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SHORT COMMUNICATION

The amino acid sensor GCN2 biases macronutrient selection during aging

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Abstract
Purpose Selection of a balanced diet has a determinant impact on human health. Individual food preferences involve socio-cultural as well as physiological factors and evolve during aging. In mammals, physiological mechanisms governing food choices appear to require the sensing of nutrient concentrations in diet. This is particularly the case for dietary amino acids that are sensed by the protein kinase GCN2. It has been reported that GCN2 is involved in the adaptive response to amino acid imbalanced diets at the level of food intake and lipid metabolism. Here, we hypothesized that GCN2 may play a role in macronutrient selection and its age-related changes.
Methods Two groups of wild-type and GCN2 knock-out mice were subjected to a food self-selection protocol at ages 6, 12, 18 and 24 months. During each test, mice were allowed to create their own diets by selecting between three separate food sources, each containing either protein, fat or carbohydrates.
Results Our results show that the absence of GCN2 had two main age-related effects. First, it exacerbated fat preference at the expense of carbohydrate consumption. Second, it prevented the increase in protein intake.
Conclusion These findings indicate that, in omnivores, the GCN2 ancient pathway participates in the control of food preference.

Keywords Diet selection · Aging · GCN2 · Macronutrient · Ingestive behavior

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Introduction

Feeding behavior can be considered as an important homeostatic mechanism that contributes to survival. This is illustrated by the innate ability of higher animals to precisely regulate their intake of macronutrients in order to ensure the consumption of a balanced diet [1–3]. This regulation is particularly important for omnivores that must choose among a variety of available food sources to recognize appropriate ones and select from among them to obtain a nutritionally adequate diet [4, 5]. In the particular case of humans living in industrialized countries, while food availability has expanded and become more diversified, there have also been significant negative consequences in terms of inappropriate dietary patterns. This has led to an increase in the occurrence of diet-related chronic diseases during aging, such as obesity, diabetes and cardiovascular diseases. Thus, understanding the underlying mechanisms of macronutrient selection is of considerable interest to improve feeding behavior and promote healthy aging.

In humans, the determination of food selection, and hence nutrient intake, is multifactorial in nature and involves both social, psychosocial, cultural factors and more primitive

physiological mechanisms [2, 6]. Studies in twins [2, 7, 8] or genetic variants [9–12] have demonstrated that macronutrient selection has an important heritable component. Similarly, studies performed in rodents have confirmed an important contribution of genetics in macronutrient selection [8, 13]. Another important factor affecting dietary selection is aging. In self-selection experiments performed in Lou/Cjall rats, aging induced a significant shift in energetic nutrient preference from carbohydrates to fat [14].

Among the physiological mechanisms governing dietary selection, post-ingestive nutritional factors have been shown to influence food intake [15–17], particularly according to the concentration of the nutrients in the diet [2, 18, 19]. Several studies performed on rats have highlighted the regulation of protein intake as a function of amino acid composition in the diet [5, 18, 20]. For example, when rats subjected to a self-selection protocol are switched from casein (an amino acid balanced protein) to wheat gluten (a lysine deficient protein), they freely select a twofold higher proportion of total energy from the protein compartment in order to obtain lysine [21, 22]. Thus, nutrient-sensing mechanisms may play an important role in food intake modulation according to the nutrient content in the diet.

The best understood mechanism for sensing intracellular amino acid availability is mediated by the highly conserved protein kinase GCN2. Genetic studies on yeast identified this eIF2α kinase as the primary sensor of amino acid starvation, involved in the regulation of amino acid homeostasis as a function of amino acid availability in the medium [23]. Through eIF2α phosphorylation, GCN2 not only regulates protein synthesis rate, but also controls stress-induced gene expression at transcriptional level. This pathway has been shown to be conserved from yeast to mammals [24, 25]. In mammals, GCN2 initiates the metabolic adaptation that provides the means for coping with inadequate amino acid composition of food. In particular, GCN2 activation initiates various physiological responses such as food intake inhibition [26, 27] or regulation of hepatic lipid metabolism [28] during nutritional deprivation of one essential amino acid.

