

# Dietary Fat, but Not Protein or Carbohydrate, Regulates Energy Intake and Causes Adiposity in Mice

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## SUMMARY

The impacts of different macronutrients on body weight regulation remain unresolved, with different studies suggesting increased dietary fat, increased carbohydrates (particularly sugars), or reduced protein may all stimulate overconsumption and drive obesity. We exposed C57BL/6 mice to 29 different diets varying from 8.3% to 80% fat, 10% to 80% carbohydrate, 5% to 30% protein, and 5% to 30% sucrose. Only increased dietary fat content was associated with elevated energy intake and adiposity. This response was associated with increased gene expression in the 5-HT receptors, and the dopamine and opioid signaling pathways in the hypothalamus. We replicated the core findings in four other mouse strains (DBA/2, BALB/c, FVB, and C3H). Mice regulate their food consumption primarily to meet an energy rather than a protein target, but this system can be over-ridden by hedonic factors linked to fat, but not sucrose, consumption.

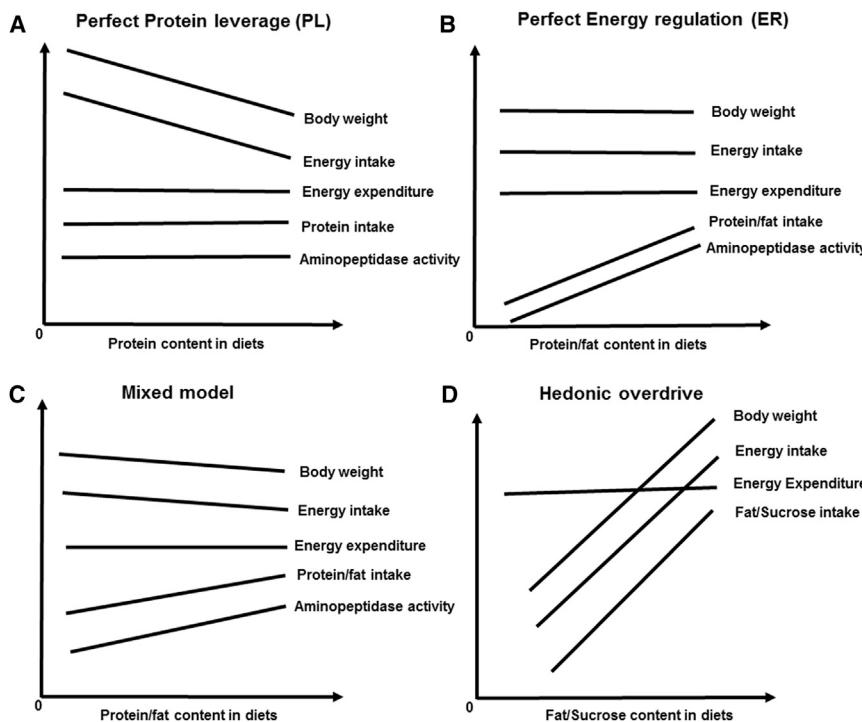
## INTRODUCTION

Obesity is a global health issue. According to the World Health Organization (WHO) in 2014, there were 1.9 billion overweight adults in the world, of which 600 million had obesity (World Health Organization, 2016). This is a major health problem because obesity is a risk factor for numerous chronic diseases (Haslam and James, 2005). It is widely agreed that obesity results from prolonged positive energy balance (Hall et al., 2012). The relative roles of reduced energy expenditure and elevated

intake in the etiology of such imbalance have been disputed (Prentice and Jebb, 1995; Swinburn et al., 2011; Westerterp and Speakman, 2008). However, data suggest that obesity is driven at least in part, if not completely, by overconsumption of energy (Swinburn et al., 2011; Westerterp and Speakman, 2008). The reasons for such overconsumption have been strongly debated (van Dam and Seidell, 2007; Willett, 1998). Changing macronutrient composition of the food may be a contributory factor, yet despite decades of study, there is still little consensus over whether high fat, high sugar, or both are responsible for the elevated intake (van Dam and Seidell, 2007; Willett, 1998). Some much needed clarity has been brought to the field recently by the “nutritional geometry” approach (Simpson and Raubenheimer, 2012). This aims to set individual dietary selection and its consequences into an n-dimensional framework, where nutritional behavior can be understood as animals attempting to reach nutritional targets that have been molded by evolution to optimize, for example, reproduction or survival (Simpson and Raubenheimer, 2012).

