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The impact of obesity on appetite regulation during nutritional ketosis

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Abstract

Obesity is a major global health concern, and sustained weight loss is often limited by increased appetite. The ketogenic diet (KD) has emerged as a potential appetite-modulating strategy. However, its neural and hormonal mechanisms remain incompletely understood. In this study, diet-induced obesity (DIO) was introduced to mice by 6-week Western chow feeding, followed by a 14-day KD intervention. Age-matched lean controls were maintained. We assessed consummatory behavior, biochemical and molecular markers of appetite regulation in the hypothalamus. Western chow feeding induced a metabolic phenotype characterized by hyperglycemia, hyperinsulinemia, hyperleptinemia, elevated HOMA-IR, reduced ghrelin and GLP-1, and decreased hypothalamic *Mc4r* expression. KD improved glucose and lipid profiles and reduced snack-directed behavior in lean and obese mice. Although food intake declined, caloric intake remained comparable due to the higher energy density of ketogenic chow. KD increased circulating β-hydroxybutyrate levels, particularly in lean mice. Hypothalamic Ghsr expression decreased, whereas *Cart* and *Mc3r* were upregulated irrespective of phenotype, indicating remodeling of melanocortin signaling. Selective *Agrp* upregulation in lean mice did not promote hyperphagia, suggesting compensatory adaptation. Overall, KD suppresses consummatory behavior and improves metabolic parameters. However, obesity-associated neuroendocrine alterations persist despite weight reduction.

Keywords Obesity · Nutritional ketosis · Appetite regulation · Hypothalamus

Abbreviations

α	MSH-α-melanocyte-stimulating hormone
Agrp	agouti-related protein
ARC	arcuate nucleus
BHB	β-hydroxybutyrate
Cart	cocaine-and amphetamine-regulated transcript
CCK	cholecystokinin
Cckar	cholecystokinin A receptor
DIO	diet-induced obesity mice model
FFA	free fatty acids

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Ghsr	ghrelin receptor
GLP1	glucagon-like peptide-1
Glp1r	glucagon-like peptide 1 receptor
Lepr	leptin receptor
LH	lateral area of hypothalamus
Mc3r	melanocortin-3 receptor
Mc4r	melanocortin-4 receptor
Npy	neuropeptide Y
PVA	paraventricular nucleus
Pomc	proopiomelanocortin
PST	palatable snack test
R/S	CT-risk-snack competition test

Introduction

Obesity is a major public health challenge characterized by profound metabolic disturbances, including hormonal dysregulation, insulin resistance, and impaired carbohydrate and lipid metabolism. Its development is closely linked to disruptions in central and peripheral mechanisms governing appetite regulation[1]. Appetite regulation is orchestrated by hypothalamic circuits, particularly the arcuate nucleus (ARC), which integrates circulating hormones and nutrients. Disruption of these systems contributes to dysregulated energy homeostasis[2].

Unhealthy eating patterns and overeating, combined with low levels of physical activity, are key drivers of overweight and obesity. Although lifestyle modification remains the first-line treatment, long-term adherence to dietary interventions is typically poor, and even individuals who initially lose weight often struggle to maintain their body mass change[3].

The ketogenic diet, a low-carbohydrate, high-fat dietary intervention, induces metabolic adaptations that shift energy utilization toward ketone bodies[4]. Ketogenic dietary interventions are established in selected forms of drug-resistant epilepsy and are being investigated as potential adjunctive strategies in metabolic, neurological, and psychiatric disorders[5,6,7]. Increasingly, they are also employed for obesity treatment, where appetite suppression is considered a key mechanism underlying the efficacy of body mass reduction. However, the neural and hormonal mechanisms underlying this effect remain incompletely understood. In particular, it is unclear whether obesity-associated metabolic alterations modify central and peripheral appetite signaling during nutritional ketosis[8].

Molecular studies of appetite regulation in the central nervous system are primarily performed in animal models. This raises the question of whether such models are appropriate for investigating appetite control in the context of nutritional ketosis. In this study, we employed a diet-induced obesity (DIO) model based on western chow feeding that reflects dietary patterns in an obesogenic food environment population, thereby facilitating mechanistic insight into its pathophysiology[9,10]. The Western diet has been shown to exert adverse health effects and strongly predispose individuals to obesity and associated metabolic disturbances[11]. Similar changes have been observed in rodents fed the equivalent of Western chow[12,13].

Thus, we hypothesized that obesity constrains the adaptive neuroendocrine response to nutritional ketosis, thereby modulating phenotype-specific pathways that regulate appetite. We evaluated the impact of a ketogenic chow feeding on consummatory behavior and biochemical parameters. Then, we examined how obesity modifies these behaviors. Additionally, the study aimed to determine whether and how nutritional ketosis alters central and peripheral mechanisms of appetite control by assessing the hypothalamic expression of appetite-regulating factors and circulating levels of appetite-regulating hormones. The experimental design is illustrated in Fig. 1.

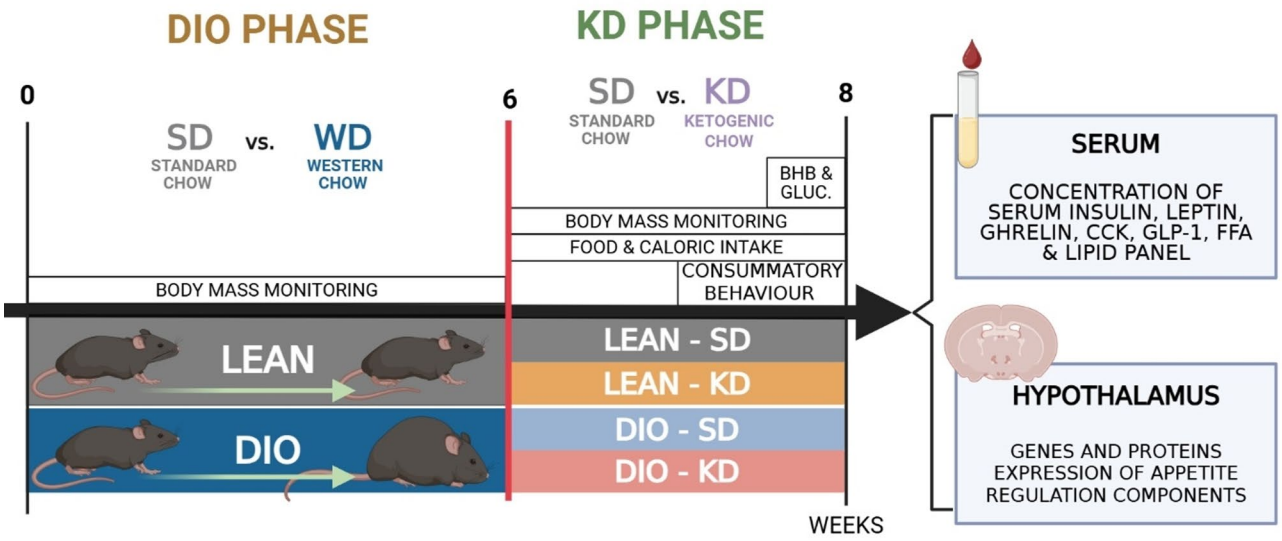

Fig. 1 Overview of the experimental design used to assess the effects of obesity on appetite-regulating factors in the mouse hypothalamus and serum. BHB - blood β-hydroxybutyrate concentration; CCK – cholecystokinin; DIO - diet-induced obesity mice model; FFA – free fatty acids; GLP1 - glucagon-like peptide-1; GLUC - blood glucose concentration; LEAN – non-obese control mice fed standard chow. Figure created by biorender.com.

Table 1 Characteristics of initial and final body mass during the DIO phase (mean ± SD) and biochemical markers after 6 weeks of western chow feeding in mice (mean ± SD).