Data previously described led us to put forward the hypothesis that sensing of intracellular amino acids might be involved in the regulation of macronutrient selection. Since (1) the food amino acid content can be detected by GCN2, (2) GCN2 is able to regulate both feeding behavior and lipid metabolism, and (3) aging affects nutrient preference and particularly protein intake, we hypothesized that GCN2 could be involved in the age-related physiological control of dietary preferences. In this study, wild-type and GCN2 −/− mice were subjected to a longitudinal macronutrient self-selection protocol. Our results revealed an important role of GCN2 with respect to protein as well as fat and carbohydrate selection during aging.

Materials and methods

Animals

The generation of GCN2-null mice has been described in detail elsewhere [24]. Mice were maintained in our animal facility in plastic cages in a temperature-controlled room (22 ± 1 °C), on a 12:12 h light–dark cycle. Maintenance of the mice and all experiments were performed in accordance with the guidelines of the appropriate ethics committee. At the beginning of the experiment, the cohort consisted in 32 six-month-old C57BL/6 J male mice (16 wild-type and 16 GCN2 −/− mice). As mice are considered as being gregarious animals, it is generally recommended to house mice in groups [29]. Here, mice were paired-housed with two wild-type or GCN2 −/− mice per cage. They were maintained in the same room from birth till the end of the experiment, in the same light and temperature conditions. During each self-selection experiment, mice were transferred to specific cages (see below). When mice were not taking part in the experiment, they had free access both to tap water and to a commercially available standard pellet diet (A03 chow from Safe: macronutrient composition by weight ≈ 55% carbohydrates, 21.4% proteins and 5.1% lipids; energy content 3.2 kcal/g; Augy, France).

Experimental diets

All experimental diets were semi-synthetic and manufactured using purified ingredients from Louis François (Saint-Maur, France) and Safe (Augy, France). They were powdered diets except for the fat component that had a creamy texture.

Powdered control diet

Before starting each self-selection phase, mice were maintained for 10 days on a powdered control chow in order to accustom them to the new form of diet and the experimental apparatus. The powdered control diet composition was close to that of the standard pellet, with a metabolisable energy content of 3.7 kcal/g. It consisted of all the ingredients used in the self-selection protocol (Table 1) in the following proportions (by weight): 22.0% protein, 52.6% carbohydrates, 5.5% fat, 5% salt mixture, 1.3% vitamins and 4.2% cellulose.

Self-selection diets

We used a self-selection diet paradigm. The three-choice macronutrient diet allowed mice to freely select from three separate dispensers, each containing a single macronutrient. The diets used in the self-selection protocol consisted

Table 1 Compositions (g/kg) and energy contents (kcal/g) of the diets

	PCD	Protein diet	Carbohydrate diet	Fat diet
Casein[a]	245 (220)	870 (780)	0	0
Cornstarch[a]	525 (476)	0	787 (713)	0
Saccharose	50	0	89	0
Lard	50	0	0	723
Sunflower oil	5	0	0	80
Salt mixture[a]	70 (49)	74 (52)	69 (48)	130 (91)
Vitamins	13	13	13	25
Fiber (cellulose)	42	42	42	42
Energy (kcal/g)	3.7	3.3	3.4	7.2

PCD powdered control diet

[a] In order to take the low purity of some ingredients into account (casein 89.6%; cornstarch 90.7%; salt mixture 70%), their weighing was overestimated. The numbers in brackets represent the real content of each ingredient

of protein (casein), carbohydrates (corn starch and saccharose), fat (lard and sunflower oil), each being supplemented with mineral salts and vitamins in proportion to the energy content of each diet, and with cellulose (Table 1). The energy content differed between the diets: protein diet = 3.3 kcal/g; carbohydrate diet = 3.4 kcal/g; fat diet = 7.2 kcal/g. This was taken into account for calculating the energy consumed from the different macronutrient compartments (Table 1).

Experimental design: longitudinal macronutrient selection study

The experimental apparatus consisted of modified plastic cages with three outer dispensers fixed on one side that allowed separation of the different diets and collection of spilled food. In addition, the cages were equipped with a grid on the bottom to avoid coprophagy. The experimental design is depicted in Fig. 1. A cohort of 16 GCN2 +/+ and 16 GCN2 −/− mice was subjected to four dietary self-selection tests at the ages of 6, 12, 18 and 24 months. In between the tests, the mice were maintained under standard conditions. For each measurement the total duration was 32 days (Fig. 1).