Applications of this approach to many species suggest that animals may eat food primarily to achieve a target intake of protein (Felton et al., 2009; Hawley et al., 2016; Raubenheimer and Simpson, 1997), which may be driven in part by fibroblast growth factor (FGF) signaling (Gosby et al., 2016). This contrasts the classical interpretation that food intake serves primarily to match energy demands. Attempting to ingest a target protein intake may lead to overconsumption of energy when the protein content of the diet declines. The protein leverage hypothesis, therefore, posits that energy consumption is driven largely by declining dietary protein content (relative to energy), and individuals overconsume energy when attempting to meet their protein target (Simpson and Raubenheimer, 2005), thereby maintaining protein intake relatively constant (Figure 1A). In contrast, the energy regulation model suggests that animals eat food primarily to match their energy demands, and hence faced with lowered





**Figure 1. Different Models of Energy Intake, Body Weight, and Energy Expenditure in Mice**

Trends of body weight, energy intake, energy expenditure, absolute protein intake, and the activity of aminopeptidase in the perfect protein leverage model (A), the perfect energy regulation model (B), a mixed model of protein leverage and energy regulation (C), and the hedonic overdrive model (D).

protein content they are not stimulated to overconsume, and may avoid elevated adiposity, but at the potential threat of protein malnutrition (Figure 1B). The hedonic over-ride hypothesis posits that normally individuals homeostatically regulate their energy intake in relation to energy demands (Figure 1D), and hence are normally in energy balance. However, this homeostatic control may be over-ridden by hedonic factors linked to consumption of various macronutrients, in particular sugars (e.g., sucrose) and fat (Berridge et al., 2010; Berthoud, 2011; Berthoud and Morrison, 2008; Berthoud et al., 2017). This hedonic effect drives individuals into positive energy balance, resulting in weight gain, and has been exceptionally characterized as food addiction (Avena et al., 2008; Gearhardt et al., 2011).

These simple predictive models belie some of the complexities that are involved in modeling the impacts of macronutrient composition of the food on resultant body composition. First, we assumed that energy expenditure is a fixed variable that is independent of the dietary composition. This is unlikely to be correct. For example, after food is ingested there is a period of elevated metabolism (variously known as the thermic effect of food, heat increment of feeding, or the specific dynamic action [SDA]). It is well established that different macronutrients have a hierarchy of impacts on SDA, with protein having the greatest and fat the smallest effect. Thus, we might anticipate post-ingestive energy demands would change in relation to the dietary composition. An additional complexity, however, is to understand how such changes in the post-ingestive period translate into total daily energy expenditure. For example, it is known that heat production from SDA and exercise may substitute for the energy demands of thermoregulation, and hence for mice that are housed below thermoneutrality there may be no net impact of SDA on total energy requirements, as our models assume. A second assumption is that the different models are appropriate under all conditions.

Hence, we assume that the mice respond to a protein target or to an energy target. Yet it is conceivable that animals may move between different targets at different stages of their lives. In particular, these “stages” may be reflected in the existing levels of fat storage. Hence, it is possible that animals normally respond to dietary composition in a manner consistent with protein leverage (Figure 1A), but as those on lower protein diets become obese they may change their regulation toward regulation of energy intake (Figure 1B). Finally, we assume that the responses to the different dietary compositions are linear. Again, this is somewhat simplistic. In particular, for example, as protein contents of the diet approach zero, in theory to achieve these requirements intake would need to expand exponentially (in the limit to infinity). This would then be constrained by the practical limitations on feeding time and intestinal absorption capacity. Over the range we are considering, however, we assume that these non-linearities are relatively small.

Despite these complexities, these models (Figure 1) illustrate a method to quantify the extent of protein leverage. If there is perfect leverage, then the protein intake plotted against protein content of the diet will extrapolate to the intercept at a value equal to the intake for the highest protein content diet (Figure 1A). This pattern might be termed 100% protein leverage. In contrast, if the animals regulate their intake of energy, then the plot of realized protein intake against dietary protein content would extrapolate to zero. This could be termed 0% protein leverage. If the animals pursue a mixed strategy (Figure 1C), then the extrapolation of the protein intake to protein content line to the intercept provides a measure of the percent effect of protein leverage in the diet choice. For example, if the gradient connecting protein intake to protein content of the diet intercepted at a point halfway between 0 and the intake at the highest protein intake, then the percent effect of protein leverage would be 50%. These models also assume that the responses are linear across the range of protein contents in the diet. Other patterns are possible and would require more complex fitting procedures. The leveraging of total intake by protein has been widely supported by empirical observations in a wide range of animals from insects to non-human primates (Felton et al., 2009; Gosby et al., 2016; Hawley et al., 2016; Raubenheimer and Simpson, 1997) and humans (Gosby et al., 2011; Martens et al., 2013; Martinez-Cordero et al., 2012; Simpson et al., 2003), although the percentage