Parameter	LEAN	DIO	*p* -value
initial body mass (g)	23.87 ± 1.48	23.85 ± 1.38	0.9571
final body mass (g)	26.69 ± 1.72	36.02 ± 4.29	< 0.0001
leptin (pg/ml)	51.73 ± 16.29	144.00 ± 88.41	< 0.0001
fasting glucose (ng/dl)	109.10 ± 17.48	170.40 ± 35.82	< 0.0001
postprandial glucose (ng/dl)	157.70 ± 21.28	200.90 ± 26.91	< 0.0001
fasting insulin (ng/ml)	51.73 ± 16.29	144.00 ± 88.41	< 0.0001
HOMA-IR	3.56 ± 1.06	16.31 ± 7.94	< 0.0001

DIO - diet-induced obesity. The Welch's t-test was applied.

The present study may contribute to a better understanding of the mechanisms underlying appetite regulation in nutritional ketosis and help clarify whether obesity-associated metabolic alterations influence the physiological response to a ketogenic diet. A more detailed characterization of these pathways may ultimately support the development of more effective strategies for obesity management, including both lifestyle-based and pharmacological approaches.

Results

Six weeks of western chow feeding leads to obesity development and metabolic disturbances

In the first phase of the experiment, to later assess changes in appetite-related factors during obesity, we verify a DIO model. At the beginning of the experiment, mean body mass did not differ between animals (Table 1). Throughout the experimental period, the body mass of the western chow-fed animals gradually increased from the third week onward, compared with the control mice fed standard chow at the same time points (supplementary material 3). Finally, after six weeks of western chow feeding, DIO mice had greater body mass than lean

animals. Also, elevated fasting ($p<0.0001$) and postprandial ($p<0.0001$) blood glucose levels were observed in DIO mice compared with the lean control group. Additionally, fasting serum insulin ($p<0.0001$) and HOMA-IR ($p<0.0001$) increased among western chow-fed animals relative to controls, indicating the development of insulin resistance. In addition, western chow-fed obese mice had elevated serum leptin concentrations ($p<0.0001$) compared to lean animals (Table 1). The observed changes confirmed the successful establishment of the DIO model, which was introduced for the subsequent phase of the experiment.

Ketogenic feeding induces metabolic remodeling independent of phenotype

Weight loss is often observed as early as two weeks after achieving nutritional ketosis[14], a period during which profound adjustments in whole-body energy metabolism occur. After two weeks on ketogenic chow, lean mice exhibited a lower body mass compared to the lean group fed a standard chow (Time x KD: $F(5, 115)=12.24$, $p<0.0001$) (Fig. 2a). Obese mice fed a ketogenic chow showed a successive significant decrease in body mass compared to the DIO-SD group (Time x KD: $F(5, 115)=14.93$, $p<0.0001$) (Fig. 2b). Two weeks of ketogenic chow treatment increased blood BHB (DIO x KD: $F(1, 47)=5.3$, $p=0.0258$) levels in both lean ($p<0.0001$) and obese animals ($p<0.0001$). Notably, the blood BHB concentration in obese mice on the ketogenic chow was lower compared to lean mice receiving the same chow ($p=0.0116$) (Table 2). The ketogenic chow elevated mean serum FFA (KD: ($F(1, 43)=84.80$, $p<0.0001$), cholesterol (KD: ($F(1, 44)=139.6$, $p<0.0001$) concentrations and improved HDL/LDL ratio (KD: ($F(1, 42)=13.74$, $p=0.0006$) compared to mice maintained on the standard chow. Obese mice, regardless of chow type, had higher cholesterol levels (DIO: ($F(1, 44)=16.15$, $p=0.0002$) than lean animals, while the serum triglyceride concentrations remained unchanged (Table 2).

After the KD phase, fasting blood glucose levels were higher in DIO mice compared to lean controls (DIO: ($F(1, 47)=38.40$, $p<0.0001$). All ketogenic chow-fed mice exhibited lower postprandial glucose levels compared to animals maintained on standard chow (KD: $F(1, 47)=152.1$, $p<0.0001$). The significant main effects of DIO and ketogenic chow on fasting insulin (DIO: $F(1, 29)=4.99$, $p=0.0332$, KD: $F(1, 29)=5.17$, $p=0.0305$) and HOMA-IR (DIO: $F(1, 29)=9.29$, $p=0.0049$, KD: $F(1, 29)=5.17$, $p=0.305$) levels were revealed, indicating that obesity elevated those parameters, and contrary ketogenic chow leads to its decreasement (Table 2).

Decreased food intake within a short period after the introduction of a ketogenic chow has been previously noted[15,16,17]. Tree-way ANOVA reveal a significant Time x DIO x KD interaction effect on food intake, suggesting that amount of consumed chow differed between lean and DIO mice, and also changed over time (Time x DIO x KD: $F(3,63)=7.28$, $p=0.0003$) (Fig. 2c). Furthermore, a reduction in food intake during the ketogenic chow feeding was noted in both lean and obese mice compared to the body mass-matched standard chow groups, but also in DIO mice shortly after switch from western to standard chow (Fig. 2c, Supplementary material 3). Significant Time x KD ($F(3,63)=4.66$, $p=0.0053$) and Time x DIO interactions ($F(3,63)=8.84$, $p<0.0001$) indicate that temporal dynamics of caloric intake differed depending on chow consumption or DIO status. However, no significant KD × DIO interaction was detected, suggesting that the overall impact of ketogenic chow on caloric intake was comparable between lean and obese mice (Fig. 2d).

Estimated cumulative food intake over the 14-day intervention was affected by both obesity status and diet. Two-way ANOVA revealed significant main effects of model ($F(1,21)=12.49$, $p=0.0020$) and diet ($F(1,21)=58.37$, $p<0.0001$), with no significant model × diet interaction (Fig. 2e). In contrast, estimated cumulative caloric intake was affected only by the model factor ($F(1,21)=9.04$, $p=0.0067$), whereas the effect of diet and the model × diet interaction were not significant (Fig. 2f). Thus, ketogenic diet feeding markedly reduced the total mass of food consumed, but this did not translate into a proportional reduction in cumulative caloric intake, likely due to the higher energy density of the ketogenic chow.