The self-selection protocol resulted in various stresses for the mice (new cages, powdered diets), that could affect food intake and body weight. So the experimental protocol consisted of suitable adaptation periods. At the beginning of each test, the mice were weighed and then placed in specific cages. They were first fed on the powdered control diet for 10 days. Mice body weights and energy intakes were assessed at the end of this period (Fig. 1). Then the diet was switched entirely to the self-selection diets, i.e. protein, carbohydrate and fat diets, each provided in a separate dispenser. Mice were accustomed to the self-selection procedure for 7 days before the measurement period. Food intake was assessed daily during the last 10 days of the self-selection diet period. Body weights were measured before the test, at the end of the powdered control diet stage, and then at the end of the self-selection diet period.

Data analyses and statistics

All data are expressed as mean ± SEM. As the mice were housed in pairs, the statistical unit for food intake was the pair of mice. A two-way ANOVA (effect of age and genotype) was performed for repeated measurements with significance at $p < 0.05$. ANOVA was followed by a mean least square test for a posteriori means comparison.

At the beginning of the experiment, the cohort consisted of 32 six-month-old C57BL/6 J male mice (16 GCN2 +/+ mice and 16 GCN2 −/− mice). From the beginning to the end of the experiment, the mice suffered a constant mortality whatever the genotype. At the age of 24 months, only 15 mice (7 GCN2 +/+ and 8 GCN2 −/− mice) had survived. It was clear that the mortality rate was identical in both genotypes (not shown). The surviving mice were repaired between the food preference measurement periods and habituated to their new counterpart for at least 1 month. Statistical analyses were performed on the data from all the paired mice that were alive at a given age. Statistical analyses were repeated on mice pairs that survived throughout the entire experiment. The results were identical between the two types of analyses. Particularly, growth curves and macronutrient selection data of mice that have survived from the beginning to the end of the study are similar to that obtained from the mean of all mice that have been included in the study (data not shown).

Results

GCN2 deletion does not affect body weight or energy intake

In order to explore the potential role of GCN2 in dietary selection and its changes during aging, we compared the macronutrient intake of wild-type and GCN2 knock-out (GCN2 −/−) mice in a longitudinal self-selection protocol described in Materials and methods. We first assessed the growth curves of wild-type and GCN2 knock-out mice during the experiment (Fig. 2). When measured at the beginning of each test, body weights significantly increased

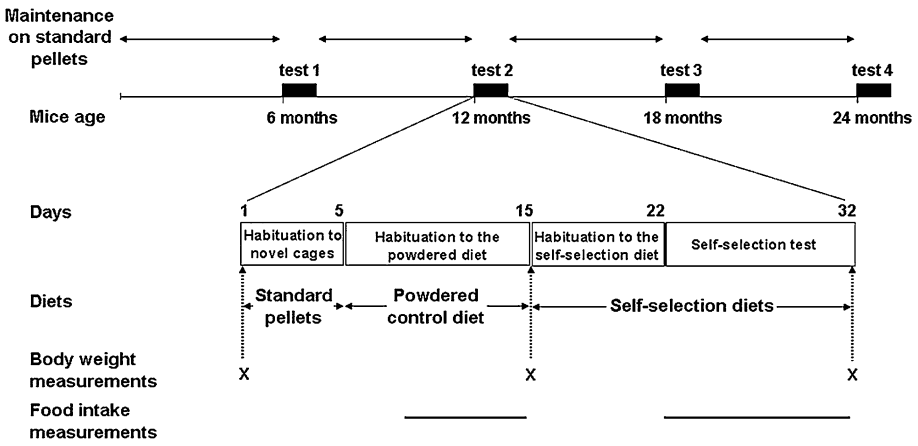

Fig. 1 Experimental procedure. The experiment was performed on a cohort of 32 male GCN2 +/+ and GCN2 −/− C57BL/6 J mice, at the ages of 6, 12, 18 and 24 months. Between each test, mice were bred under standard conditions. For each test, mice were accustomed to new cages, powered diets and self-selection procedure before measuring macronutrient selection. Mice body weights were recorded before beginning the test and at the end of the powdered control and the self-selection diet periods. Caloric intake was measured during the last 7 days of the powdered control diet period and during the last 10 days of the self-selection test