**Table 1. Summary of Experiments Performed and Main Findings**

Experiments	Design	Main Findings
Experiment 1: manipulation of dietary protein levels at fixed fat contents	<ul style="list-style-type: none"> <li>two series of 6 diets with 6-fold variation in protein content by energy (5%–30%)</li> <li>series 1 had 60% fat and series 2 20% fat (by energy)</li> <li>C57BL/6 mice exposed to all 12 diets</li> <li>BALB/c, C3H, DBA/2, and FVB strains exposed to 6 diets with high fat only (series 2)</li> <li>main outcomes, food intake, adiposity, and energy expenditure</li> <li>RNA-seq performed on brain and adipose tissues</li> </ul>	<ul style="list-style-type: none"> <li>protein levels were unrelated to energy intake in C57BL/6 mice</li> <li>adiposity increased as protein level increased on 20% fat (series 1)</li> <li>no major changes in hypothalamic gene expression levels</li> <li>no evidence for white adipose tissue browning or changes in energy expenditure</li> <li>same patterns were observed in the other four strains</li> <li>protein leverage was less than 5% in all strains except BALB/c (12%)</li> </ul>
Experiment 2: manipulation of dietary fat levels at fixed protein contents	<ul style="list-style-type: none"> <li>two series of 6 diets with 8-fold variation in fat content (10%–80% and 8.3%–66.6%)</li> <li>series 3 had 10% protein and series 4 had 25% protein (by energy)</li> <li>C57BL/6 mice exposed to all 12 diets</li> <li>BALB/c, C3H, DBA/2, and FVB strains exposed to 6 diets with high protein only (series 4)</li> <li>main outcomes, food intake, adiposity, energy expenditure</li> </ul>	<ul style="list-style-type: none"> <li>weight of food ingested was stable at fat contents up to 50% and thereafter declined</li> <li>food energy ingested progressively increased to 50% fat content and then stabilized</li> <li>adiposity reached a peak on diets with 50%–60% fat content (by energy)</li> <li>hypothalamic gene expression shows increase in expression of genes in reward pathways in relation to dietary fat</li> <li>AgRP and NPY both downregulated in relation to dietary fat levels</li> <li>no evidence for white adipose tissue browning or effects on energy expenditure</li> <li>same patterns were observed in the other four strains</li> <li>dietary fat is a key driver of energy intake and adiposity</li> </ul>
Experiment 3: manipulation of dietary sucrose levels at fixed fat and protein contents	<ul style="list-style-type: none"> <li>C57BL/6 mice exposed to 6 diets with 6-fold variation in sucrose levels (5%–30%) at fixed protein (25%) and fat (41.7%) (all percentage values by energy) (series 5)</li> <li>main outcomes, food intake, adiposity, energy expenditure</li> </ul>	<ul style="list-style-type: none"> <li>food intake and adiposity unrelated to sucrose levels in diet</li> </ul>

contribution of protein leverage to total intake has generally not been quantified as explicitly as indicated by the above approach. Nevertheless, protein leverage is increasingly being used to understand aspects of comparative feeding biology and the human obesity epidemic (Bekelman et al., 2017).

