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Brief Communication

MOLECULAR METABOLISM

A gene–diet interaction controlling relative intake of dietary carbohydrates and fats

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ABSTRACT

Objective: Preference for dietary fat vs. carbohydrate varies markedly across free-living individuals. It is recognized that food choice is under genetic and physiological regulation, and that the central melanocortin system is involved. However, how genetic and dietary factors interact to regulate relative macronutrient intake is not well understood.

Methods: We investigated how the choice for food rich in carbohydrate vs. fat is influenced by dietary cholesterol availability and agouti-related protein (AGRP), the orexigenic component of the central melanocortin system. We assessed how macronutrient intake and different metabolic parameters correlate with plasma AGRP in a cohort of obese humans. We also examined how both dietary cholesterol levels and inhibiting *de novo* cholesterol synthesis affect carbohydrate and fat intake in mice, and how dietary cholesterol deficiency during the postnatal period impacts macronutrient intake patterns in adulthood.

Results: In obese human subjects, plasma levels of AGRP correlated inversely with consumption of carbohydrates over fats. Moreover, AgRP-deficient mice preferred to consume more calories from carbohydrates than fats, more so when each diet lacked cholesterol. Intriguingly, inhibiting cholesterol biosynthesis (simvastatin) promoted carbohydrate intake at the expense of fat without altering total caloric consumption, an effect that was remarkably absent in AgRP-deficient mice. Finally, feeding lactating C57BL/6 dams and pups a cholesterol-free diet prior to weaning led the offspring to prefer fats over carbohydrates as adults, indicating that altered cholesterol metabolism early in life programs adaptive changes to macronutrient intake.

Conclusions: Together, our study illustrates a specific gene–diet interaction in modulating food choice.

Keywords Dietary preference; Cholesterol; AgRP; Simvastatin

1. INTRODUCTION

The relative consumption of carbohydrates vs. fats over time varies tremendously among free-living individuals, but the genetic and environmental factors affecting the relative intake of particular macronutrients remain ill-defined. Throughout evolution, general food scarcity and the unpredictability of food availability have selected for metabolic adaptations that confer survival advantages. One such adaptation by mammals could have been to prefer calorically dense, fat-rich foods. Indeed, food preference is heavily influenced by energy status; mice selectively increase consumption of fats over carbohydrates during refeeding after a fast compared to the *ad lib* fed state [1]. In humans, babies born to mothers who were pregnant during the Dutch famine grew up to prefer fatty foods, in support of a potential developmental adaptation to food scarcity during fetal life [2]. Together, these findings suggest that a preference for fat, the most energy-dense macronutrient, is an adaptive response engaged under conditions when calories are limited to restore energy balance whenever food becomes available.

Genetics is a strong intrinsic determinant of macronutrient selection. Mice from 13 inbred strains exhibited widely variable, strain-specific patterns of macronutrient preference, with some strains preferring fats and others preferring carbohydrates [3]. In humans, the central melanocortin system has been implicated in regulating carbohydrate and fat preferences. The central melanocortin system consists of Proopiomelanocortin (POMC), AgRP, and their CNS receptors — Melanocortin 3 and 4 receptors (MC3R and MC4R). AgRP is an antagonist and inverse ligand for melanocortin receptors; it is expressed by a group of neurons in the mediobasal hypothalamus that co-express NPY and GABA. The role of AgRP neurons in regulating feeding is well established, as optogenetic or chemogenetic stimulation of AgRP neurons leads to voracious feeding, whereas acute

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ablation of these neurons leads to severe anorexia and weight loss [4–6]. However, most of the orexigenic effects of AgRP neurons are attributed to NPY and GABA, but not to AgRP [4–7]. Paradoxically, although transgenic overexpression of *Agrp* or infusion of a pharmacologic level of AgRP into the brain stimulates feeding [8–11], $Agrp^{-/-}$ mice have normal feeding and body weight [12,13]. However, central administration of AgRP (83–132) in rodents selectively increases consumption of fats over carbohydrates [14]; wild-type mice prefer a high-fat diet, whereas AgRP-deficient mice do not [15]. A polymorphic allele in the third exon of the *AGRP* gene (Ala67Thr) is associated with lower fat intake and higher carbohydrate intake in humans [16]. Notably, MC4R-deficient human subjects have an increased preference for a high-fat diet and a reduced preference for high-sucrose food when compared with lean or equally obese subjects without MC4R mutations [17].

Thus, available evidence indicates that food choice is under strong genetic and physiological control and that the central melanocortin system is involved in this regulation. However, how environmental and dietary factors interact with this regulatory system is unclear. One important dietary component is cholesterol. Cholesterol is essential for life, but it is energetically costly to synthesize and cytotoxic when produced in excess. Thus, cholesterol production is under tight homeostatic regulation. The body normally obtains cholesterol from two sources: the diet and *de novo* biosynthesis mainly by the liver. When dietary cholesterol content is high, hepatic cholesterol synthesis is suppressed by robust negative feedback mechanisms [18]; however, when dietary cholesterol is absent, as in plant-based diets, hepatic cholesterol synthesis is increased to meet the body's cholesterol needs [19]. This process is driven primarily by regulating the mRNA levels of *Hmgcr*, which encodes HMG-CoA reductase (HMGCR) [19]. Little is known about the functional relationship between cholesterol and hypothalamic neuronal functions, however, acute antagonism of the central melanocortin receptors in rodents increases circulating cholesterol levels independent of food intake and body weight [20,21]. AgRP-deficient mice also showed reduced circulating cholesterol levels [22]. Moreover, the human *AGRP* gene, located on the long arm of chromosome 16, is in the vicinity of *CETP* and *LCAT*, two key genes involved in cholesterol metabolism. Furthermore, a recent quantitative trait locus (QTL) study links *AGRP* expression to HDL cholesterol levels in humans [23]. These findings suggest that there may be a functional interaction between the central melanocortin system involving AgRP and cholesterol metabolism. However, whether altering cholesterol homeostasis impacts relative fat vs. carbohydrate intake, including any interaction with the central melanocortin system, has not been explored.

In this study, we shed light on an important connection between dietary cholesterol, systemic cholesterol homeostasis, AgRP, and macronutrient preference. We provide evidence in mice that disturbances in cholesterol homeostasis, through dietary or pharmacological manipulation, lead to changes in relative preference for carbohydrates over fats and show that this behavioral adaptation is mediated in part through hypothalamic AgRP. Together, our study suggests that food choice is influenced by an interaction among genes, diets, and even medications impacting AgRP and cholesterol metabolism.

2. RESEARCH DESIGN AND METHODS

2.1. Mice and diets

All mice were housed in the animal barrier facility at UCSF in a room with constant temperature and a 7 AM–7 PM light/dark cycle. $Agrp^{-/-}$ mice were originally generated by Dr. Gregory Barsh at Stanford University and previously studied [13]. $Agrp^{+/-}$ mice were backcrossed to the C56BL/6J background for more than 6 generations and were interbred to generate $Agrp^{+/+}$ (WT) and $Agrp^{-/-}$ (KO) mice. Whenever possible, littermates were compared to account for variabilities in genetic background and litter dynamics. The macronutrient composition of different diets are described in each experiment. Cholesterol (Sigma–Aldrich St. Louis, MO, CAS: 57-88-5) or Simvastatin (0.01% w/w; Zocor, Merck) were supplemented to specific diets as specified in relevant experiments. Animal care and all experiments were approved by the University of California at San Francisco Institutional Animal Care and Use Committee.