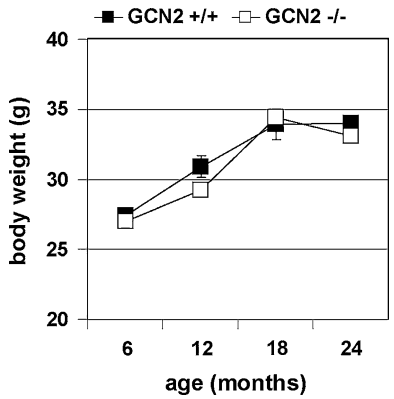

Fig. 2 Growth curves of wild-type and GCN2 knock-out mice during the longitudinal study. GCN2 +/+ and GCN2 −/− mice body weights were assessed at the beginning of each self-selection test after breeding on standard pellets

as a function of advancing age ($F(3.36) = 37.0$; $p = 0.0001$) until 18 months, then remained constant between 18 and 24 months of age. The growth curves of GCN2 +/+ and GCN2 −/− mice appeared identical, suggesting that GCN2 is not involved in growth regulation of mice fed on a standard diet.

We also assessed mice body weights during the macronutrient selection tests. Table 2 shows that food changes only slightly affected the body weight of young mature (6 months) and middle-aged (12 months) animals. Conversely, in older mice (18 and 24 months), diet shifts from standard pellets to the powdered control diet, then to self-selection diets, resulted in a marked decrease in body weight that never recovered initial values until the end of the test. However, during maintenance on lab chow between the last two self-selection tests, 18-month-old mice regained their initial body weight, so that the body weights of 18- and 24-month-old mice were identical. Overall, the GCN2 genotype did not affect changes in body weights resulting from modifications of either age or diets.

In order to check the effects of the experimental diets on total energy intake, food consumption was recorded daily during the last 7 days of the powdered control diet phase and during the last 10 days of the self-selection diet phase (see Fig. 1). We first checked that food consumption was not affected by the texture of the diet. Indeed we did not observed any significant change in the total energy intake from PCD compared to the standard pellet that were given to the mice between each selection test (data not shown). Data are reported in Table 3. Consistent with body weight values, energy intake increased as a function of age ($F(3.18) = 10.3$; $p = 0.0004$). The ANOVA showed an effect of the diet ($F(1.6) = 97.5$; $p = 0.0001$) on energy intake. Indeed, whatever the genotype or age, we observed a significant decrease in energy intake on the self-selection diet when compared to the powdered control diet (−10% at 6 months, −17% at 12 months, −11% at 18 months, and −22% at 24 months). At least two hypotheses could explain this result: (1) By self-selecting macronutrients, mice may accurately ingest macronutrients according to their needs. On the other hand, the nutrient composition of the powered control diet (PCD) may not be perfectly adequate, so mice have to ingest more food to provide an adequate amount of one essential nutrient. (2) During the self-selection diet period, the three separate food sources containing either protein, fat or carbohydrates have a consistency (palatability) or taste that may not be optimum for mice. The consequence could be a lower food intake.

Table 2 Changes in wild-type and GCN2 knock-out mice body weights (g) during the self-selection tests

	6 months			12 months			18 months			24 months		
	SP	PCD	SSD	SP	PCD	SSD	SP	PCD	SSD	SP	PCD	SSD
GCN2 +/+	27.4 ± 05	26.7 ± 0.4	26.2 ± 0.4	30.9 ± 0.8	30.5 ± 0.4	30.7 ± 0.6	33.9 ± 1.1	30.4 ± 0.6	29.9 ± 1.1	34.0 ± 0.5	27.0 ± 0.7	28.1 ± 1.2
GCN2 −/−	27 ± 0.5	26.4 ± 0.4	25.3 ± 0.5	29.2 ± 0.5	28.1 ± 0.4	29 ± 0.4	34.4 ± 0.6	29 ± 0.7	28.8 ± 0.9	33.1 ± 0.5	27.3 ± 0.5	29.1 ± 0.9

Mice body weights were assessed at the beginning of each self-selection test after breeding on standard pellets (SP) (see also Fig. 2). Body weight measurements were also performed at the end of the powdered complete (PCD) and the self-selection (SSD) diet periods

Furthermore, it is noteworthy that when mice were fed on self-selection diets, the energy intake was lower at 24 months than at 18 months of age, while body weights were identical. This may result either from differences in the energy efficiencies of diets self-selected by 18- and 24-month-old mice, respectively, or from modifications of metabolic rate between 18- and 24-months of age. In addition, although energy intake was slightly lower in GCN2 −/− mice than in wild-type mice fed either on PCD or SSD (Table 3), the difference did not reach statistical significance. Taken together these results demonstrate that energy intake and body weight are not significantly modified by the GCN2 genotype.