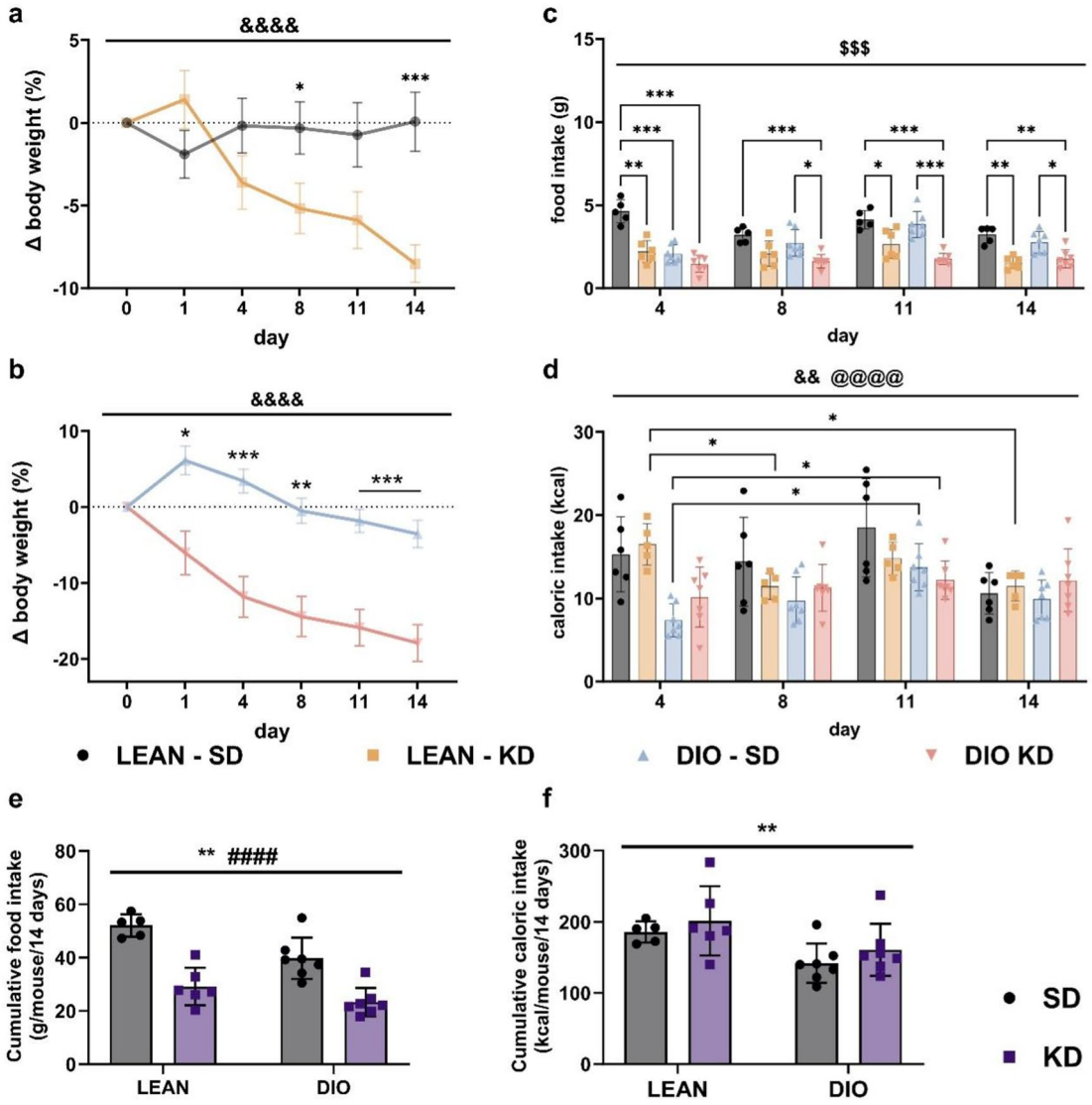

Fig. 2 Ketogenic chow feeding reduced body mass and altered food and caloric intake in lean and diet-induced obese (DIO) mice. Changes in body mass were monitored over 14 days in lean (a) and DIO mice (b) fed standard chow (SD) or ketogenic chow (KD). KD feeding was associated with a progressive reduction in body mass, which was particularly pronounced in DIO mice. Food intake (c) and caloric intake (d) were assessed throughout the intervention period to evaluate feeding-related adaptations to KD. Estimated cumulative food intake (e) and estimated cumulative caloric intake (f) over the 14-day intervention were calculated from interval-based measurements of mean daily food intake. The final day was extrapolated from the last recorded interval. Each data point in panels (e) and (f) represents one cage, with intake values expressed per mouse. Data are presented as mean ± SD. Lines relate to the significance of the main effects in three-way ANOVA, and connectors indicate Sidak post-hoc comparison (*$p<0.05$; **$p<0.01$; ***$p<0.001$) between the specific groups: Interaction (Time × DIO × KD): $$$ $p<0.001$; Interaction (Time × KD): && $p<0.01$, &&&& $p<0.0001$; Interaction (Time × DIO): @@@@ $p<0.0001$. For panels (e) and (f), lines indicate significant main effects from two-way ANOVA: model factor: **$p<0.01$; diet factor: ####$p<0.0001$.

Nutritional ketosis suppresses appetite regardless of obesity phenotype

During two behavioral tests – the palatable snack (PST) and the Risk–Snack Competition Tests (R/S-CT) - the assessment of ketogenic chow feeding on consummatory behavior was performed. Feeding with the ketogenic chow reduced snack intake (PST: KD: $F(1, 46)=15.61$, $p=0.0003$; R/S-CT: KD: $F(1, 46)=19.12$, $p<0.0001$)

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Table 2 Effects of 2 weeks of administration of the ketogenic chow on serum hormones and biochemical parameters in lean and DIO mice (mean ± SD).

Parameter	LEAN-SD	LEAN-KD	DIO-SD	DIO-KD
ghrelin (ng/ml)	1305 ± 843.6	1299 ± 941.3	449.5 ± 335.7	248.2 ± 209.1
leptin (pg/ml)	56.07 ± 19.8	65.5 ± 27.37	99.35 ± 26.21	90.06 ± 20
GLP-1 (ng/ml)	4952 ± 1489	4492 ± 1781	2926 ± 1298	580.9 ± 836.2
CCK (ng/ml)	172.7 ± 5.453	170.9 ± 4.85	167 ± 6.5	163.9 ± 5.59
fasting insulin (ng/ml)	0.59 ± 0.31	0.44 ± 0.12	1 ± 0.57	0.58 ± 0.23
fasting glucose (g/dl)	117.3 ± 10.4	129 ± 13.48	149.3 ± 17.95	148.6 ± 16.24
postprandial glucose (g/dl)	174 ± 28.35	90.77 ± 15.41	173.8 ± 28.02	100.7 ± 15.65
HOMA-IR	4.3 ± 2.52	3.40 ± 1	9.24 ± 5.2	5.40 ± 2.49
BHB (mM)	0.367 ± 0.14	2.50 ± 0.57****	0.38 ± 0.13	1.92 ± 0.69****$####
FFA (mM)	0.11 ± 0.03	0.24 ± 0.05	0.12 ± 0.03	0.27 ± 0.08
cholesterol (mg/dL)	85.16 ± 11.94	132.2 ± 11.89	95.40 ± 20.66	160.00 ± 18.72
HDL/LDL	0.86 ± 0.216	1.05 ± 0.19	0.76 ± 0.34	1.04 ± 0.11
triglycerides (mg/dL)	117.5 ± 54.59	130 ± 55.68	110.4 ± 43.95	122.50 ± 39

Two-way ANOVA followed by Tukey's multiple comparisons test: ****$p < 0.0001$ vs. LEAN-SD; $ $p < 0.05$ vs. LEAN-KD; #### $p < 0.0001$ vs. DIO-SD. BHB - β-hydroksybuterate; CCK - cholecystokinin; FFA - free fatty acids; GLP-1 - glucagon-like peptide-1.

