Melanocortin-4 receptor mutations paradoxically reduce preference for palatable foods

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Haploinsufficiency of the melanocortin-4 receptor (MC4R) results in melanocortin obesity syndrome, the most common monogenic cause of severe early onset obesity in humans. The syndrome, which produces measurable hyperphagia, has focused attention on the role of MC4R in feeding behavior and macronutrient intake. Studies show that inhibition of MC4R signaling can acutely increase the consumption of high-fat foods. The current study examines the chronic feeding preferences of mice with deletion of one or both alleles of the MC4R to model the human syndrome. Using two-choice diet paradigms with high-fat or high-carbohydrate foods alongside normal chow, we show, paradoxically, that deletion of one allele has no effect, whereas deletion of both alleles of the MC4R actually decreases preference for palatable high-fat and high-sucrose foods, compared with wild-type mice. Nonetheless, we observed hyperphagic behavior from increased consumption of the low-fat standard chow when either heterozygous or homozygous mutant animals were presented with dietary variety. Thus, decreased MC4R signaling in melanocortin obesity syndrome consistently yields hyperphagia irrespective of the foods provided, but the hyperphagia appears driven by variety and/or novelty, rather than by a preference for high-fat or high-carbohydrate foodstuffs.

food preference | food intake | reward

Food preference in humans is highly complex, involving cultural, sociological, psychological, and physiological factors. Physiological inputs to food preference include both homeostatic and hedonic drives, with the latter referring to the effects of sensory and reward pathways that control the desire to consume highly palatable energy-dense foods (1). Profound hyperphagia has been demonstrated in several of the monogenic obesity syndromes (2), and it is important to determine the mechanisms that drive hyperphagia, including the relative contributions of homeostatic versus hedonic drives and their impact on food preference.

Melanocortin obesity syndrome, resulting from null or hypomorphic mutations in one allele of the melanocortin-4 receptor (MC4R), is the most common monogenic cause of severe early onset obesity in humans (3, 4). The obese phenotype is due in large part to hyperphagia, which has been documented in humans (3) and in mouse (4, 5) and rat (6) models of the syndrome. Data also suggest that central melanocortin signaling may specifically regulate the consumption of dietary fats. The *lethal yellow* (A^y/a) agouti mouse, in which ectopic expression of the agouti protein is presumed to block the central melanocortin-3 receptor (MC3R) and MC4R, shows a preference for fat consumption that is not seen in wild-type (WT) C57BL/6J mice on a three-choice macronutrient diet of carbohydrate, fat, or protein (7). Intracerebroventricular (ICV) administration of agouti-related protein (AgRP), an endogenous CNS antagonist of the MC3R and MC4R, preferentially increases acute consumption of high-fat chow in a two-choice paradigm providing high-fat and low-fat chow in Long–Evans rats (8). Similar experiments in the mouse demonstrate that the MC3R/MC4R agonist MTII acutely decreased intake of fat, but not carbohydrate or protein, in a three-choice diet model (9). The consumption of dietary fat in a two-choice model may also be increased by administration of MC3R/MC4R antagonists AgRP or SHU9119 directly into the central nucleus of the amygdala (CeA) (10). The amygdala has been demonstrated to play a role in emotion, reward, and motivation (11), and several studies link the amygdala to macronutrient preference and intake (10, 12, 13). Indeed, behavioral studies demonstrate that ICV injection of AgRP increases the appetitive response to a fat, but not to a carbohydrate, stimulus in both operant and Pavlovian conditioning paradigms (14).

Previous studies have also characterized a significant stimulation of hyperphagia, persisting for up to 2 wk, in the $MC4R^{-/-}$ and $^{+/-}$ mice, following a switch from normal rodent chow (13.5% of kilocalories from fat) to a high-fat diet (HFD) (45–60% of kilocalories from fat) (15, 16). In contrast, WT mice return to isocaloric intake within ∼4 d after the switch to HFD. The reports described above show that inhibition of melanocortin signaling stimulates an increase in consumption of dietary fat. This could be valuable information for the design of specific dietary recommendations for children with melanocortin obesity syndrome. However, the majority of these model systems do not mimic human melanocortin obesity syndrome in two regards. First, most studies have used broad agonists or antagonists that act at both MC3R and MC4R. Second, most studies involve acute treatment, whereas the syndrome results from a chronic deficit in MC4R activity. In this report, we use WT, $MC4R^{+/-}$, and $MC4R^{-/-}$ mice to characterize chronic macronutrient preference in melanocortin obesity syndrome.

Results

$MC4R^{-/-}$ Mice Underconsume Palatable Sucrose Solutions in an ad Libitum Access Paradigm. To determine if the previously described high-fat hyperphagia in the $MC4R^{-/-}$ and $^{+/-}$ mice was specifically due to fat content rather than to caloric density, we studied feeding behavioral responses to added sucrose in adult male WT mice or littermates with MC4R-null mutations ($^{+/-}$ and $^{-/-}$) on a C57BL/6J background. We replaced the cage water with a sapid 5% (wt/vol) sucrose solution and monitored feeding and drinking behavior in single-housed mice before and after the switch (Fig. 1). Because plain water was not left as an option, a 5% sucrose solution was chosen to elicit elevated drinking without causing excessive thirst (as would be seen with higher concentrations). Body weights were inversely related to the number of WT MC4R alleles, as shown previously (Fig. 1*A*). Intake of standard chow (SC) (Fig. 1*B*) and fluid (Fig. 1*C*) were also measured daily before and after water was replaced with 5% sucrose.