2.2. Dietary choice study

Mice were singly housed and given two different diets simultaneously, with macronutrient composition, cholesterol level, and concomitant statin supplementation described under each experiment. The diets were presented in 100 mL L-shaped glass feeders that had a circular opening at the top of the leading end (BioServ). The feeders were suspended with a metal hanger along the perimeter of the cage, and their relative position was alternated regularly to prevent the development of side preference. The mice were allowed to habituate to the diet pair for one week during which the relative intake of each diet was stabilized. Body weights were measured daily when fresh diets were added.

2.3. Body composition, metabolic measurements, and meal pattern analysis

Metabolic studies and body composition measurements were performed at the UCSF Mouse Metabolism Core. Briefly, body composition (lean and fat mass) was measured using Echo-MRI. Indirect calorimetry, locomotor activity, food intake, and energy expenditure were measured using a 12-chamber comprehensive lab animal monitoring system (CLAMS; Columbus Instruments, Inc.). Measurements over multiple days were recorded, and data from the first day were excluded from analysis. Meal pattern analysis was carried out using procedures modified from a prior study [24]. Briefly, meal patterns were analyzed from CLAMS feeding event files (BDTA files). A meal is defined as a minimum of 0.02 g of food consumption in a single bout with a minimum of 10 min between bouts.

2.4. Dietary manipulation during mouse postnatal development

Breeding was set up with 1 adult male and two female C57BL/6 mice, which were maintained on PicoLab 5058 diet (23.2 Kcal% protein, 21.6 Kcal% fat, and 55.2 Kcal% carbohydrate; 200 ppm cholesterol; 3.56 Kcal/g). Upon being visibly pregnant, female mice were singly housed with ample nesting materials. On postnatal day 1 (P1), pups were culled to a litter size of 6 pups, and litters were randomly divided into 2 groups: one group was given a cholesterol-free, plant-based chow diet (Teklad 2018, Envigo), while the other group given the same chow diet supplemented with 0.2% cholesterol (TD.07798, Envigo). The use of 0.2% cholesterol mimics the cholesterol content in typical western rodent diets (TD.88137, Envigo). Upon weaning, pups in each group were further randomly divided into two sub-groups and maintained on the same chow diet, with or without cholesterol supplement, until adulthood.

2.5. Gene expression analysis

RNA isolation from mouse hypothalamic tissues was performed using the RNeasy plus mini kit (Qiagen). qPCR was performed using Taqman gene expression assay probes: Hmgcr, Mm01282499_m1; Agrp, Mm00475829_g1; Npy, Mm00445771_m1; Pomc, Mn00435874_m1.

2.6. Metabolic and dietary evaluation of human subjects

This study involved analysis of samples and data obtained from de-identified obese individuals who are members of the UCSF Inflammation, Diabetes, Ethnicity and Obesity (IDEO) cohort, which recruits participants from a variety of clinics at the UCSF Medical Center and Zuckerberg San Francisco General Hospital. The IDEO cohort, and its development and growth over time, is approved by the UCSF Committee on Human Research (IRB approval 14-14248). Detailed information about this cohort is provided in the Supplementary Methods.

2.7. Measurement of plasma AGRP levels in humans

Fasting plasma AGRP concentrations were measured in duplicate using the human AGRP Quantikine ELISA Kit (R&D Systems, Minneapolis, MN), per the manufacturer's instructions.

2.8. Statistical analysis

Human data are expressed as mean ± SD, while mouse data are expressed as mean ± SEM. Statistical methods for specific experiments are described in the relevant figure legends. In general, a Student's t-test was used to assess differences between two independent groups. Repeated-measures two-way ANOVA was used for data acquired by repetitively testing animals over time. A non-parametric paired t-test was used when comparing the same mice on diets with or without cholesterol. For human data, the Pearson correlation was used to compute r and p values. Differences were regarded as statistically significant if $p < 0.05$. All analyses were two-tailed and were performed using Prism software (GraphPad Software, Inc, La Jolla, CA).

3. RESULTS

3.1. Plasma AGRP levels are inversely correlated with intake of dietary carbohydrates in a cohort of obese human subjects

AgRP is detectable in the plasma of rats and humans [25–27], rising with caloric restriction and falling after meals in a manner mirroring what is seen for hypothalamic *Agrp* mRNA levels [26,27]. These studies suggest that plasma AGRP levels can be used as a surrogate marker for hypothalamic AGRP expression in humans [26,27]. We analyzed plasma AGRP levels in obese women [body fat ≥30% by DEXA [49]] from an established multi-ethnic cohort of human subjects living freely in the San Francisco Bay Area, termed "IDEO" (Inflammation, Diabetes, Ethnicity, and Obesity). In doing so, we found that fasted plasma AGRP levels did not correlate with BMI, body fat content, blood glucose, HbA1c, plasma insulin, HOMA-IR, plasma cholesterol, or triglycerides (Figure 1A–J). However, plasma AGRP levels in these subjects did correlate with a specific pattern of macronutrient intake as assessed by the Automated Self-Administered 24-hour (ASA24) Dietary Assessment Tool [50,51]; people with relatively low AGRP levels reported consuming more carbohydrates, whereas those with relatively high AGRP levels reported consuming fewer carbohydrates by comparison ($r = -0.5015$, $p = 0.001$, Figure 1M). Normalizing daily carbohydrate intake with total caloric intake showed a similar trend (Figure 1L). Thus, consistent with findings in humans with MC4R mutations [17], our results raise the intriguing possibility that AGRP may influence relative carbohydrate intake in humans and that plasma AGRP levels could be a biomarker of this relationship.

3.2. AgRP deficiency leads to increased carbohydrate intake at the expense of fat in mice, a process modulated by dietary cholesterol

We next examined whether *Agrp*$^{-/-}$ mice exhibited a similar preference for dietary carbohydrate intake relative to fat. Briefly, weight-matched adult *Agrp*$^{-/-}$ and control mice were given simultaneous access to two diets during refeeding, one higher in carbohydrates (HCD, 52.8 Kcal% carbohydrate, 30.6 Kcal% fat, 16.6 Kcal% protein) and the other higher in fat (HFD, 62.6 Kcal% fat, 28.4 Kcal% carbohydrate, 8.9 Kcal% protein). The relative location of the two feeding tubes within the cage was alternated to prevent the development of any specific food preference based on its location. To simultaneously evaluate whether dietary cholesterol may influence carbohydrate vs. fat intake, both the HCD and HFD diets were prepared with cholesterol (⊕C) and without cholesterol (∅C), with the other dietary components being identical.