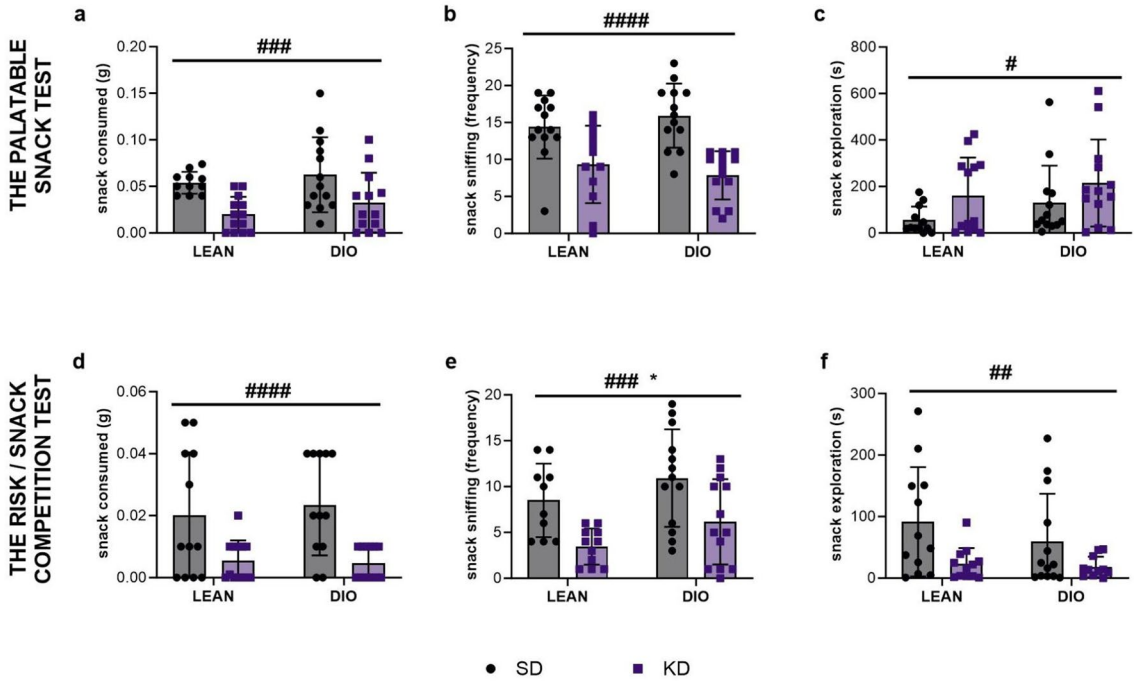

Fig. 3 Ketogenic chow feeding reduced consummatory behavior in lean and diet-induced obese (DIO) mice. Palatable snack consumption (a), snack sniffing frequency (b), and snack exploration time (c) were assessed in the palatable snack test after two weeks of standard chow (SD) or ketogenic chow (KD) feeding in lean and DIO mice. The same behavioral parameters were then evaluated in the risk/snack competition test, including snack consumption (d), snack sniffing frequency (e), and snack exploration time (f). KD feeding was associated with reduced consummatory behavior, whereas DIO mice showed increased snack-related responses in selected measures. Data are presented as mean ± SD. Lines relate to the significance of the main effects in two-way ANOVA: DIO: * $p < 0.05$, KD: #$p < 0.05$; ## $p < 0.01$; ### $p < 0.001$; #### $p < 0.0001$. SD – standard chow, KD – ketogenic chow.

(Fig. 3a and d), and decreased the frequency of snack sniffing (PST: KD: $F(1, 47) = 29.49$, $p < 0.0001$; R/S-CT: KD: $F(1, 43) = 15.37$, $p < 0.0001$) among mice regardless of obesity phenotype (Fig. 3b). In the PST, a significant main effect of ketogenic chow (KD: $F(1,47) = 4.98$, $p = 0.0304$) was observed on snack exploration time, with ketogenic chow-fed mice spending more time in the snack area than standard chow-fed animals, independent of obesity status (Fig. 3c). In the R/S-CT, snack exploration time was significantly reduced by ketogenic chow feeding (KD: $F(1, 44) = 9.29$, $p = 0.0039$) (Fig. 3f). Additionally, a significant main effect of the DIO ($F(1,43) = 4.19$,

$p=0.0469$) was detected on sniffing frequency, indicating that obese mice exhibited higher sniffing frequency than lean animals under adverse light stimuli (Fig. 3e). Moreover, no significant differences in the studied parameters of anxiety-related behaviours were observed in the OF and EPM tests (Supplementary material 3).

Obesity and ketogenic chow differentially alter circulating appetite-regulating hormones

To determine whether nutritional ketosis and obesity affect central and peripheral mechanisms of appetite regulation, we assessed circulating levels of appetite-related hormones.

Ghrelin is an orexigenic hormone primarily secreted by the stomach and acts on the hypothalamic ghrelin receptor (Ghsr), promoting food intake and fat storage[18]. Obesity may lead to metabolic changes, including reduced levels of ghrelin[19]. A significant main effect of the DIO on ghrelin concentration ($F(1,36)=20.73$, $p<0.0001$) was observed, with DIO mice exhibiting lower mean ghrelin concentrations than lean controls (Table 2).

Leptin is an adipocyte-derived anorexigenic hormone that signals energy sufficiency via Lepr, thereby inhibiting food intake and regulating energy homeostasis[20]. Two-way ANOVA revealed a significant main effect of the DIO ($F(1,41)=22.69$, $p<0.0001$) on circulating leptin levels, with obese mice exhibiting higher leptin concentrations than lean controls (Table 2).

Cholecystokinin (CCK) is a satiety hormone primarily released from the small intestine in response to nutrient ingestion, particularly fats and proteins, and promotes satiety through Cckar-mediated signaling in hypothalamic nuclei that regulate appetite and energy balance. Two-way ANOVA revealed a significant main effect of the DIO on circulating CCK levels ($F(1,42)=14.35$, $p=0.0005$), with DIO mice exhibiting lower CCK concentrations than lean controls (Table 2).

Glucagon-like peptide 1 (GLP-1) is a gastrointestinal satiety hormone that modulates energy balance by reducing hunger-driven feeding and food motivation. It is secreted in response to postprandial nutrient availability and, through Glp1r activation, plays a critical role in maintaining glucose homeostasis[21]. Two-way ANOVA showed significant main effects of DIO ($F(1,30)=33.71$, $p<0.0001$) and ketogenic chow feeding ($F(1,30)=7.53$, $p=0.0101$) on serum GLP-1 levels, with both factors associated with lower circulating GLP-1 concentrations (Table 2).

Ketogenic feeding modulates central appetite-regulating factors signaling in a phenotype-dependent manner

Appetite regulation in the ARC results from a dynamic balance between the activity of orexigenic neurons (Agrp/Npy), whose stimulation promotes food intake, and anorexigenic neurons (Pomc/Cart), which promote satiety signals. Stronger stimulation of a given neuronal population leads to a behavioral response to hunger or satiety, respectively[22]. We next assessed hypothalamic receptor expression to determine whether peripheral hormonal changes were accompanied by alterations in central appetite-related signaling. In the hypothalamus of DIO mice, Ghsr protein levels were significantly lower than in the LEAN-SD group, as indicated by a significant DIO × KD interaction ($F(1,20)=8.41$, $p=0.0089$; LEAN-SD vs. DIO-SD, $p=0.0016$) (Fig. 4a). Furthermore, all ketogenic chow-fed mice exhibited lower hypothalamic Ghsr protein levels than lean control animals, regardless of body mass (LEAN-SD vs. LEAN-KD, $p=0.0104$; LEAN-SD vs. DIO-KD, $p=0.0059$) (Fig. 4a). No changes were observed in hypothalamic *Ghsr* mRNA expression after two weeks of ketogenic chow feeding (Table 3). Ketogenic chow significantly affected hypothalamic Lepr mRNA expression ($F(1,12)=10.38$, $p=0.0073$), with higher mean Lepr mRNA expression observed in ketogenic chow-fed mice than in standard chow-fed mice, regardless of obesity status (Table 3). At the protein level, significant main effects of DIO ($F(1,20)=7.10$, $p=0.0149$) and ketogenic chow ($F(1,20)=11.66$, $p=0.0027$) were observed for hypothalamic Lepr levels. Accordingly, DIO mice exhibited lower hypothalamic Lepr protein levels than lean mice, whereas ketogenic chow-fed mice showed lower levels of this protein than standard chow-fed mice (Fig. 4b). A significant main effect of DIO was also detected for hypothalamic Cckar mRNA expression ($F(1,17)=8.53$, $p=0.0096$), indicating that DIO mice