Food and fluid intake were further analyzed as daily averages of the period before and following the presentation of 5% sucrose. WT and $MC4R^{+/-}$ mice significantly decreased food intake after 5% sucrose was given, whereas $MC4R^{-/-}$ mice did not significantly adjust food intake (Fig. 1 *B* and *D*). All mice increased fluid intake dramatically when given 5% sucrose, although $MC4R^{-/-}$ mice consumed significantly less than WT and $MC4R^{+/-}$ littermates. Surprisingly, WT and $MC4R^{+/-}$ consumed fluid amounts nearly equal to their body weights (Fig. 1*E*). During the 5-d period when mice had SC and 5% sucrose, there was a MC4R gene dose-dependent increase in total caloric intake. However, the portion of intake coming from sucrose was smaller in the $MC4R^{-/-}$ animals compared with WT and $MC4R^{+/-}$ (Fig. 1*F*). The sucrose

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The authors declare no conflict of interest.

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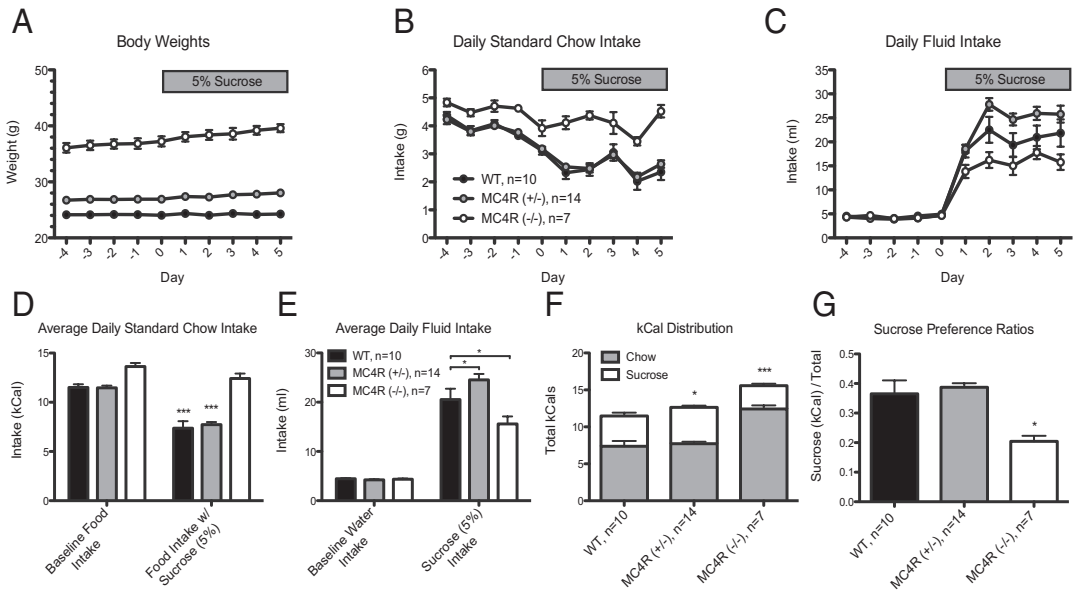

Fig. 1. MC4R$^{-/-}$ mice underconsume sucrose and have low-sucrose preference. Three-month-old male WT, MC4R$^{+/-}$, and MC4R$^{-/-}$ mice were singly housed for dietary studies. Daily measurements of (*A*) body weight, (*B*) SC intake, and (*C*) fluid intake were taken while the mice were given SC and water (days −4–0) or SC and 5% sucrose (days 1–5). Water was replaced with sucrose solution immediately after the measurement on day 0 as indicated by the gray bar on each graph. (*D*) Average daily SC intake for each genotype during days −4–0 (*Left*) and days 1–5 when 5% sucrose is given (*Right*). Statistical significance for each genotype is compared with its own baseline. (*E*) Average daily fluid intake of water for each genotype during days −4–0 (*Left*) and of 5% sucrose during days 1–5 (*Right*). (*F*) Total caloric consumption during days 1–5 including a breakdown of calories obtained from either SC or 5% sucrose solution. Statistical significance is for total kilocalorie value from both diets compared with that of WT mice. (*G*) Sucrose preference ratios, calculated as the ratio of sucrose calories consumed divided by total calories consumed. Statistical significance is compared with that of WT mice. WT: $n = 10$; MC4R$^{+/-}$: $n = 14$; MC4R$^{-/-}$: $n = 7$. Results are expressed as mean ± SEM, and statistical analyses were done by unpaired *t*-test. $^{*}P < 0.05$, $^{***}P < 0.001$.

preference ratio was also significantly decreased in MC4R$^{-/-}$ mice compared with WT and MC4R$^{+/-}$ littermates.