In one cohort (Cohort 1), the mice were given access to cholesterol-free HCD∅C & HFD∅C diets, followed by a resting period, and then cholesterol-containing HCD⊕C & HFD⊕C diets. To minimize any confounding effects due to the order in which the mice were presented with their diets, another cohort of mice (Cohort 2) was analyzed in the same way except that the sequence of exposure to cholesterol supplementation was reversed (Figure 2A). The data from both cohorts were then combined for analyses. Wild-type control and *Agrp*$^{-/-}$ mice consumed similar amount of calories whether fed the HCD⊕C or the HFD⊕C (Figure 2B). However, when the comparison was made in the absence of dietary cholesterol (i.e., using the HCD∅C and HFD∅C), *Agrp*$^{-/-}$ mice consumed significantly more of the carbohydrate-rich than the fat-rich diet (Figure 2C). These results indicate that dietary cholesterol and an intact AgRP function each contributes to the selective consumption of fat over carbohydrate; when dietary cholesterol is lacking and when given a choice of both HCD and HFD, AgRP-deficiency leads to increased HCD intake at the expense of HFD.

3.3. Hypothalamic *Agrp* expression is sensitive to dietary cholesterol content

Seeing the metabolic phenotypes manifested by *Agrp*$^{-/-}$ mice when dietary cholesterol is absent, we wondered whether cholesterol availability influenced AgRP expression. To investigate, we divided eight-week-old weight-matched male wild-type C57BL/6J mice into 2 groups and provided each with a diet that differed only in cholesterol content (0% or 1%, with 1% cholesterol content being like that in egg yolk). Body weights and food intake of the mice were similar between groups during the two weeks on either diet (Figure 2D–E), after which the mice were dissected under *ad-lib* feeding conditions before the onset of the dark cycle. As expected, hepatic *Hmgcr* mRNA levels were markedly suppressed by 1% dietary cholesterol (Figure 2F). Interestingly, hypothalamic *Agrp* mRNA levels, but not those of *Npy*, were also significantly suppressed by 1% dietary cholesterol (Figure 2G–H). These results indicate that hypothalamic *Agrp* expression is responsive to signals that reflect dietary cholesterol availability.

3.4. Inhibiting *de novo* cholesterol synthesis stimulates carbohydrate intake at the expense of fat intake, and this regulation is disrupted in AgRP-deficient male mice

Cholesterol is essential for cellular function, and its availability in mammals arises both from dietary consumption and via *de novo* synthesis, mostly in the liver. When cholesterol is absent from the diet, as in plant-based diets, cholesterol is produced entirely by the liver to supply bodily needs. To this end, we sought to determine if inhibiting cholesterol synthesis using statins, a class of cholesterol-lowering drugs that inhibit the activity of HMGCR, may affect carbohydrate and fat intake. To evaluate if statins might alter the palatability of the diet, we first conducted a preference test in which we provided mice with two identical diets, one containing 0.01% simvastatin (Zocor, Merck) and the other without. This dose of simvastatin is several-fold

A

Characteristics of the human obese subjects (obesity is defined as %fat ≥ 30% by DEXA)
Gender: Female (n=40); Ethnicity: Caucasian n=13, Chinese n=13, Hispanic n=14. Values are mean ± standard deviation.

Age (year)	BMI (kg/m^2)	Weight (kg)	Fat mass (%)	Lean mass (%)	Insulin (mU/L)	Glucose (mg/dL)	Homa-IR	Triglycerides (mg/dL)	Total-C (mg/dL)	LDL-C (mg/dL)	HDL-C (mg/dL)
45.0±12.3;	30.6±10.8;	78.3±26.8;	40.7±5.3;	59.3±5.3;	20.8±22.5;	105.3±43.2;	6.0±7.4;	102.5±57.5;	201.5±41.0;	120.0±39.0;	56.3±12.8

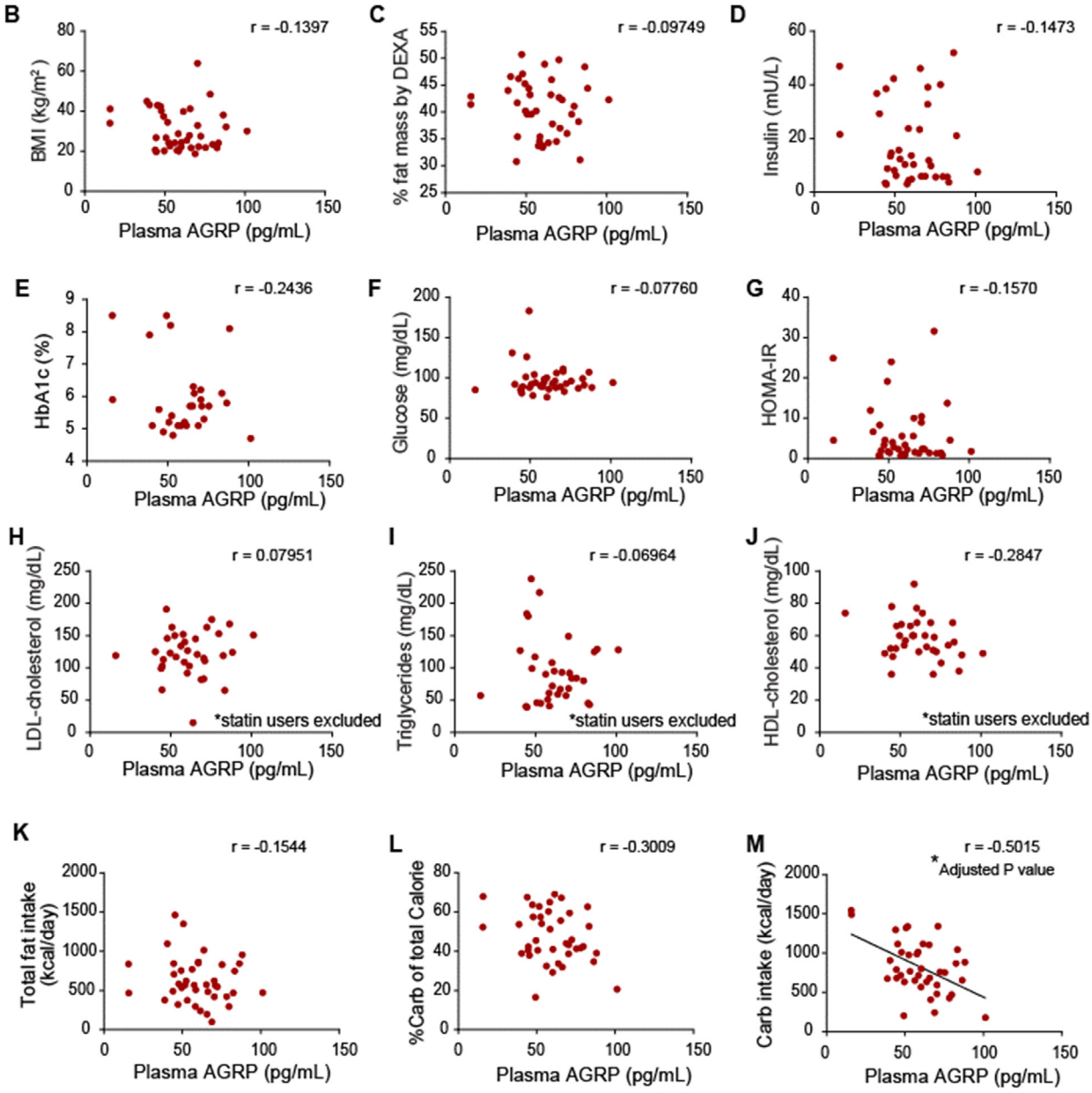

Figure 1: AGRP levels in obese humans correlated inversely with intake of dietary carbohydrates. (**A**) Characteristics of obese human subjects (n = 40 females). Obesity was defined as body fat ≥30% by DEXA. Values are expressed as mean ± SD. (**B**–**J**) Scatter plots show individual AGRP plasma concentration did not correlate with various available metabolic parameters or serum lipid levels in subjects. (**K**–**M**) Relationships between plasma AGRP levels and carbohydrate consumption. Correlation coefficient r and *p* values were computed using Pearson Correlation function in GraphPad Prism. All analyses were two-tailed. Statistical significance cutoff of the *p* value is adjusted by applying Bonferroni correction. **p* indicates that the correlation is statistically significant after Bonferroni correction.