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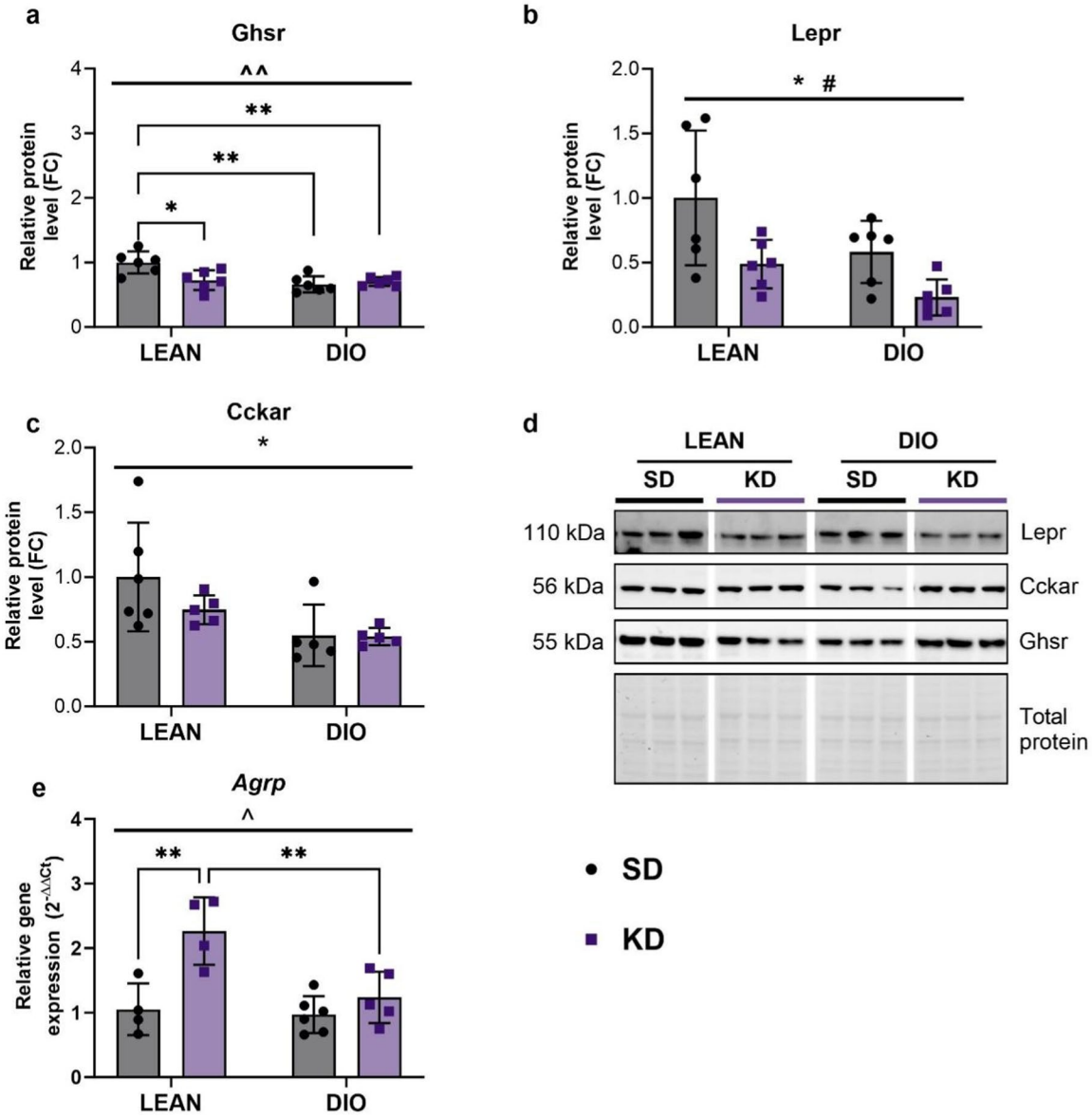

Fig. 4 Effects of obesity and ketogenic chow feeding on hypothalamic appetite-related receptor protein levels and *Agrp* gene expression. Relative protein levels of the ghrelin receptor (Ghsr) (a), leptin receptor (Lepr) (b), and cholecystokinin A receptor (Cckar) (c) were assessed in the hypothalamus after 14 days of standard chow (SD) or ketogenic chow (KD) feeding in lean and diet-induced obese (DIO) mice. Relative hypothalamic *Agrp* gene expression is shown in panel (e). Obesity was associated with lower hypothalamic levels of Ghsr, Lepr, and Cckar, while KD feeding affected selected receptors, including Ghsr and Lepr. In addition, KD feeding selectively increased hypothalamic *Agrp* expression in lean mice. Representative immunoblots and total protein staining used for normalization are shown in panel (d). Representative cropped immunoblots are shown. The full-length, uncropped blots are provided in Supplementary Materials 2. Data are presented as mean ± SD. Lines relate to the significance of the main effects in two-way ANOVA, and connectors indicate Tukey post-hoc comparison (*$p<0.05$; **$p<0.01$) between the specific groups: model factor: *$p<0.05$, diet factor: #$p<0.05$; interaction model × diet: ^$p<0.05$, ^^$p<0.01$.

exhibited lower expression levels than lean animals, regardless of nutritional ketosis status (Table 3). Consistently, hypothalamic Cckar protein levels were reduced in DIO mice compared with lean animals ($F(1,17)=8.27$, $p=0.0106$) (Fig. 4c). In contrast, hypothalamic Glp1r mRNA expression remained unchanged across the experimental groups (Table 3). A significant main effect of the interaction of DIO and ketogenic chow on hypothalamic

Table 3 Effects of 2 weeks of ketogenic chow administration on selective gene expression levels in the hypothalamus of lean and DIO mice.

Gene symbol	LEAN-SD	LEAN-KD	DIO-SD	DIO-KD
Cart	1.18 ± 0.9	3.38 ± 1.54	1.88 ± 0.18	3.36 ± 0.77
Cckar	1 ± 0.36	0.85 ± 0.09	0.60 ± 0.28	0.63 ± 0.07
Ghsr	1.03 ± 0.3	1.1 ± 1.19	1.19 ± 1.38	1.64 ± 1.35
Glp1r	1.01 ± 0.22	0.75 ± 0.4	0.6 ± 0.32	0.72 ± 0.23
Lepr	1.04 ± 0.31	2.1 ± 0.75	0.90 ± 0.53	2.88 ± 1.59
Mc3r	1.14 ± 0.69	1.87 ± 0.64	0.57 ± 0.12	1.53 ± 0.51
Mc4r	1 ± 0.4	0.88 ± 0.41	0.48 ± 0.24	0.55 ± 0.1
Npy	1.03 ± 0.29	0.70 ± 0.24	0.76 ± 0.54	0.54 ± 0.18
Pomc	1.07 ± 0.47	1.22 ± 0.8	7.55 ± 13.29	3.95 ± 5.22

Data are presented as fold expression relative to the LEAN-SD group (mean ± SD). Statistical analysis was performed using two-way ANOVA.

Agrp mRNA expression (DIO x KD: $F(1, 15) = 6.57$, $p = 0.0216$) was revealed. In lean mice, the ketogenic chow increased *Agrp* mRNA expression compared to those on a standard chow (LEAN-SD vs. LEAN-KD, $p = 0.0030$). Conversely, in DIO mice on a ketogenic chow, *Agrp* mRNA levels were lower than those observed in lean mice consuming the same chow (LEAN-KD vs. DIO-KD, $p = 0.0074$). In all mice, ketogenic chow elevated the mean *Cart* mRNA expression level ($F(1, 12) = 14.66$, $p = 0.0024$) (Table 3). It was previously shown that increased *Agrp* expression may inhibit POMC neuron activity in the anorexigenic system, leading to a sensation of hunger[23,24,25]. After two weeks of ketogenic chow feeding, no significant differences in the hypothalamic gene expression levels of *Pomc* and *Npy* were observed (Table 3). Agrp neurons may secrete Agrp into the PVA and compete with α-melanocyte-stimulating hormone (α-MSH) for binding sites on Mc3r and Mc4r. The mean hypothalamic *Mc3r* mRNA expression was higher among mice fed the ketogenic chow compared to animals on standard chow (KD: $F(1, 13) = 10.14$, $p = 0.0072$). There was a significant main effect of the DIO ($F(1, 16) = 13.43$, $p = 0.0021$) on *Mc4r* mRNA expression, indicating that obese mice had lower hypothalamic *Mc4r* mRNA levels than lean animals on standard chow.

Discussion

In the present study, we showed that nutritional ketosis was associated with reduced snack-directed behaviours, suggesting a suppressive effect on the consummatory behaviours, regardless of metabolic status. However, the behavioral effects were not uniformly reflected peripherally. Peripheral hormone levels did not change consistently. Hormone receptor expression also showed no uniform pattern. The same applied to hypothalamic neuropeptides in both phenotypes. Obesity induce persistent metabolic disturbances. These alterations affected appetite-regulating pathways. Short-term weight reduction did not fully reverse them. Ketogenic feeding only partially normalized these changes. The mechanisms underlying appetite regulation in obesity, as well as the impact of obesity itself on the functioning of appetite-regulating pathways, remain poorly understood[26]. Here, we performed an experiment employing a DIO model established by western-style chow feeding rich in salty and sweet snacks. This design has strong translational relevance, as it mimics obesogenic dietary patterns characterized by high consumption of palatable foods. We observed that mice fed western chow showed a remarkable increase in body mass. Similar observations have previously been reported in female[12,13] and male rodents[27]. The western-style chow not only induced the phenotypic changes but also contributed to the development of metabolic disturbances associated with obesity, as evidenced by hyperglycemia, hyperinsulinemia, hyperleptinemia, and elevated HOMA-IR, as previously demonstrated[27,28,29,30].