MC4R $^{-/-}$ and WT Mice Exhibit Equivalent Consumption of Calorie-Free Sucralose-Sweetened Water in an ad Libitum Access Paradigm. To determine if defective sweet-taste sensation was responsible for underconsumption of sucrose solution in MC4R$^{-/-}$ mice, we tested whether naive WT, MC4R$^{+/-}$, and MC4R$^{-/-}$ mice would consume a solution containing a nonnutritive sweetener, sucralose. Body weight, SC intake, and fluid intake (Fig. 2 *A*–*C*) were measured daily to assess changes in consumption after sucralose presentation. MC4R$^{-/-}$ consistently consumed the largest amount of SC before and after 2 mM sucralose was provided. For all genotypes, sucralose presentation caused no large changes in total SC intake, although a modest reduction was deemed to be significant for the MC4R$^{+/-}$ (Fig. 2 *B* and *D*). Sucralose presentation did cause significant but modest increases in fluid consumption; however, sucralose was consumed equally by all genotypes (Fig. 2 *C* and *E*), suggesting that the differential response to sucrose in the MC4R$^{-/-}$ group (Fig. 1) was dependent on the caloric value of the macronutrient.

MC4R$^{-/-}$ Mice Have Low Preference for a Palatable High-Sucrose Solid Diet Under an ad Libitum Two-Choice Paradigm. We next used a solid high-sucrose diet (HSD) in a two-choice paradigm to allow us to comprehensively study macronutrient preference while avoiding the potential limitations of fluid consumption, including polyuria

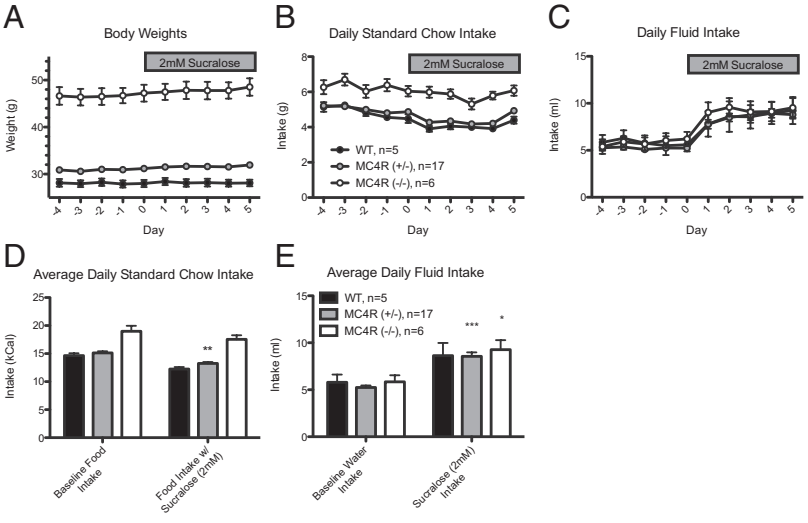

Fig. 2. MC4R$^{-/-}$ and $^{+/-}$ mice exhibit normal taste-mediated sucralose consumption. Four-month-old male WT, MC4R$^{+/-}$, and MC4R$^{-/-}$ mice were singly housed for dietary studies. (*A*) Body weight, (*B*) SC intake, and (*C*) fluid intake were measured daily before (days −4–0) and after (days 1–5) 2 mM sucralose was given following measurement on day 0 as indicated on the graphs. (*D*) Average daily SC intake for each genotype during baseline days −4–0 (*Left*) and after sucralose presentation days 1–5 (*Right*). (*E*) Average daily fluid intake of water on days −4–0 (*Left*) and 2 mm sucralose on days 1–5 (*Right*). Statistical significance for each genotype is calculated compared with baseline period in *D* and *E*. WT: $n = 5$; MC4R$^{+/-}$: $n = 17$; MC4R$^{-/-}$: $n = 6$. Results are expressed as mean ± SEM, and statistical analyses were done by unpaired *t*-test. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