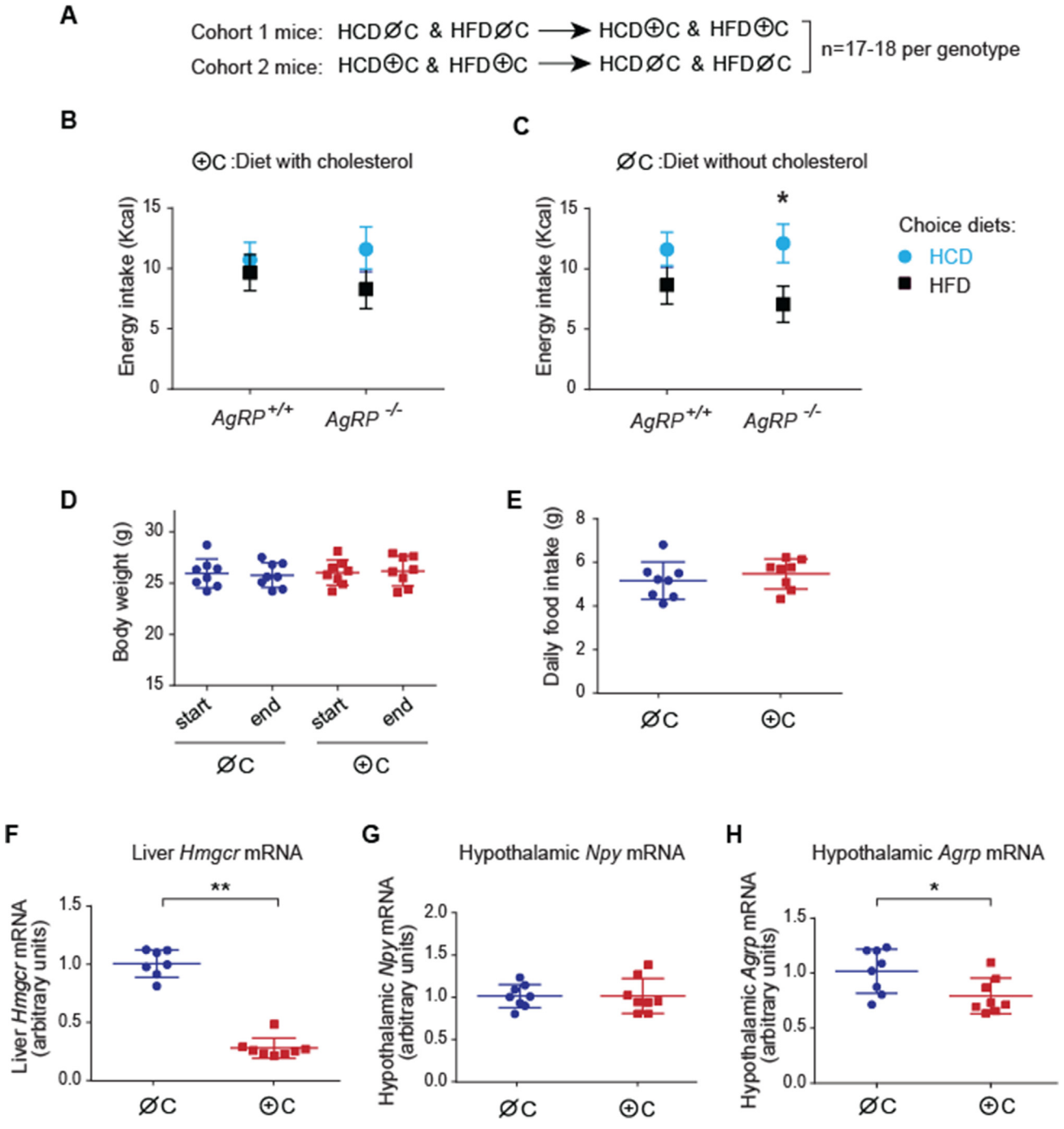

Figure 2: AgRP and dietary cholesterol promote preferential overconsumption of dietary fats during refeeding. (**A**–**C**) Individually housed, male *Agrp*$^{+/+}$ and *Agrp*$^{-/-}$ mice were given *ad-lib* access to two diets. The diets were made by modifying the amount of olive oil mixed with TD88122 basal mix (Teklad). HCD: 52.8 Kcal% carbohydrate, 30.6 Kcal % fat, 16.6 Kcal% protein. HFD: 28.4 Kcal% carbohydrate, 62.6 Kcal% fat, 8.9 Kcal% protein. Diet pairs were either supplemented with 280 ppm (0.028%) cholesterol or without. After acclimatization on the HCD and HFD, the mice were fasted for 24 h and then re-fed on the same diet pairs for 24 h. 24 h food intake during the re-feeding period was compared between *Agrp*$^{+/+}$ mice and *Agrp*$^{-/-}$ mice (n = 17–18 per genotype) on indicated choice diets with or without dietary cholesterol. $^{*}p < 0.05$ comparing HCD and HFD in the choice diets. Data are mean ± SEM. (**D**–**H**) Eight-week-old male C57BL/6J mice were weight-matched and divided into two groups (n = 8/group). Group 1 was fed with a cholesterol-free HFD (43.5 Kcal% fat, 40 Kcal% carbohydrate, and 16.6 Kcal% protein), and group 2 was fed with the identical diet supplemented with 1% cholesterol. Two weeks later, mice were sacrificed under *ad-lib* fed condition. (**D**–**E**) There were no differences in body weight and food intake between the groups. (**F**) Hepatic *Hmgcr* mRNA was reduced by 1% dietary cholesterol. (**G**–**H**) Hypothalamic *Agrp* mRNA, but not *Npy* mRNA, was suppressed by 1% dietary cholesterol. Data are mean ± SD. ∅C: diet without cholesterol. ⊕C: diet with cholesterol supplementation. $^{*}p < 0.05$; $^{**}p < 0.01$ by two-tailed student's t-test.