Low-carbohydrate diets, including the ketogenic diet, are effective for weight loss and the treatment of obesity, with potential appetite-suppressing effects[31,32]. It has been demonstrated that BHB can influence both orexigenic and anorexigenic hypothalamic signals, thereby inhibiting or promoting food intake[27]. Here, nutritional ketosis

was confirmed by an increase in blood BHB concentration in all ketogenic chow-fed mice. Notably, lean mice exhibited higher circulating BHB concentrations than their DIO counterparts, indicating that obesity development may reduce the efficiency of ketogenesis or increase peripheral utilization of ketone bodies.

Consistent with previous reports, we observed that obesity development was associated with elevated circulating leptin levels, which may contribute to hypothalamic Lepr resistance[33]. Nutritional ketosis reduced hypothalamic Lepr protein expression in all mice, whereas gene expression showed the opposite pattern. Thus, gene expression diverges from protein expression, likely due to more dynamic transcriptional responses to metabolic stimuli, such as changes in lipidogram, as well as to post-translational modifications. Previously, Lee et al.l. (2022) confirmed that activation of Lepr neurons in the LH promotes seeking or consummatory behaviors, whereas their inhibition reduces those behaviours[34]. We suggest that the reduction in Lepr protein levels in ketogenic chow-fed mice was accompanied by decreased snack consumption and its sniffing frequency. Moreover, during nutritional ketosis, snack exploration time increased under neutral conditions but decreased under aversive light stimuli, suggesting diminished hedonic and risk-driven food-seeking behavior. We confirmed that the reduced interest in palatable snacks in ketogenic-fed mice was not related to anxiety-like behavior. Moreover, open-field data did not indicate marked alterations in general locomotor activity. This suggests that the observed changes in snack-directed behavior were unlikely to reflect nonspecific reductions in activity. These findings support that the ketogenic chow exerts an appetite-suppressing effect in both obese and lean mice. However, we performed assessments on whole hypothalamus homogenates. Therefore, the neuronal populations from LH, ARC, and PVA were not assessed separately, and the results are not specific to a particular nucleus and should be carefully interpreted in the context of receptor activation and its consummatory effects.

We observed that obesity-induced positive energy balance reduced ghrelin secretion and hypothalamic sensitivity to ghrelin, as confirmed previously[35,36]. In line with earlier observations[31,37,38], we showed that nutritional ketosis was also associated with decreased hypothalamic Ghsr levels. This change may result in reduced food consumption and hypothalamic adaptation to altered energy balance[39]. Here, all mice fed the ketogenic chow consistently reduced food intake, and among lean mice, caloric intake declined progressively during ketogenic chow administration. Cumulative intake analysis further showed that KD reduced the total amount of food consumed, but this was not accompanied by a corresponding diet-related reduction in cumulative caloric intake. Therefore, the marked reduction in body mass observed after ketogenic chow feeding cannot be explained solely by reduced food intake. Differences in macronutrient composition, energy density, substrate utilization, and energy expenditure may also have contributed to this effect. However, because body composition, indirect calorimetry, thermogenesis, and adipose tissue metabolic markers were not assessed, this mechanism should be interpreted with caution.

In obesity, reduced Ghsr levels in the hypothalamus may result from an adaptive response to chronic energy overconsumption and elevated satiety hormone levels[36,40]. Here, in DIO mice, characterized by reduced ghrelin and increased leptin levels, inhibition of orexigenic signals through Ghsr may result from prior chronic energy overconsumption during western chow feeding. The observed decrease in Ghsr expression in obese mice fed a ketogenic chow may not be a direct consequence of the diet itself but rather a secondary effect of preexisting obesity-related disturbances.

Consumption of a high-fat, high-carbohydrate diet has been reported to attenuate the anorexigenic efficacy of CCK despite its elevated peripheral levels, suggesting the development of CCK resistance[41,42]. Although circulating CCK levels were not elevated in DIO mice at the time of assessment, this may reflect normalization of hormone concentrations following cessation of western-style chow feeding rather than the absence of prior CCK pathway activation. Previous exposure to a western-style diet may have promoted persistent adaptations in CCK-responsive circuits. Consistent with this possibility, DIO mice exhibited reduced hypothalamic Cckar expression regardless of nutritional ketosis, suggesting diminished central responsiveness to CCK-mediated satiety signaling. This impairment may contribute to altered appetite control. It is supported by the increased frequency of snack sniffing observed in DIO mice under mild aversive light conditions. It can suggest enhanced food-seeking behavior.

Huang et al. (2022) demonstrated that the central effects of Glp1r activation depend on nutritional status, showing that the receptor agonist exenatide exerts stronger glucose-lowering effects under conditions of nutrient abundance than under nutrient deficiency[43]. In the present study, standard chow-fed lean mice had higher Glp1r levels in the hypothalamus than other groups. In association with low carbohydrate intake in all ketogenic chow-fed mice, postprandial glucose and circulating GLP-1 levels were reduced, as previously shown[44,45]. Thus, during nutritional ketosis, reduced glucose availability may attenuate hypothalamic Glp1r–mediated signaling as part of the adaptive mechanisms that maintain glucose homeostasis[21]. Obesity often leads to reduced GLP-1 secretion and impaired hypothalamic Glp1r signaling[46]. Here, Glp1r gene expression was not substantially altered by obesity or ketogenic feeding. This may indicate that hypothalamic GLP-1 receptor signaling was relatively preserved under the present experimental conditions. However, these changes occurred in the context of reduced circulating GLP-1 concentrations. This discrepancy may reflect the relatively short duration of ketogenic chow, which may have been sufficient to alter peripheral GLP-1 availability but not long enough to induce detectable changes in hypothalamic Glp1r expression. However, DIO and lean mice fed ketogenic chow were less engaged with palatable snacks during behavioral testing, thus further supporting the thesis that a ketogenic chow can suppress appetite through other mechanisms or potentially enhance GLP-1 activity in other parts of the body[47].

In the present study, hypothalamic *Agrp* expression increased selectively in lean ketogenic mice, despite reduced hypothalamic Lepr and Ghsr protein levels in both lean and DIO animals, suggesting attenuation of hormone-dependent orexigenic signaling[22]. The selective upregulation of *Agrp* in lean mice coincided with higher circulating BHB levels, raising the possibility that enhanced ketone availability may contribute to transcriptional regulation through epigenetic mechanisms. BHB has been identified as an endogenous epigenetic modulator that regulates neuronal expression of genes related to metabolism[48,49]. Therefore, it is plausible that enhanced ketone availability may contribute to *Agrp* upregulation through transcriptional mechanisms involving epigenetic modification. Another, non-mutually exclusive explanation may involve glucose sensing by AgRP neurons. Recent studies indicate that AgRP neurons integrate peripheral glucose-related signals and that their activity is linked to glycemic responses to nutrient availability[50,51]. In our study, selective Agrp upregulation in lean KD-fed mice occurred in parallel with lower postprandial glucose levels. It is suggesting a possible inverse relationship between glycemic status and hypothalamic Agrp expression under ketogenic feeding. However, this interpretation remains associative, as we measured whole-hypothalamus Agrp expression rather than AgRP neuron activity. Importantly, this *Agrp* upregulation has not been related to increased food intake, supporting the interpretation of an adaptive and compensatory transcriptional response rather than functional orexigenic signaling[52]. Nevertheless, whether such epigenetic mechanisms appear in Agrp neurons requires further investigation. Furthermore, obesity has been associated with DNA methylation changes within the *Agrp* promoter, leading to transcriptional repression. This also may partly explain the absence of *Agrp* expression induction in DIO mice fed ketogenic chow. However, our analyses were performed on whole hypothalamic homogenates, whereas *Agrp* expression and its appetite-regulating effects are nucleus-specific. Therefore, those effects may have been masked by the composite transcriptional profile of the hypothalamus[53]. Downstream of anorexigenic neurons, melanocortin receptor signaling plays a central role in energy homeostasis. Activation of Mc3r and, particularly, Mc4r suppresses food intake and increases energy expenditure, whereas Agrp counteracts this pathway by inhibiting melanocortin signaling[54,55]. DIO mice, two weeks after cessation of western chow, were characterized by reduced hypothalamic *Mc4r* expression, suggesting impaired melanocortin responsiveness. In contrast, ketogenic feeding induced upregulation of *Cart* and *Mc3r* expression irrespective of obesity status, indicating diet-driven remodeling of melanocortin signaling. Collectively, these findings suggest that metabolic flexibility in lean animals may permit adaptive transcriptional responses to ketosis, whereas obesity-associated dysfunctions may constrain such plasticity.