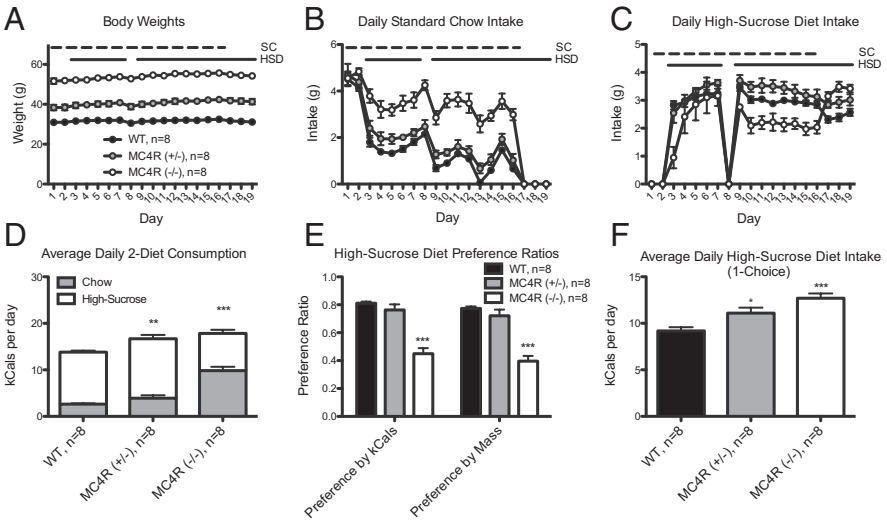

Fig. 3. MC4R$^{-/-}$ mice exhibit low preference for HSD in a two-choice diet. Seven-month-old male WT, MC4R$^{+/-}$, and MC4R$^{-/-}$ mice were singly housed for dietary studies. (*A*) Body weight, (*B*) SC intake, and (*C*) HSD intake were measured daily. Mice were given SC, HSD, or both diets simultaneously as indicated by the lines above each graph. The HSD presentation was temporarily disrupted on day 8, but resumed normally afterward. (*D*) Average caloric consumption during the second week of the two-choice diet when steady consumption behavior is reached. Caloric contributions from each diet provided are included. Statistical significance is calculated for total intake of both diets. (*E*) HSD preference ratios by kilocalories (*Left*) and by mass (*Right*) for each genotype during the second week of the two-choice diet (days 10–16). (*F*) Average daily one-choice HSD intake during the final 3-d period when SC is removed. WT: $n = 8$; MC4R$^{+/-}$: $n = 8$; MC4R$^{-/-}$: $n = 8$. Statistical significance is tested against the corresponding WT values by unpaired *t*-test. $*P < 0.05$, $**P < 0.01$, $***P < 0.001$.

and saturation of consumption levels. In addition to SC, mice were provided with HSD for 2 wk while body weight and intake of both diets were monitored daily. During the final three study days (days 17–19), SC was removed and one-choice HSD consumption was measured to contrast feeding responses to each diet with or without choice (Fig. 3). As expected, body weights were dependent on the MC4R genotype and increased steadily during two-choice diet presentation (Fig. 3*A*). The daily SC intake measurements showed that, although both diets were presented simultaneously, all genotypes continued to consume some amount of SC. However, the MC4R$^{-/-}$ mice consistently consumed more SC than their WT and MC4R$^{+/-}$ littermates (Fig. 3*B*). Following the initial presentation of HSD, it took several days for all mice to reach a steady state of consumption, which seemed to be unaffected by a brief removal of HSD on day 8. By the second week of the two-choice diet (days 10–16), all mice consumed steady daily levels of HSD. Interestingly, upon switching from a two-choice diet to a one-choice HSD paradigm, MC4R$^{-/-}$ mice went from consuming the smallest amount of the HSD to the largest amount (Fig. 3 *C* and *F*).

Further analyses of feeding behaviors on these diets were conducted by averaging daily intake values during steady-state consumption on the two-choice diet (days 10–16) and the one-choice diet of HSD (days 17–19) (Fig. 3 *D*–*F*). Total caloric intake increased as MC4R signaling was decreased. However, the portion of caloric intake coming from the HSD was notably smaller in the MC4R$^{-/-}$ group (Fig. 3*D*). Furthermore, the HSD preference ratio was significantly reduced in the MC4R$^{-/-}$ group compared with both the WT and MC4R$^{+/-}$ littermate groups. There appears to be an intermediate reduction in HSD preference in the MC4R$^{+/-}$ group, although the difference is not significant (Fig. 3*E*).

High-Sucrose Diet Consumption Does Not Cause Hyperphagia or Fasting Hyperglycemia in MC4R$^{-/-}$ Mice. The previous studies of high-fat–induced feeding behaviors included a dietary switch that induced a brief and universal novelty hyperphagia followed by a sustained hyperphagia in MC4R$^{-/-}$ and $^{+/-}$ mice. Using this paradigm, we sought to determine if these behaviors would occur following a similar switch from standard chow to HSD. Because the MC4R$^{-/-}$ and $^{+/-}$ groups consumed more HSD than their WT counterparts when the other choice was removed (Fig. 3*F*), we expected that this dietary switch might elicit hyperphagia similar to that seen previously with HFD (16). We raised WT, MC4R$^{+/-}$, and MC4R$^{-/-}$ mice on SC and switched them to HSD while measuring body weight and food intake every 24 h (Fig. 4). Although there was the expected MC4R gene dose-dependent effect on baseline body weight across groups, the weights remained steady through the study (Fig. 4*A*). In contrast with a dietary switch to HFD, the dietary switch to HSD alone had no effect on steady-state consumption in any genotype (Fig. 4*B*). When daily consumption levels are averaged between SC consumption (days –6–0) and HSD consumption (days 1–7), all genotypic groups appear to maintain isocaloric dietary behavior across diets. For both diets, the MC4R$^{-/-}$ mice consume significantly more calories than their WT littermates (Fig. 4*C*). These isocaloric dietary behaviors are very different from the dramatic hyperphagia noted during the HFD studies in MC4R$^{-/-}$ and $^{+/-}$ mice (15, 16). Because MC4R$^{-/-}$ mice are severely obese, we questioned