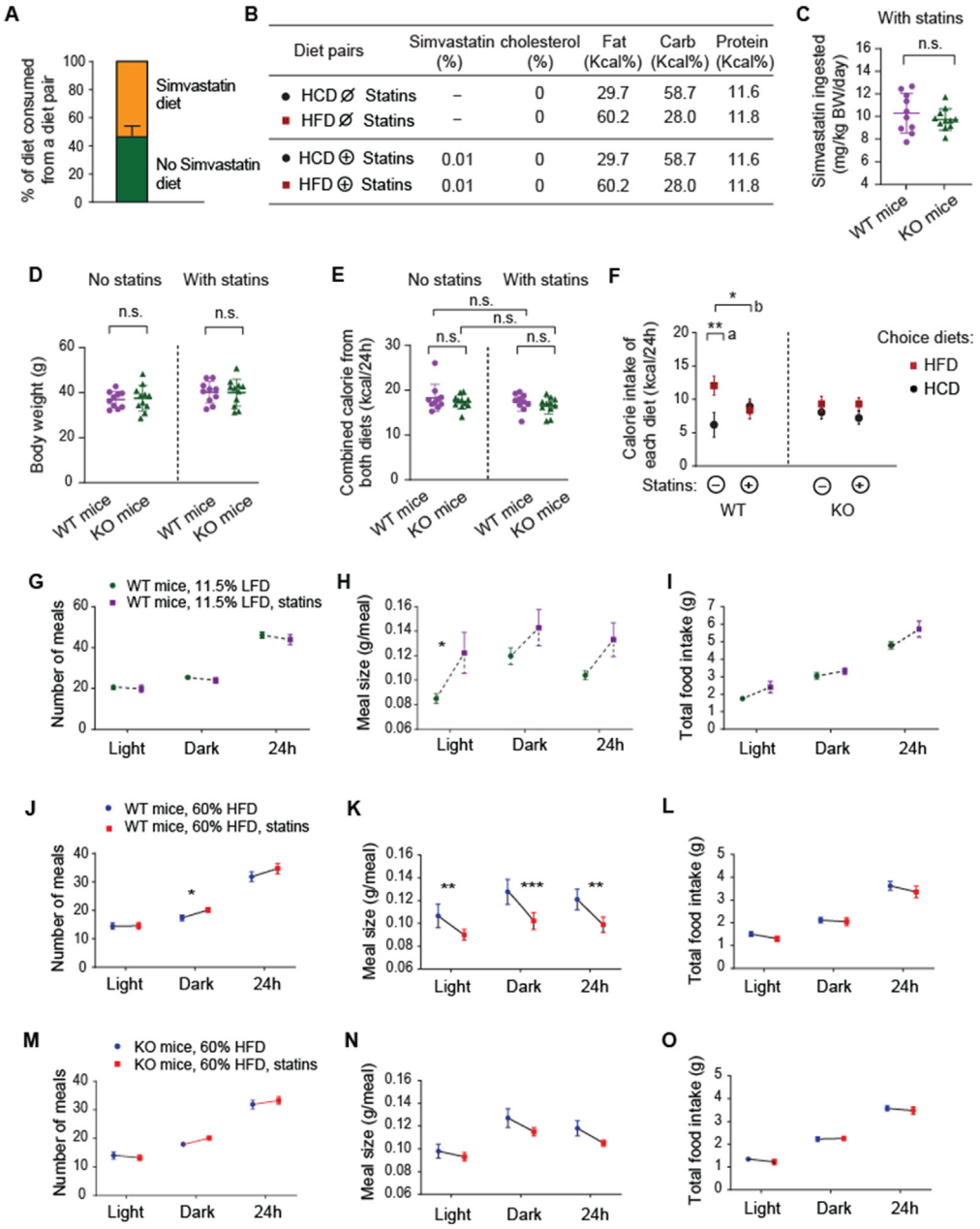

Figure 3: Statin treatment led to increased consumption of carbohydrates at the expense of fat in wild-type mice but not in AgRP-deficient mice. (**A**) C57BL/6J mice (n = 10) were simultaneously provided with two identical diets, one supplemented with statins (Simvastatin 0.01% w/w) and one without. Mice did not show a preference for either diet. (**B**) Diet pairs were made so that the diet was either high in carbohydrates (HCD) or fat (HFD), while maintaining similar protein content as shown. HCD: 58.7 Kcal% carbohydrate, 29.7 Kcal% fat, 11.6 Kcal% protein. HFD: 28.0 Kcal% carbohydrate, 60.2 Kcal% fat, 11.8 Kcal% protein. These diets were custom-made by adding a calculated amount of fat (olive oil) or carbohydrate (sucrose) to a cholesterol-free, plant-based diet (Teklad 2018). Both HCD and HFD diets were either supplemented with 0.01% Simvastatin or not. (**C**–**F**) Singly housed 10-to-16-month-old *Agrp*$^{-/-}$ (KO, n = 11) and *Agrp*$^{+/+}$ (WT, n = 10) male mice were simultaneously offered HCD and HFD diets in their home cages. WT and KO mice did not show differences in amount of statins ingested (**C**), combined caloric intake from HCD and HFD with or without statins (**D**), or body weights on diets with or without statins (**E**). At baseline, *Agrp*$^{+/+}$ mice consumed significantly more HFD than HCD, but KO mice did not show significantly different intake between the two diets. Two weeks after supplementing the same HCD and HFD with Simvastatin (0.01% w/w), WT mice reduced HFD intake and increased HCD intake. In contrast, statin treatment had no effect on KO mice (**F**). (**G**–**I**) Eight-week-old C57BL/6J male mice were randomly divided into two weight-matched groups; one was given a LFD (D10001, Research Diets, Inc., 67.7 Kcal% carbohydrate, 11.5 Kcal% fat, 20.8 Kcal% protein), and the other group was given the same diet supplemented with 0.01% Simvastatin (D19022509, Research Diet, Inc). Meal number, meal size, and total food intake were analyzed six weeks after being on the respective diets. A meal was defined as a minimum of 0.02 g in meal size with a minimum inter-meal interval of 10 min. Dotted lines signify that two distinct groups of mice were used. (**J**–**O**) Four-to-six-week-old male *Agrp*$^{-/-}$ (KO) and their *Agrp*$^{+/+}$ (WT) littermates were provided *ad libitum* access to a HFD (28.0 Kcal% carbohydrate, 60.2 Kcal% fat, 11.8 Kcal% protein) for 3 consecutive days, followed by the same HFD containing 0.01% Simvastatin for 3 days. Data from the first day when the animals were acclimatizing to the diet were excluded. In WT mice, simvastatin resulted in reduced meal size and increased number of meals in the dark cycle, without altering total food intake (**J**–**L**). In contrast, simvastatin treatment had no effects on KO mice (**M**–**O**). Solid lines signify the same group of mice was used. Data are presented as mean ± SEM and analyzed by repeated measures ANOVA with Sidak's multiple comparisons test or by unpaired t-test. n.s. not significant, $*p < 0.05$; $**p < 0.01$; $***p < 0.001$.

lower than in a prior study using atorvastatin in C57BL/6 mice [28] and similar to a dose of simvastatin that is well tolerated [29]. The mice similarly consumed the simvastatin-free or simvastatin-containing diet (Figure 3A), indicating that including simvastatin in the diet did not alter its palatability.

