] Pressey JC, de Saint-Rome M, Raveendran VA, Woodin MA. Chloride transporters controlling neuronal excitability. *Physiol Rev* 2023;103(2):1095–135.
- [65] Sorensen AT, Ledri M, Melis M, Nikitidou Ledri L, Andersson M, Kokaia M. Altered chloride homeostasis decreases the action potential threshold and increases hyperexcitability in hippocampal neurons. *eNeuro* 2017;4(6).
- [66] Garfield AS, Shah BP, Burgess CR, Li MM, Li C, Steger JS, et al. Dynamic GABAergic afferent modulation of AgRP neurons. *Nat Neurosci* 2016;19(12):1628–35.
- [67] Thaler JP, Guyenet SJ, Dorfman MD, Wisse BE, Schwartz MW. Hypothalamic inflammation: marker or mechanism of obesity pathogenesis? *Diabetes* 2013;62(8):2629–34.