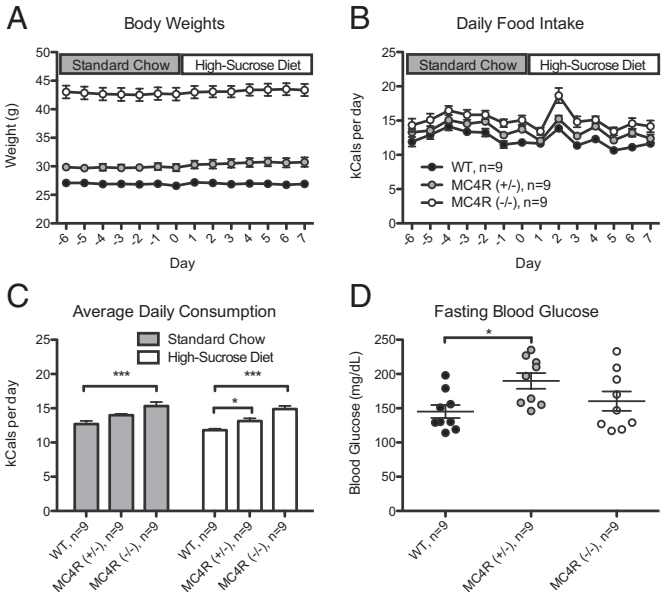

Fig. 4. Switch to a HSD without choice does not cause hyperphagia or fasting hyperglycemia in MC4R$^{-/-}$ mice. Four-month-old male WT, MC4R$^{+/-}$, and MC4R$^{-/-}$ mice were singly housed and studied under a diet-switch paradigm described previously (16). (*A*) Body weight and (*B*) food intake were measured daily as the mice were switched from a one-choice SC to a one-choice HSD, as indicated above these graphs. (*C*) Average daily consumption of each diet by genotype with SC consumption (*Left*) and HSD consumption (*Right*). (*D*) Blood glucose measurements taken from the tail vein following a 6-h daytime fast. Each blood glucose data point shown is the value for a single mouse. WT: $n = 9$; MC4R$^{+/-}$: $n = 9$; MC4R$^{-/-}$: $n = 9$. All graphs indicate mean ± SEM, and statistical significance is tested against the corresponding WT values by unpaired *t*-test. $*P < 0.05$, $***P < 0.001$.

whether the lack of preference and hyperphagic behaviors was due to potential diabetic side effects exacerbated by excessive sucrose consumption. Following the 7 d of HSD consumption, we measured blood glucose following a 6-h daytime fast and noted no significant difference in fasting blood glucose between the WT and MC4R$^{-/-}$ groups (Fig. 4*D*).

MC4R$^{-/-}$ Mice Exhibit Low Preference for Palatable High-Fat Diet Under an ad Libitum Two-Choice Paradigm. Having shown that MC4R deficiency confers reduced dietary preference for sucrose-rich food, we sought to test whether MC4R deficiency causes dietary fat preference consistent with the high-fat hyperphagia noted previously (15, 16). To study these feeding behaviors, we used groups of WT, MC4R$^{+/-}$, MC4R$^{-/-}$, and severely obese, leptin receptor deficient *db*/*db* mice (Fig. 5). All mice were raised on SC and then given a choice of SC and HFD for 2 wk. The cage position of the two diets was then switched for a period of 3 d to control for positional preferences. For the final 3 d of study, the SC was removed and only HFD was provided. The body weights increased steadily during the study and were as expected according to MC4R genotypes. The *db*/*db* mice, although close in age, were much heavier than even the MC4R$^{-/-}$ mice, consistent with expectations (Fig. 5*A*). Following the initial period of novelty associated with the HFD presentation, all groups reached a steady level of intake of both SC and HFD by the second week of the two-choice diet (days 16–22). The intake levels for both diets also remained unchanged after the position of each was switched (days 23–25), suggesting that the feeding preferences are not dependent upon location of the diets within the cage (Fig. 5 *B* and *C*).

Further analyses were done by averaging daily intake values during the steady-state consumption period of the second week of the two-choice diet (days 16–22). Total caloric intake was elevated in MC4R$^{-/-}$ and $^{+/-}$ animals as functional MC4R alleles were lost, whereas the *db*/*db* group consumed the largest total amount calories (Fig. 5*D*). Contrary to our expectations, removal of MC4R signaling caused a significant decrease in preference for HFD whereas the WT and *db*/*db* groups maintained a very strong preference for the diet. Analysis of preference by intake mass showed that MC4R$^{-/-}$ mice exhibited only a mild (~0.6) preference for HFD compared with a nearly full preference (~1.0) in WT and *db*/*db* mice (Fig. 5*E*). Although the preference for HFD was low in MC4R$^{-/-}$ mice in a two-choice diet paradigm, the one-choice intake of HFD following removal of SC was still higher in the MC4R$^{-/-}$ mice compared with WT (Fig. 5*G*), much like the baseline intake of SC or HSD in a one-choice paradigm (Figs. 3*F* and 5*F*).

Dietary Variety Drives Hyperphagia in MC4R$^{-/-}$ Mice Under Multiple Diet Regimens. Using the data gathered from our one-choice and two-choice diet studies, we were able to more closely examine the changes in total caloric intake conferred by the presentation of multiple dietary choices (Fig. 6). When mice were given 5% sucrose in place of cage water, genotype-dependent effects on caloric regulation were evident in that MC4R$^{-/-}$ mice became hypercaloric whereas WT mice remained isocaloric (Fig. 6*A*). There was a gene dose-dependent increase in caloric difference with MC4R$^{-/-}$ mice exhibiting the largest caloric change when given 5% sucrose (Fig. 6*B*). This effect is mimicked when the mice are given the two-choice diet of SC and HSD (Fig. 6*C*). The WT mice, when given both solid diets, remain isocaloric, whereas the MC4R$^{+/-}$ and $^{-/-}$ mice increase their total caloric intake (Fig. 6*D*, left side). Furthermore, removal of SC causes hypophagia on a one-choice HSD paradigm (compared with baseline), although MC4R$^{-/-}$ mice maintain a significantly smaller drop in caloric intake compared with their WT littermates (Fig. 6*D*, right side). It should be noted that the hypophagia on the one-choice HSD paradigm was seen only after removal of SC following a two-choice diet. When mice were switched from SC to HSD without choice, all genotypes remained isocaloric (Fig. 4*C*).