To determine the effects of statin treatment on relative consumption of dietary carbohydrate vs. fat, weight-matched 10-to-16-month-old wild-type and *Agrp*$^{-/-}$ mice were given equal access to a pair of HCD (58.7 Kcal% carbohydrate, 29.7 Kcal% fat, 11.6 Kcal% protein, 0% cholesterol) and HFD (60.2 Kcal% fat, 28.0 Kcal% carbohydrate, 11.8 Kcal% protein, 0% cholesterol), with or without supplementing with simvastatin (0.01%, Zocor, Merck) (Figure 3B). No differences in simvastatin intake, combined caloric intake from HCD and HFD, or body weights were observed (Figure 3C—E). However, in the absence of statin treatment, wild-type male mice consumed significantly more HFD than HCD, whereas male *Agrp*$^{-/-}$ mice did not (Figure 3F). When the diets were supplemented with simvastatin, the wild-type mice did rapidly lower their HFD ⊕ Statin intake while simultaneously increasing their HCD ⊕ Statin intake; by contrast, *Agrp*$^{-/-}$ mice did not alter their relative macronutrient preferences in response to statin treatment (Figure 3F). Together, these results suggest that blocking *de novo* cholesterol biosynthesis both potently suppresses HFD preference while accordingly increasing HCD preference in a choice paradigm; moreover, the mechanism by which statin treatment impacts relative fat vs. carbohydrate intake depends on intact AgRP function, as mice lacking AgRP were resistant to the effects of simvastatin. A sexually dimorphic response was observed, as statin treatment did not affect dietary preference in female mice (Supplementary Figure 1).

3.5. Statin supplementation in the setting of a HFD leads to reduced meal size in wild-type mice but not in AgRP-deficient mice

Given the effect of statin treatment on relative carbohydrate vs. fat preference, we sought to determine if statins differentially impact *ad lib* consumptive patterns in mice fed a HCD vs. a HFD. Wild-type C57BL/6 male mice were fed a HCD that was low in fat (67.7 Kcal% carbohydrate, 11.5 Kcal% fat, 20.8 Kcal% protein) with or without simvastatin supplementation. After 6 weeks on these diets, no significant difference in meal numbers or total food intake was observed between statin-treated and control mice, although meal size was elevated in the light cycle but not in the dark cycle in statin-treated mice (Figure 3G—I).

We then evaluated if statin treatment may affect meal pattern when mice are fed a HFD (60.2 Kcal% fat, 28.0 Kcal% carbohydrate, 11.8 Kcal% protein). To minimize any potential confounding effects of chronic HFD-induced obesity on meal pattern, we evaluated the effects of statin treatment after only a short-term exposure to the HFD. After collecting feeding data on the diet without simvastatin, the mice were switched to the same HFD, supplemented with 0.01% simvastatin. Notably, wild-type mice increased their meal frequency but reduced individual meal size while maintaining a consistent overall caloric intake (Figure 3J—L). By contrast, when *Agrp*$^{-/-}$ mice were fed the same HFD, the presence or absence of simvastatin had no impact on either meal frequency or size (Figure 3M—O). Together, these findings indicate that limiting *de novo* cholesterol biosynthesis is sufficient to reduce meal size when mice are fed a fat-rich diet and that the mechanism by which this occurs requires AgRP.

3.6. Mice with early exposure to cholesterol-free diets prefer fats over carbohydrates as adults

The first two years of life in humans are a critical window of time during which an infant's food preferences develop. Breastfed children have a head start in developing preferences for a wider variety of healthy foods compared with formula-fed children [30]. Notably, natural breast milk contains substantial amounts of cholesterol, whereas baby formula contains less. Importantly, dietary preference is subjected to developmental compensation as human babies who were born during the Dutch famine grew up preferring fatty foods [2]. Thus, we evaluated if early exposure to a diet lacking cholesterol may affect macronutrient preferences later in life.

To this end, pregnant C57BL/6J female dams were maintained on a standard breeder chow diet, (LabDiet 5058) and upon giving birth their litters were culled to a fixed size of 6. The litters were randomly divided into two groups, one in which the lactating dams were fed a cholesterol-free plant-based chow diet (Teklad 2018, Envigo) and another in which the dams were continued on the same plant-based chow diet, except supplemented with 0.2% cholesterol (TD.07798, Envigo), levels resembling the content in a typical western diet. Upon weaning, pups in each group were further randomly divided into two sub-groups, maintained on the same chow diet—with or without cholesterol supplementation—until adulthood (Figure 4A).

At weaning (3 weeks of age), there were no differences in lean or fat mass in male pups raised by dams on cholesterol-free or cholesterol-rich diets, although female pups did display a small reduction in body weight and lean mass when raised by dams on the cholesterol-free diet (Figure 4B—C). At 8 weeks of age, however, no differences in lean or fat mass were observed in any of the four groups, male or female, regardless of the presence or absence of cholesterol in the diets (Figure 4D—E). Together, these results suggest that the absence of cholesterol during lactation does not pose any lasting impact on body composition when pups reach adulthood.

Once the pups reached 4—5 months of age, we examined the impact of early-life dietary cholesterol availability on their relative preference for dietary carbohydrates vs. fats by letting the mice choose between a pair of HCD and HFD with identical protein contents using our established protocol. Male progeny fed a cholesterol-free diet since birth consumed more HFD but less HCD when compared with 3 other groups that were put on cholesterol-free diets either during lactation or post-weaning (Figure 4F). Female mice showed a similar trend, although the data were more variable (Figure 4G).

To evaluate if hypothalamic neuropeptide expression in mouse pups is affected by cholesterol content in the maternal diet, we analyzed the male pups, at the time of weaning, that were raised by dams subjected to the dietary protocol above. We found that pups raised by dams fed a cholesterol-free diet had higher hypothalamic mRNA levels of *Agrp*, but not *Npy* or *Pomc*, as compared with pups raised by dams fed a cholesterol-rich diet, consistent with the fat-preferring phenotypes of these mice as adults (Supplementary Figure 2). Together, these results suggest that dietary cholesterol, especially during early development, impacts the central melanocortin pathway in association with a lasting effect on relative dietary carbohydrate vs. fat preference.

4. DISCUSSION

In this study, we show that the relative intake of fats and carbohydrates can be impacted by alteration of cholesterol metabolism and that part of this action is mediated by interaction with AgRP. We further show that alteration of cholesterol availability early in life has a long-lasting impact on fat and carbohydrate selection in adulthood. Together, these findings suggest that dietary preference is a complex interaction among genes, diets, and even medications.