The relatively short duration of ketogenic chow feeding should be considered when interpreting these findings. Although two weeks of nutritional ketosis induced measurable changes in selected appetite-related markers, this period may not have been sufficient to reveal the full extent of central and peripheral adaptations. Longer interventions may therefore be required to determine whether additional regulatory mechanisms emerge over time.

Another limitation is that body composition, indirect calorimetry, thermogenesis, and adipose tissue metabolic markers were not assessed. Therefore, the mechanisms underlying ketogenic diet-induced body mass reduction cannot be fully determined. In addition, blood and tissue samples were collected under ketamine/xylazine anesthesia, which may affect circulating hormone levels and hypothalamic protein signaling. However, the same anesthesia protocol was applied to all experimental groups, reducing the likelihood that this factor explains the observed between-group differences.

In summary, western-style chow feeding and obesity induce persistent peripheral and central metabolic alterations that are not fully normalized by dietary intervention. Obesity development was associated with impaired gastrointestinal satiety signaling despite elevated adiposity signals. This dysregulation of appetite-related hormones and carbohydrate metabolism persisted despite dietary modification. In contrast, ketogenic chow improved the lipid profile and reduced consummatory behavior in both lean and obese mice. However, the reduced hypothalamic Ghsr protein levels observed in obese mice fed ketogenic chow likely reflect obesity-associated maladaptation rather than a direct effect of ketosis. At the central level, ketogenic chow consistently upregulated *Cart* and *Mc3r*, while DIO downregulated *Mc4*r expression, suggesting remodeling of melanocortin signaling. The selective increase in *Agrp* expression in lean mice fed ketogenic chow appears compensatory to weight loss rather than indicative of functional orexigenic dominance. Overall, these findings suggest that metabolic flexibility enables adaptive neuroendocrine remodeling in lean animals, whereas obesity-associated dysfunction constrains this plasticity.

Materials and methods

Animals

The young adult (10-week-old) male C57BL/6NCrL mice were provided by the Medical University of Silesia, Katowice, Poland. During the experiment, mice were housed in standard cages (3–5 individuals per cage) with food and water *ad libitum*. All procedures were carried out in a climate-controlled room (22 ± 2 °C, humidity: 40–60%) with a 12 h:12 h light/dark cycle starting at 07:00 AM. All experimental procedures were approved by the Local Ethics Committee for Animal Experiments in Katowice, Poland (Polish name: Lokalna Komisja Etyczna do Spraw Doświadczeń na Zwierzętach w Katowicach; approval No. 3/21, 15 February 2021). All experiments and methods were performed in accordance with relevant guidelines and regulations. The minimum number of mice required to obtain consistent data was used, and every effort was made to minimize animal suffering.

Experimental Design

The experiment was divided into two phases: first, the establishment of the DIO model (DIO phase), and then maintenance of animals on the ketogenic chow (KD phase). The study included 51 young adult male mice, randomly divided into two groups. Animals in the first group were fed *ad libitum* for six weeks with a western chow enriched with salty and sweet snacks (DIO, $n = 26$). During the same period, control mice received standard chow (LEAN, $n = 25$). Following the DIO phase, animals were divided into four groups in a body mass-matched manner. For the next 14 days, lean and obese animals were either maintained on standard chow, as control groups (LEAN-SD, $n = 12$ and DIO-SD, $n = 13$), or switched to a ketogenic chow (LEAN-KD, $n = 13$ and DIO-KD, $n = 13$) (Fig. 1).

Throughout the experiment, body mass was monitored weekly (DIO phase) or every 3–4 days (KD phase). Also, during the KD phase, food and caloric intake were measured every 3–4 days.

Cumulative food intake was estimated over the 14-day intervention based on interval measurements of mean daily food intake. The final day was extrapolated from the last recorded interval. Cumulative caloric intake was calculated by multiplying cumulative food intake by the energy density of each diets. Because food intake was

measured at the cage level, each cage was treated as the experimental unit for cumulative intake analyses, with values expressed per mouse. Consummatory and anxiety-related behavioral tests were conducted during the last week of the experiment. A day before euthanasia, blood samples from the tail tip were collected. On the euthanasia day, mice were anesthetized, and then the retro-orbital blood, and the hypothalamus were dissected.

Chow administration

To obtain the DIO model, male mice were subjected to standard chow (Labofeed B, Wytwórnia Pasz Morawski) enriched with salty and sweet snacks for six weeks. The dietary components were chosen to closely mimic the taste, diversity, and caloric content of the western-style human diet, as described previously by Grabowska et al. (2025)[56]. Briefly, mice were given one of two sets of snacks on alternate days, providing them with a diversified diet. The first set included: candy bar (Mars by Mars Inc.), crackers (Lajkonik Snacks), cookies (Lajkonik Snacks), and dry pork sausage (Tarczyński). The second set contained: a candy bar (Bounty by Mars Inc.), potato chips (Lays Salt by PepsiCo), and Tilsit cheese (Hochland). Moreover, mice received clean water and a 10% fructose solution (Consweet Sweetener) in a second container. At the same time, control lean mice were administered with the same standard chow, but without enrichment with snacks (LEAN).

Following the DIO phase, two groups of male mice were switched to ketogenic chow (Wytwórnia Pasz Morawski) for the next 14 days. One group consisted of mice that developed diet-induced obesity (DIO-KD), while the other group included lean mice (LEAN-KD). The control groups, including lean (LEAN-SD) and obese (DIO-SD) mice, were maintained on standard chow (Labofeed B, Morawski). Both types of chow were provided ad libitum. The energy values of the standard, ketogenic, and western chows were 3.57 kcal/g, 6.9 kcal/g, and 4.84 kcal/g, respectively (Supplementary material 1).

Behavior assessment

Behavioral tests were conducted on days 10 to 14 of the KD phase, with each test occurring at one-day intervals. The tests were performed between 8:00 AM and 3:00 PM under dim light conditions (~ 10 lx) and low noise levels. Before each test, the mice underwent a habituation period, during which they stayed in the testing room for 3 h and were deprived of food. Before each trial, the surfaces of the behavioral test areas were thoroughly cleaned. Mouse behavior during the tests was recorded and analyzed using EthoVision XT 10 (Noldus Information Technology).

Open field test

The open field test was conducted in a white plastic box (40.7 × 40.7 cm). The locomotor and exploratory activities of the mice were assessed using three parameters: total distance traveled, time spent in the center of the area, and time spent in the periphery of the area. At the beginning of the trial, each mouse was placed individually in the center of the open field, and its exploratory behavior was recorded for 10 min.

Elevated plus maze test

The elevated plus maze test was conducted using a crossed maze consisting of two opposing open arms (30 × 5 cm), two opposing closed arms (30 × 5 × 15 cm), and the central zone (5 × 5 cm). The maze was positioned 40 cm above the floor. Time spent in closed and open arms and in the head-dip area was calculated to assess locomotor and exploratory activities. Each mouse was placed individually in the central zone, facing an open arm, and its behavior was recorded for 5 min. These tests served as screening procedures to identify potential individual anxiety-related alterations.