Upon presentation of a two-choice SC and HFD, all genotypes exhibited hyperphagia in that they maintained a caloric intake higher than SC alone under both HFD regimens (two-choice and one-choice, Fig. 6 *E* and *F*). Interestingly, the greatest level of hyperphagia was still evident in the MC4R$^{-/-}$ mice. Whereas WT and *db*/*db* mice had the smallest caloric increase when switched

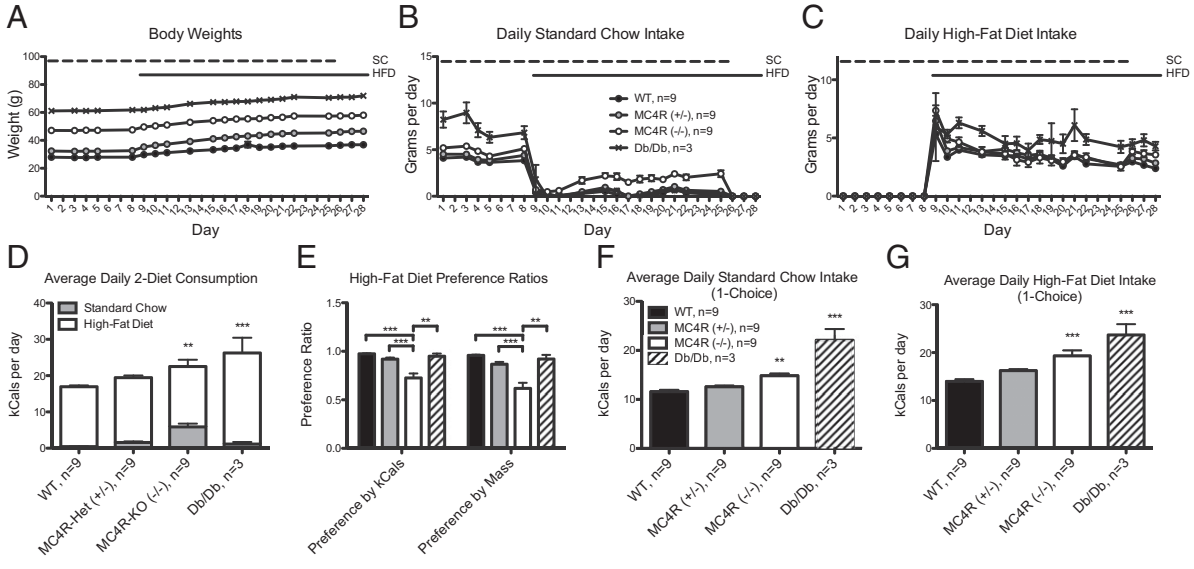

Fig. 5. MC4R$^{-/-}$ mice exhibit low preference for HFD in a two-choice diet. Five-month-old male WT, MC4R$^{+/-}$, MC4R$^{-/-}$, and Db/Db mice were singly housed for dietary studies. (*A*) Body weight, (*B*) SC intake, (*C*) and HFD intake were measured daily. All mice were given SC, HFD, or both diets simultaneously as indicated above each graph. (*D*) Average daily caloric consumption during the second week of the two-choice diet (days 16–22) for each genotype. Contributions from each diet are included. Statistics are calculated from the total two-diet caloric values. (*E*) HFD preference ratios for each genotype during the second week of the two-choice diet. Preference ratios are calculated by kilocalories (*Left*) and by mass (*Right*). (*F*) Average daily intake of SC under one-choice paradigm (days 2–8). (*G*) Average one-choice daily intake of HFD during the final 3 d after SC is removed (days 26–28). WT: $n = 9$; MC4R$^{+/-}$: $n = 9$; MC4R$^{-/-}$: $n = 9$; Db/Db: $n = 3$. Statistical significance is tested against the corresponding wild-type values, unless otherwise indicated, by unpaired *t*-test. $^{**}P < 0.01$, $^{***}P < 0.001$.