Food has both nutritional and hedonic values, and post-ingestive effects can influence food preferences independent of palatability or

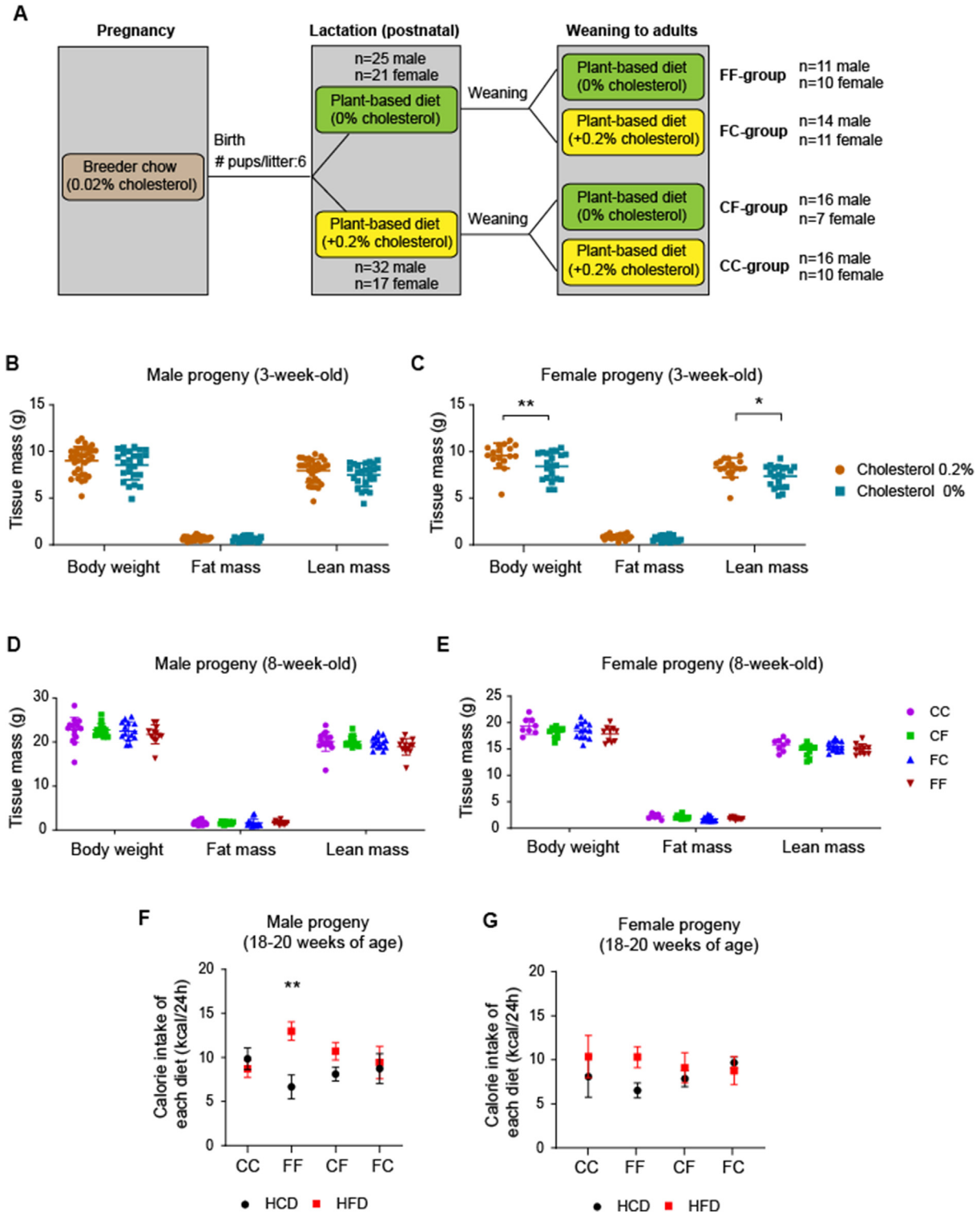

Figure 4: Dietary cholesterol intake early in life affects food choice in adult mice. (**A**) Experimental design to alter dietary cholesterol intake during postnatal and early adulthood. (**B**–**E**) Body weights, lean mass, and fat mass at 3 weeks and 8 weeks of age. (**F**–**G**) Male progeny that were exposed to a cholesterol-free diet both pre-weaning and post-weaning preferred a HFD (28.0 Kcal% carbohydrate, 60.2 Kcal% fat, 11.8 Kcal% protein) over a HCD (58.7 Kcal% carbohydrate, 29.7 Kcal% fat, 11.6 Kcal% protein), whereas other groups that were exposed to cholesterol-containing diets pre-weaning, post-weaning, or both prefer did not show the phenotype. Female mice showed a similar trend. FF group: cholesterol-free diet both pre-weaning and post-weaning; FC group: cholesterol-free diet pre-weaning and 0.2% cholesterol diet post-weaning; CF group: 0.2% cholesterol diet pre-weaning and cholesterol-free diet post-weaning; CC group: 0.2% cholesterol diet both pre-weaning and post-weaning. Data are presented as mean ± SEM and analyzed by one-way ANOVA (**B**–**E**) or two-way ANOVA with Sidak's multiple comparisons test (**F**–**G**). $*p < 0.05$; $**p < 0.01$.

taste transduction. For example, mice lacking sweet taste receptor signaling still develop strong preference for sucrose that is based on caloric content [31]. In addition, separate neural circuits are known to encode the hedonic and nutritional values of sugar [32]. How the AgRP-MC4R neuronal pathway controls dietary preference is still not completely understood, but AgRP neuronal circuits have been shown to interact with the dopamine neuronal circuits, demonstrating the cross-talks between neuronal circuits governing homeostatic and hedonic feeding [33–37].

When mice are offered two different diets simultaneously, depletion of dietary cholesterol or treatment with statins promotes carbohydrate intake at the expense of dietary fat without affecting overall caloric intake. In addition, when fed a fat-rich diet, mice treated with statins reduce meal size while increasing meal number. As maintenance of overall energy intake is still intact in AgRP deficiency or during statin treatment, the shortening of HFD meals is likely compensated by larger meals on HCD, which may result in increased HCD intake and decreased HFD intake.

Mechanisms underlying the effects of statins on meal size have yet to be determined. Cholesterol is the precursor for bile acid biosynthesis. Notably, recent studies have shown that postprandial bile acids can reach the brain and exert anorectic effects [38,39]. AgRP neurons express TGR5, a G-protein coupled receptor for bile acids, and deletion of TGR5 from AgRP neurons leads to increased food intake [38]. Thus, disruption of cholesterol metabolism by statins could alter bile acid production and release and subsequently TGR5 agonism in AgRP neurons. In addition, bile acids and bile acid-stimulated FGF15/19 have been shown to suppress *AgRP* expression, highlighting a liver-gut-brain crosstalk via cholesterol-bile acid-AgRP interactions [40–43]. Future experiments are required to determine if secretion of bile acids is modulated by cholesterol in a macronutrient-dependent manner. Other potential mechanisms such as epigenetic modification of the *Agrp* gene will also need to be evaluated.

In this study, we show that plasma AGRP levels in obese human subjects are negatively correlated with consumption of carbohydrates, raising the possibility that dietary preference in people can be influenced by the cholesterol-AgRP pathway. In a preliminary study, we examined whether the use of statins may affect plasma AGRP levels in a cohort of human subjects. In 79 individuals in the Cholesterol and Pharmacogenetics (CAP) clinical trial treated with 40 mg/d simvastatin for 6 weeks [44], there was a small but significant reduction of AGRP levels (paired t-test: 66.5 vs. 63.5 pg/mL, $p = 0.004$). Body weights were not significantly altered by statin treatment. We also explored whether acute treatment with statins may affect AgRP mRNA expression in the mouse hypothalamus. However, after two days of simvastatin treatment, body weight of the male mice decreased, which confounded interpretation as weight loss in-and-of itself will likely lead to changes of hormonal profiles such as leptin levels, which could secondarily affect AgRP levels. More experiments are needed to evaluate the impact of statins on AgRP expression.