Palatable snack and the risk–snack competition tests

To assess snack-directed consummatory behavior, the palatable snack test (PST) and the risk–snack competition test (R/S-CT) were applied. These behavioral paradigms were based on rodents' natural tendency to explore and sample novel food items[57]. Before testing, mice were habituated to the experimental room for 3 h and were food-deprived during this period to increase motivation to approach and consume the snack. Gouda cheese was used as the palatable snack in order to minimize disruption of nutritional ketosis. The snack was selected on the basis of a pilot preference assessment comparing three types of cheese. Mice had not been exposed to this type of cheese before the behavioral tests. For each trial, a pre-weighed piece of Gouda cheese was used, and snack consumption was calculated as the difference between the pre- and post-test snack mass.

In the PST, each mouse was placed individually in a transparent plastic arena (40.7 × 40.7 cm) and allowed to freely explore the empty arena for 10 min. After this habituation period, the pre-weighed snack was placed in the peripheral area of the arena and remained freely available for an additional 10 min. Snack-directed behavior was assessed by measuring snack consumption, sniffing frequency, and snack exploration time.

The R/S-CT was used to assess snack-directed behavior under a mild aversive illumination. The test was performed in the same type of plastic arena divided into light and dark zones of equal size (50/50), with free access between compartments. Each mouse was initially placed in the centre of the illuminated compartment and allowed to explore both compartments for 10 min. Subsequently, the pre-weighed snack was placed in the peripheral area of the illuminated compartment, where it was exposed to mild aversive illumination at 100 lx for an additional 10 min. This illumination level was selected to create an approach–avoidance conflict without preventing access to the snack.

Snack consumption was defined as the amount of cheese consumed during the test. Sniffing frequency was defined as the number of discrete episodes in which the mouse directed its nose toward the snack and investigated it without biting or chewing. Snack exploration time was defined as the cumulative time spent in direct proximity to, or physical interaction with, the snack, including eating, sniffing and close investigation. Behavioral scoring and verification were performed by an observer blinded to the experimental groups.

Euthanasia and tissue collection

Sample collection was conducted between 9:00 AM and 12:00 PM. Sequentially, with each mouse processed individually, animals were anesthetized and euthanized with an overdose (IP injection: ketamine > 100 mg/kg/b.m., xylazine > 10 mg/kg/b.m.). Following anesthesia, blood was collected via retroorbital sinus puncture and then centrifuged (4000 × g, 20 min, 4 °C). The hypothalamus (0 to −2 anterior/posterior to bregma coordinates) encompassing the ARC, the paraventricular nucleus (PVA), and the lateral area (LH), and the ventromedial and dorsomedial hypothalamic nuclei was then dissected. Tissues intended for protein-level analysis were immediately snap-frozen, while those for gene expression analysis were preserved in RNAlater (Sigma-Aldrich). All obtained samples were stored at −80 °C until further analysis.

Biochemical analyses

One day before euthanasia, blood and serum were collected from the tail tip after a 6-hour fasting period, and following a 2-hour exposure to a fresh portion of chow. Fasting blood glucose, postprandial blood glucose and β-hydroxybutyrate (BHB) level were measured using the Optium Xido Neo glucometer (Abbott Laboratories). Fasting serum insulin and leptin concentrations were assessed with commercially available ELISA kits (90080, Crystal Chem; RAB0334, Sigma-Aldrich). The serum lipid panel, obtained postprandially from retroorbital blood, was determined with an Epoll 200 (Alpha Diagnostics) using cholesterol (C6608-100, Alpha Diagnostics) and triglycerides (T6630-100, Alpha Diagnostics) reagents, according to the manufacturer's instructions. Postprandial serum concentrations of ghrelin (EIAM-GHR, RayBiotech), GLP-1 (EIAM-GLP1, RayBiotech),

cholecystokinin (EIAM-CCK, RayBiotech), and free fatty acids (FFA) (Ab65341, Abcam) were quantified using commercially available ELISA kits following the manufacturer's protocols.

Western blotting

The hypothalamus sample was homogenized in the RIPA buffer (300 µl for every 10 mg of tissue; Sigma-Aldrich Corp.) containing phosphatase and protease inhibitors (cOmplete™ ULTRA Tablets, Roche Holding AG; PhosSTOP™, Roche Holding AG). The homogenates were then centrifuged (12,000 × g, 20 min, 4 °C). The protein concentration was measured using the BCA Reagent (ab102536, Abcam). Equal amounts of protein (10 µg) were denatured in Laemmli buffer with β-mercaptoethanol, separated on 4–15% Stain-Free polyacrylamide gels (#5678085, Bio-Rad), and transferred onto PVDF membranes. After blocking with casein buffer (B6429, Sigma-Aldrich), membranes were incubated with primary antibodies (Supplementary material 1) overnight at 4 °C. Following TBST washes, membranes were incubated with a secondary antibody (1:5000, ab97051, Abcam). Detection was performed using the Clarity™ Western ECL substrate (Bio-Rad Laboratories Inc.), and chemiluminescent signals were visualized with a ChemiDoc-Touch Imaging System (Bio-Rad Laboratories Inc.). Densitometric analysis was performed with Image Lab software (Bio-Rad Laboratories Inc.). Expression levels were normalized to total lane protein (Stain-Free technology, Bio-Rad Laboratories Inc.) and presented as relative fold change to the LEAN-SD control group. Original full-length gels and blots were included in Supplementary Material 2.

Gene expression analysis

Total RNA was isolated from the hypothalamus using the miRNeasy Mini Kit (217004, Qiagen) according to the manufacturer's protocol. Reverse transcription (250 ng of RNA) was performed with the Maxima H Minus First Strand cDNA Synthesis Kit with dsDNase (K1682, Thermo Fisher Scientific). qPCR was performed using the QuantiTect SYBR® Green PCR Kit (20414, Qiagen) along with target-specific primers (Genomed, Poland) (Supplementary material 1) in the LightCycler 96 system (Roche Diagnostics). The relative expression levels of target genes were calculated using the 2^−ΔΔCt method, normalizing to the geometric mean of *Atp5j* and *Actb* expression.

Statistical analysis

Statistical analyses were performed using GraphPad Prism software (version 10.1.1, GraphPad Software, Inc.). Normality of the data distribution was assessed with the Shapiro–Wilk test. To compare two independent experimental groups (LEAN vs. DIO), Welch's t-test was applied. Two-way repeated-measures ANOVA with the Greenhouse–Geisser correction, followed by Sidak's post hoc test (body weight gain over time) and three-way repeated-measures ANOVA with the Greenhouse–Geisser correction, followed by Tukey's post hoc test (food intake across time points), were performed. To assess the main effects of DIO and ketogenic chow (KD), and the interaction between these factors (Interaction), a two-way ANOVA was performed on data from four experimental groups (biochemical parameters, behavioral outcomes, gene expression, protein levels). The same two-way ANOVA approach was used to analyses estimated cumulative food intake and estimated cumulative caloric intake, using cage-level values expressed per mouse. If a significant interaction effect was detected, Tukey's multiple-comparison tests were used to identify differences among groups. Data are presented as mean ± standard deviation (SD). In all analyses, p-values less than alpha 0.05 were considered statistically significant (Supplementary material 3).

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Author contributions M.G. and K.G. performed the experiments, contributed to the methodology, prepared the visualizations, and wrote the original draft of the manuscript. M.G. and D.L. conceptualized the study. M.N.-Ch. performed the experiments, contributed to the methodology, and reviewed and edited the manuscript. A.M. and H.J.Sz. provided resources and reviewed and edited the manuscript. J.J.B. provided resources and supervised the study. D.L. contributed to the methodology, supervised the study. All authors reviewed and approved the final manuscript.

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Data availability All data generated or analyzed during this study are available from the corresponding author upon reasonable request.

Declarations

Competing interests The authors declare no competing interests.

Approval for animal experiments Animals were treated using the protocols approved and monitored by the Local Committee for the Care and Use of Laboratory Animals in Katowice, Poland (permission no. 3/21, 15th February 2021), and all experiments were performed following relevant guidelines and regulations. Manuscripts presenting studies that have been reported in accordance with the ARRIVE guidelines

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