GCN2 deletion affects macronutrient selection changes during aging

Macronutrient selection measurements were made on the last 10 days of each self-selection test. Data resulted from the energy consumption records for each dispenser (protein, lipid, carbohydrate). Food weights were converted into energy intakes by taking the caloric content of each macronutrient into account (see Table 1). Results are expressed as the percentages of total calories that account for fat, carbohydrate and protein intakes in order to reflect the diet composition (Fig. 3).

At the age of 6 months, the protein/carbohydrate/fat selection profile was not influenced by the GCN2 genotype, and was fairly comparable to the powdered control diet composition (Fig. 3): about 54% carbohydrates, 25% lipids and 21% proteins, by caloric content. As mice grew older, statistical analysis revealed a strong effect of age on macronutrient selection ($F(6.36) = 27.1$; $p = 0.0001$). Interestingly, these changes were greatly affected by the GCN2 −/− genotype. First, all mice underwent an age-related shift from carbohydrates to fat as an energy source, an effect that was heightened for GCN2 −/− mice when compared to their wild-type counterparts (Fig. 3). Second, whereas protein selection increased with aging in wild-type mice, this tendency was reversed in GCN2 knock-out mice ($p = 0.028$). Indeed, protein intake of GCN2 +/+ mice increased from 12- to 24-months of age (+25%, $p = 0.05$), but significantly decreased for GCN2 −/− mice during the same period (−25%, $p = 0.021$) (Fig. 3). At the age of 24 months, the GCN2 knock-out mice selected 42% more fat, 33% less carbohydrates and 40% less protein as compared to wild-type mice.

Discussion

Up to now, the described role of GCN2 was to orchestrate the response to amino acid deficiencies in organisms from

Table 3 Caloric intake (kcal/day/mouse) of GCN2 +/+ and GCN2 −/− mice fed on powdered complete (PCD) and self-selection diets (SSD)

Age	6 months		12 months		18 months		24 months	
Diet	PCD	SSD	PCD	SSD	PCD	SSD	PCD	SSD
GCN2 +/+	14.2 ± 0.5	13.1 ± 0.4	16.9 ± 0.6*	14.0 ± 0.5*	17.2 ± 0.6*	15.2 ± 0.6*	17.2 ± 0.6*	13.3 ± 0.4
GCN2 −/−	13.7 ± 0.5	12.0 ± 0.5	16.3 ± 0.4*	13.4 ± 0.2*	16.0 ± 0.2*	14.2 ± 0.5*	17.1 ± 0.6*	13.4 ± 0.5*

Total energy intake was recorded daily by weighing food dispensers during the last 7 days of the powdered complete diet phase and during the last 10 days of the self-selection diet phase. A posteriori tests: $^{*}p < 0.05$ versus 6 months

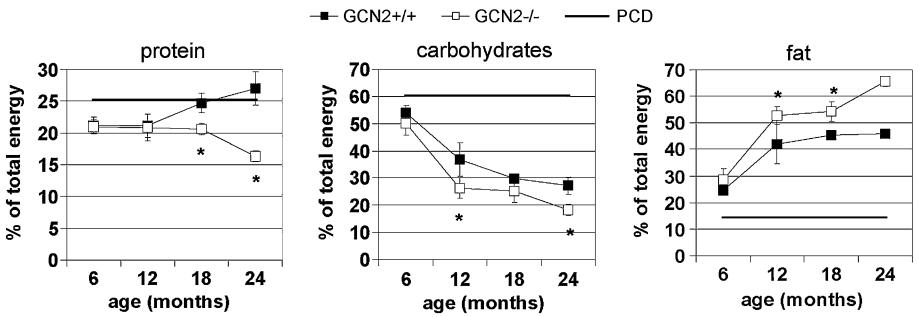

Fig. 3 GCN2 affects age-related changes in macronutrient selection. Mice macronutrient selection was measured at 6, 12, 18 and 24 months of age, with cages equipped with separate dispensers each containing protein, carbohydrates or fat. Results are expressed as % of total caloric intake derived from protein, carbohydrates and fat. The percentage of consumption of each macronutrient when the animal is fed on powdered complete diet is represented with a *solid line*. A posteriori tests: $^{*}p < 0.05$ versus GCN2 +/+