A previous study showed that plasma AGRP is increased in obese men [45]. In this study, we only analyzed obese subjects. Thus, AGRP levels, which could be altered in subjects with marked difference of body adiposity (e.g., lean vs. obese or fed vs. food-deprived), might not show such changes in a group of subjects who are all obese. Despite this, our study found that low plasma AGRP levels are associated with higher carbohydrate intake relative to fat. This finding is consistent with findings that human subjects with MC4R deficiency show altered dietary preference when compared with equally obese control subjects [17].

In addition, our study shows that male mice, but not female mice, were responsive to the effects of statins on dietary preference under current experimental conditions. Due to the limited number of male subjects in our cohort, the relationship between plasma AGRP levels and dietary carbohydrate intake requires further study. Sex-specific differences in cholesterol homeostasis and bile acid synthesis are recognized, and female mice are known to have a larger bile acid pool size than male mice [46]. Statin treatment is less effective in preventing stroke and all-cause mortality in women compared with men [47]. The mechanisms underlying such sexual dimorphic responses remain to be determined.

The first 2 years of life are a critical time for the development of food preferences, and breastfeeding during this period is associated with healthier dietary patterns compared with formula feeding [30]. This study reveals that limiting dietary cholesterol in the postnatal period, which likely leads to chronic elevation of endogenous cholesterol biosynthesis, results in increased consumption of fats over carbohydrates in these mice during adulthood. This observation suggests that alteration of cholesterol metabolism early in life has induced developmental adaptation in dietary preference, a concept consistent with the increased fat preference in mice that are food-restricted [1] or in human infants who were born during famine [2]. As cholesterol metabolism is under homeostatic regulation, limitation of cholesterol intake in pups is expected to lead to chronic elevation of cholesterol biosynthesis and other adaptive changes which may involve the CNS feeding circuits. Breastfeeding is associated with lower rates of obesity and type-2 diabetes in adulthood [48]. This finding raises the possibility that reducing cholesterol intake via the use of baby formula may influence food choice later in life.

AUTHOR CONTRIBUTIONS

N.G.N., M.T.M., S.K.K., and A.W.X. designed the experiments. N.G.N., L.W., M.T.M., D.L., R.C., D.A., A.H., D.A.N., T.H., E.V., G.S.B., M.W.M., and R.M.K. performed the experiments, analyzed data, or generated key experimental reagents. N.G.N., M.T.M., S.K.K., and A.W.X. wrote the manuscript.

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CONFLICT OF INTEREST

None declared.

APPENDIX A. SUPPLEMENTARY DATA

Supplementary methods

IDEO cohort participants. This study involved a sample of 40 obese individuals from the UCSF Inflammation, Diabetes, Ethnicity and Obesity (IDEO) cohort, which includes human subjects recruited from clinics at the University of California San Francisco (UCSF) Medical Center and Zuckerberg San Francisco General Hospital. A subset of patients from this cohort was recruited from the Bariatric Surgery Center at UCSF and met clinical criteria for bariatric surgery. All subjects signed consent forms, approved by the UCSF Institutional Review Board, to participate in the IDEO cohort and other subsequent studies such as this one. Individuals were excluded if they were active

smokers, were not weight-stable for at least 3 months prior to enrollment (change >3%), had any inflammatory or infectious disease, had a history of cancer or heart, liver, or renal failure, or consumed >20 g per day of alcohol. We also excluded subjects who were on any medications likely to affect inflammation, including glucocorticoids and PPARg agonists.

Human subject selection criteria for this study. Of all the subjects participating in the IDEO cohort at the onset of this project, we chose only obese subjects, as our goal was to evaluate the potential relationship between plasma AGRP and metabolic dysregulation, e.g., hyperglycemia in obesity. As cohort participants were mostly women, female obese subjects were chosen for this study. Since BMI can often be a poor measure of body adiposity, obesity in this study was defined as a body fat content of ≥30% by DEXA [49]. Three subjects were also excluded for the following reasons: one had a plasma triglyceride level of 821 mg/dL and consequently incalculable LDL levels. Another subject had a plasma AGRP concentration of 296.3 pg/mL. These two individuals were also identified as statistical outliers (GraphPad Outlier Detection). The third subject had a high coefficient of variation (CV) in the duplicate measurements of plasma AGRP.

Anthropometric and body composition measurements in human subjects. Body mass index (kg/m^2) was calculated from standardized measurements of height and weight taken from each subject. Body composition was estimated by dual-energy X-ray absorptiometry (DEXA) using a Hologic Horizon/A scanner (additional details in Supplementary Information) (3-minute whole body scan, <0.1 G mGy). Individuals up to 450 lbs can be accurately measured by this device, and high-performance and "offset" scanning techniques were used to ensure complete coverage for those whose bodies were wider than the table width. Subsequent analyses used Hologic 12.4 software, following International Society for Clinical Densitometry guidelines, with precision error (1 SD) for total body fat and percent body fat of approximately 0.3 kg and 1%, respectively (calibration to correct for drifts using device-specific whole-body phantoms). Downstream analysis of these data can accurately estimate subcutaneous, visceral, gynoid, and android fat masses and percentages.

Clinical, biological, and plasma AGRP measurements in human subjects. After a 10-hour overnight fast, blood samples were drawn the UCSF Clinical Laboratory, processed for serum and plasma, and stored at −80 °C. Plasma glucose, insulin, total cholesterol (TC), high-density lipoprotein cholesterol (HDL-C), low-density lipoprotein cholesterol (LDL-C), and triglycerides (TAG) were later measured using standard, clinically approved instruments. Plasma AGRP concentrations were measured in duplicate using the human AGRP Quantikine ELISA Kit (R&D Systems, Minneapolis, MN) per the manufacturer's instructions.

Dietary data from human subjects. All IDEO cohort participants provide detailed dietary information at the time of initial enrollment using the Automated Self-Administered 24-hour (ASA24) Dietary Assessment Tool (version 2016) developed by the National Cancer Institute, Bethesda, Maryland (further details on the ASA24 presented in Supplementary Information and at https://epi.grants.cancer.gov/asa24). The ASA24 system is a publicly available web-based software tool that enables automated and self-administered 24-hour dietary recalls for clinical researchers to analyze 65 nutrients and 37 food groups [50,51]. A trained interviewer completed the 24-hour dietary recall with each cohort participant, either online or using pen/paper. If performed with pen/paper, the data were entered into the computer-based analytical system later. Of the 28 subjects selected for this study, 2 were excluded from ASA24 analysis, as they were on prescribed liquid diets. ASA24 data were acquired from individuals well before any information about their AGRP levels had been obtained.

Human subjects with statin treatment. AGRP levels were measured in plasma samples obtained after overnight fast before and after six weeks treatment with simvastatin 40 mg/d from a subset of 79 healthy non-smoking participants (36 men, 43 women, 60 Caucasian, 19 African-American, mean age 56.4 yr, mean BMI 28.8 kg/m^2) in the Cholesterol and Pharmacogenetics (CAP) Study [44]. Mean pre- and post-treatment LDL cholesterol concentrations were 130.1 and 73.2 mg/dL, respectively. There was no significant correlation between changes in AGRP and LDL cholesterol levels.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.molmet.2022.101442.

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