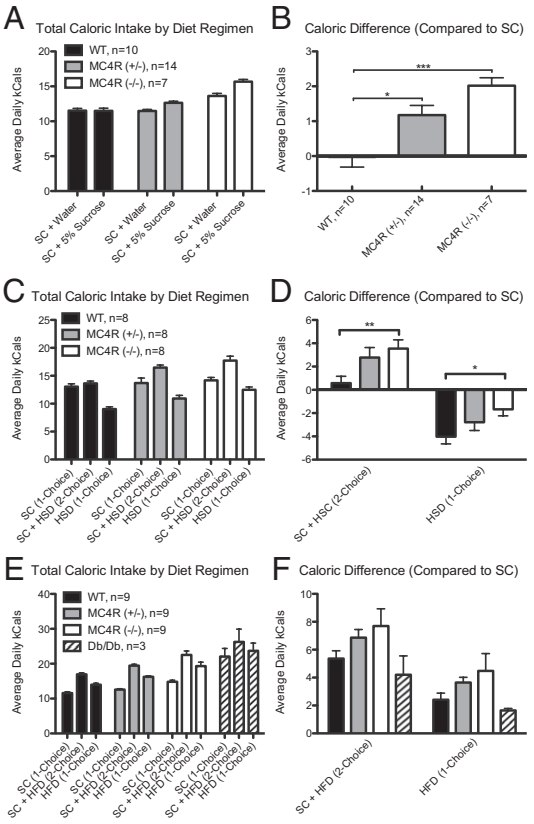

Fig. 6. Two-choice diets drive additional hyperphagia in MC4R$^{-/-}$ mice. *A*, *C*, and *E* display the total caloric intake under diet regimens using (*A*) SC + 5% sucrose liquid, (*C*) SC + HSD, and (*E*) SC + HFD. One-choice and two-choice intake totals are shown for each diet and genotype used. *B*, *D*, and *F* display the caloric differences obtained by subtracting baseline caloric intakes from the caloric intakes under two-choice diets in *A*, *C*, and *E*, respectively. (*B*) Caloric differences caused by SC + 5% sucrose diet compared with SC alone. (*D*) Caloric differences caused by two-choice SC + HSD and one-choice HSD, compared with SC alone. (*F*) Caloric differences caused by two-choice SC + HFD and one-choice HFD, compared with SC alone. Statistical significance of caloric differences (*B*, *D*, and *F*) is compared with caloric differences in corresponding WT mice using unpaired *t*-test. $*P < 0.05$, $**P < 0.01$, $***P < 0.001$.

to diet regimens containing HFD, MC4R$^{+/-}$ and MC4R$^{-/-}$ mice incrementally exhibited the highest increases (Fig. 6*F*).

Discussion

Developing a clear understanding of the mechanism driving the hyperphagia in melanocortin obesity syndrome may be important for treatment of the disease (3). Our initial studies demonstrated significant increases in hyperphagia, relative to WT mice, following exclusive presentation of high-fat chow, suggesting an increased preference for high-fat foods (15, 16). In this report, we describe a paradoxical loss in preference for energy-dense palatable foods, enriched with either fat or carbohydrates, in mice lacking both alleles of the MC4R and no increase in preference in heterozygotes. Interestingly, although long-term preference for palatable diets decreased in MC4R knockout mice, total caloric intake increased whenever MC4R$^{+/-}$ or MC4R$^{-/-}$ mice were presented with multiple types of foods. Under a chronic two-choice diet model, WT mice developed strong preferences for HSD and for HFD when either diet is measured against SC consumption. These preferences developed quickly upon presentation of the diets and persisted for the duration of study. In our observations with WT mice, we noted an ~80% preference for HSD and an ~100% preference for HFD compared with SC. MC4R$^{-/-}$ mice, when placed under the same feeding paradigms, showed a temporary preference for HSD or HFD over SC. However, after the period of dietary novelty ended and feeding behavior reached a steady state during week 2, the MC4R$^{-/-}$ displayed a distinct lack of preference for either the HSD or the HFD. This lack of preference was characterized by the continued consumption of SC, which drove the preference ratio for HSD and HFD to a level lower than that in WT mice. No increased preference for HSD or HFD over chow could be observed in the MC4R$^{+/-}$ mice, despite their hyperphagia lending additional relevance of these results to human MC4R haploinsufficiency.

The divergent results of this study compared with previous studies necessitate a careful comparison of the experimental methods. In general, many previous studies have concluded that loss of melanocortin signaling results in an elevated preference for fat and a decreased preference for carbohydrates. Ubiquitous overexpression of *agouti*, as in the A^y/a mouse, was shown to cause elevated fat consumption at the expense of carbohydrate consumption in a chronic three-choice diet through blockade of MC3R and MC4R signaling (7). Furthermore, ICV administration of AgRP, the endogenous antagonist of MC3R and MC4R, also acutely increases fat consumption in Long–Evans rats (8). Although these models addressed food preference in chronic and acute models of inhibition of central melanocortin signaling, they can be assumed to be models of dual MC3R and MC4R inhibition. ICV administration of a variety of nonspecific MC3R/MC4R agonists such as MTII has also been shown to specifically decrease fat intake (17). These studies are generally interpreted to argue that inhibition of MC4R stimulates preference for, or the reward value of, dietary fat and that this plays a role in the hyperphagia seen in MC4R-deficiency states. However, in contrast with previous pharmacological models of dual MC3R/MC4R inhibition, MC4R$^{-/-}$ mice on a mixed genetic background on a chronic three-choice diet showed no clear preference for fat, protein, or carbohydrate (9). This observation most closely aligns with our results, although the latter study may be confounded by potential stress-induced anorexia from handling. Recently, shRNA blockade of MC4R signaling in the nucleus accumbens was shown to prevent stress-mediated anhedonia, measured as a lack of sucrose preference (18). Although this result seems to conflict with our findings, we must note that our studies involved acclimatized, rather than stressed, animals. Furthermore, we focused our studies on the global deletion of one or both alleles of the MC4R to model the human melanocortin obesity syndrome. We noted significant hyperphagia in the MC4R$^{-/-}$ and MC4R$^{+/-}$ mice contemporaneous with the low preference for palatable diets, ruling out a generalized stress-induced anorexia.