yeast to mammals. In yeast, extensive studies have demonstrated that GCN2 regulates biosynthesis and transport of amino acids as a function of their availability in the medium [23]. In mammals, GCN2 has been shown to regulate gene expression and physiological functions making it possible to cope with inadequate amino acid composition of the food [26, 27]. Here we provide evidence that GCN2 activity is involved in the regulation of macronutrient selection as a function of age.

The determination of food selection is a complex process involving several parameters. Among these factors, the role of aging has been well characterized in rats [14]. Our results show that aging is also an important determinant of macronutrient selection in C57BL/6 mice. Indeed, whatever the genotype, aged mice selected more and more fat in their diet at the expense of carbohydrates. The origin of changes in food preference occurring in old animal is unclear. Metabolic changes that appear with aging may be related to this behavior. The age-related opposing changes between decreasing lean mass and increasing fat content could lead to changes in dietary needs. In these circumstances, lipids may become a better energy source than carbohydrates in aged mice, and that could explain the higher consumption of fat. Furthermore, aging results also in a reduced anabolic response to dietary proteins [30]. Therefore, a higher amount of amino acids is required to adequately stimulate post-prandial protein synthesis and could explain why wild-type mice gradually increase their protein intake as they become older. These metabolic changes occurring with aging could modulate GCN2 activity and by this way influence food preference. However, we cannot exclude that other mechanisms could also be involved in this behavior.

The main conclusions obtained from our experiments are that GCN2 plays a role in macronutrient selection. Indeed, GCN2 deletion had two main age-related effects: first, it prevented the increase in protein intake, and second, it exacerbated fat preference at the expense of carbohydrate consumption. Contrary to wild-type mice that increased their protein intake during aging, protein consumption was decreased in old GCN2–/– mice. Several findings in the literature point out that animals do not select for proteins, but rather for amino acids as indispensable building substrates [5, 21, 31]. These data suggest that in order to maintain protein metabolism during aging, GCN2 may play a rectifying role at the level of dietary choices to fit amino acid needs. With respect to fat and carbohydrate choices, we can make the hypothesis that GCN2 could play a direct role not only in protein but also in fat and carbohydrate dietary selection during aging. This hypothesis could be connected to recent data showing that GCN2 activity controls lipid metabolism in response to a leucine free diet [28]. The observation that GCN2 deletion effects on protein selection appeared after the shift from carbohydrate to fat preference had started, suggests that these two effects could be independent.

The GCN2 protein kinase is activated in vivo by nutritional amino acid deficiencies. In our protocol, mice are not exposed to a nutritional stress that could activate GCN2. It can be hypothesized that either the basal activity of GCN2 could be involved in the control of food selection or, during certain periods of the animal's life, amino acid availability could become limited and activate GCN2.

Another question that can be raised relates to the identification of the tissue(s) involved in the GCN2-dependent control of dietary selection. As GCN2 is ubiquitously expressed (at variable levels), different tissues could be involved in the GCN2-dependent regulation of food preferences. Literature reports that nutrient-sensing mechanisms involved in the control of food intake implicate mainly the brain but also peripheral tissues. The identification of the tissue(s) involved in the GCN2-dependent control of macronutrient selection will require further investigations.

As there is good evidence to suggest that digressions from diets with a healthy composition may have genetic origins [10, 12], mechanisms promoting the search for essential nutrient containing-food may be targeted. Therefore, identifying those genes involved in dietary selection mechanisms should provide important clues to the biology behind the occurrence of metabolic age-related diseases. Our present data raise the possibility that nutrient sensors may serve as dietary selection regulators. We show that, in addition to limiting food intake when the food is amino acid-imbalanced [27], GCN2 is required to control dietary selection towards food sources that fit the body's needs. Consequently, nutrient sensors may represent interesting targets to modulate energy intake as well as food selection in humans.

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