Melanocortin signaling in the amygdala is suspected to be at least partially responsible for the effects on fat preference. MC4R expression is relatively high in the CeA, a brain region involved in food reward and macronutrient selection. Stereotaxic injections of the MC3R/MC4R antagonists AgRP or SHU9119 into the CeA have been shown to acutely increase fat consumption and overall food consumption in rats. Conversely, MTII injections had the opposite effect (10). These effects on dietary reward are supported by evidence that ICV AgRP injections in rats increased fat-associated motivation in behavioral tests. Although motivation for sucrose reinforcers was not affected in a progressive ratio test, the Pavlovian response to macronutrient paired stimuli switched from sucrose to fat following AgRP injection (14). However, just like agonism or antagonism of MC3R and MC4R, site-specific modulation of melanocortin receptors also may not replicate the biology of hyperphagia in humans with global MC4R haploinsufficiency.

In previous reports, a clear and sustained gene dose-dependent hyperphagic response to a high-fat diet was noted when MC4R$^{-/-}$ and $^{+/-}$ animals were switched from SC to HFD in a one-choice study (16). In the current study, we still observed this fat-induced hyperphagia during the first few days of novel fat presentation and also whenever the SC choice is removed from

the cage after the mice become accustomed to both diets. We consistently observed MC4R$^{-/-}$ mice eating the most of any single diet under one-choice studies; however, the ratio of calories from either HFD or HSD consumed by MC4R$^{-/-}$ mice is significantly reduced whenever there are two dietary choices. Although this finding is paradoxical considering the tendency for MC4R$^{-/-}$ and $^{+/-}$ to overeat, we consistently observed an exaggerated gene dose-dependent hyperphagia in MC4R$^{-/-}$ and $^{+/-}$ caused by the introduction of dietary variety (Fig. 6). Although WT mice were generally resistant to chronic hyperphagia under two-choice diet regimens, the MC4R$^{-/-}$ mice consistently exhibited the most dramatic increases in calorie intake when given dietary variety. This increase was even greater than that seen in *db*/*db* mice under a high-fat/SC choice diet.

Several potential explanations may underlie the unique observations in this study, relative to the prevailing model. MC3R signaling, which is also affected by treatment with nonspecific melanocortin agonists and antagonists, may play a role in dietary preference that confounds results previously attributed to MC4R. Second, the current study uses a palatable solid two-choice model that has not been used previously in the study of dietary preference. In using HSD and HFD as the alternative choice from standard chow, we use diets that are both high in their respective macronutrient contents and highly palatable to WT mice, as evidenced by the observed prolonged preference. The low palatability for WT mice of the high-fat and high-carbohydrate diets used in previously reported studies may also have confounded tests of the effects of the MC4R genotype. By studying feeding behavior in a chronic model, we were able to isolate the effects of MC4R deficiency on preference from the influence of dietary novelty and stress. Finally, ICV administration of melanocortin agonists/antagonists may not produce the organism-wide diminution of MC4R signaling present in the human haploinsufficiency syndrome. Outside of the brain, MC4R expression has been described in the gastrointestinal tract, including in vagal nerves and myenteric ganglia (19), which also play a role in dietary behaviors.

In summary, the findings presented here highlight an important phenotype that may hold particular relevance to the human melanocortin obesity syndrome. Although aspects of reward and hedonic drive can clearly be modulated by administration of nonspecific melanocortin compounds intracerebroventricularly or into specific brain regions, our data demonstrate that global loss of MC4R in an animal does not cause hyperphagia by increasing preference for palatable high-carbohydrate or high-fat foods (1). Although these studies did not specifically measure the reward value of the given food choices, they imply that hyperphagia in this model is not driven by an increased reward value attached to palatable foods.

Methods

Experimental Animals. All studies used adult (aged 3–7 mo) male WT, MC4R$^{+/-}$, and MC4R$^{-/-}$ sibling mice derived from the original MC4R-null colony (4) and backcrossed onto a C57BL/6J background for 20+ generations. *db*/*db* mice lacking the leptin receptor were from The Jackson Laboratory (stock no. 00697). Mice were raised on a 12-h light, 12-h dark cycle and given ad libitum access to standard chow (Laboratory Rodent Diet 5001) and water. All experiments were approved by the Animal Care and Use Committee of Vanderbilt University.

Body Weight, Food, and Liquid Intake Measurements. Daily measurements of body weight, food intake, and fluid intake were taken by hand. Food intake measurements were obtained by weighing food every 24 h around 1400 hours and subtracting the difference to obtain the amount consumed. The cage was inspected daily for fragments of food that fell from the hopper, which were then accounted for in the measurements. Fluid intake was obtained by providing a preweighed water bottle with a gravity-fed sipper tube. The difference in weight in grams is presented as a fluid consumption equal to that value in milliliters.

Dietary Preference Studies. For preference studies, mice were single-housed and subjected to daily handling for up to 1 wk before the experimental start to reduce experimental stress known to affect feeding behaviors (20). Baseline measurements of SC intake were taken for multiple days before a petri dish containing a measured amount of a second diet was added to the floor of the cage. The diets used were as follows: SC [LabDiet 5L0D/5001: 58% carbohydrate (by energy content), 28.5% protein, 13.5% fat, 3.02 kcal/g]; HFD (Research diets D12492i: 20% carbohydrate, 20% protein, 60% fat, 5.24 kcal/g); and HSD (Kellogg's Froot Loops: 88.2% carbohydrate, 3.6% protein, 8.2% fat, and 3.79 kcal/g). To test for locational preference, the positions of the diets were switched between the hopper and the dish with no notable effect. Preference ratios were calculated by taking the intake of the diet in question, by mass or caloric value, and dividing it by total intake.

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