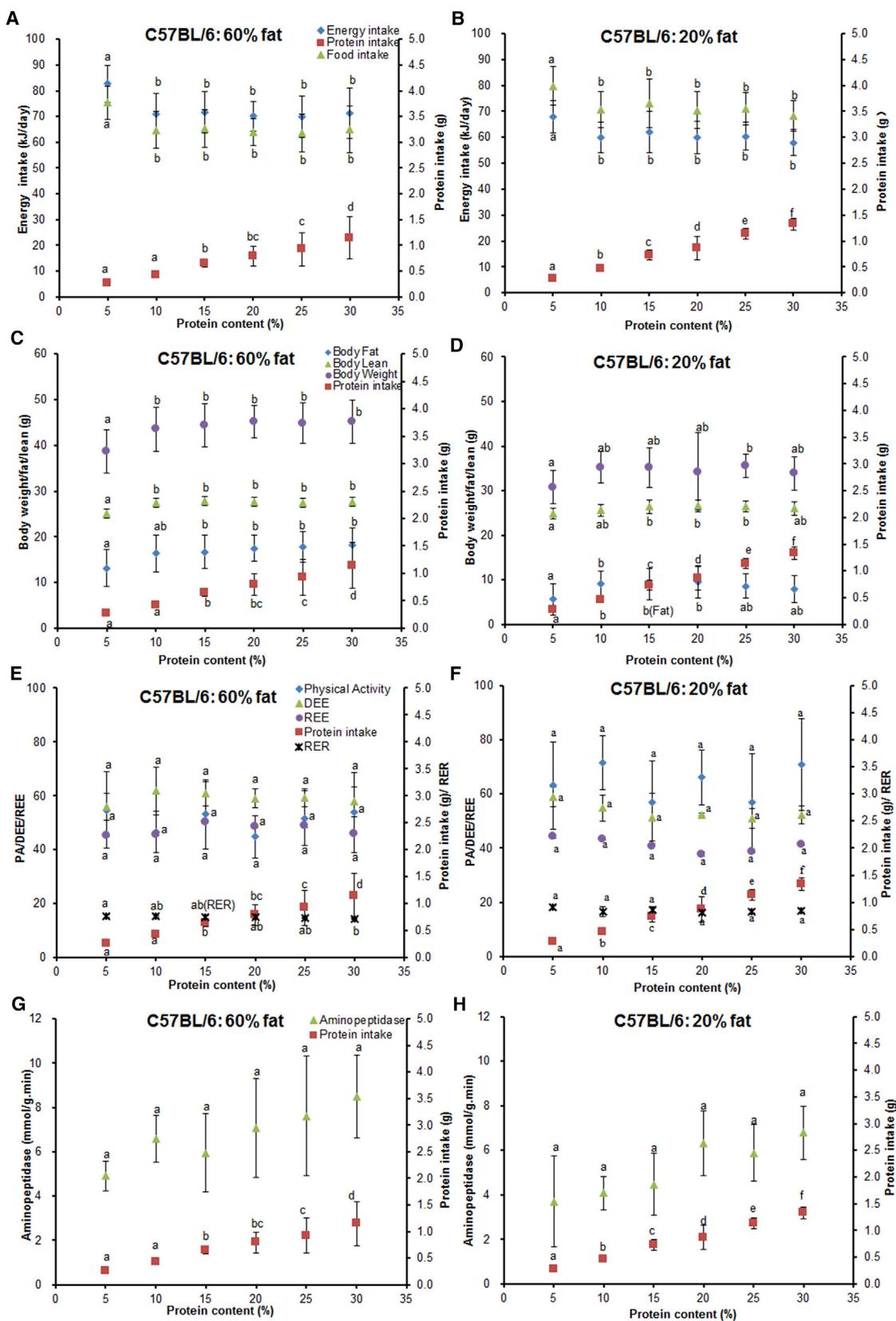
Since the mouse is a widespread model used to understand human obesity, we aimed to explore how changes in macronutrient composition of the diet impact food intake. In particular, how changes in protein, carbohydrate (sugar), and fat contents of the diet leverage intake and cause adiposity. In total, we used 29 different diets varying orthogonally in their macronutrient composition to allow separation of the different macronutrient effects. We combined these observations with gene expression profiling of the hypothalamus and adipose tissue to assess if FGF pathways were stimulated, and measurements of aminopeptidase activity in the alimentary tract, to explore the underlying mechanisms by which protein, carbohydrate, or fat may exert its effects on appetite. The most extensive work was performed in C57BL/6 mice, and then the core observations were repeated in four other strains (BALB/c, C3H, DBA/2, and FVB), which include strains classically regarded as “resistant” to obesity.

## RESULTS

The overall experimental designs and the outcomes are summarized in Table 1.

### Protein Leverage Hypothesis Is Not Supported

C57BL/6 mice were fed for 3 months on two series of six diets with variable protein (5%–30% by energy) and constant high (60%) or low fat (20% by energy) (12 diets in total with 20 mice per diet; diet details in Table S1). The energy intakes of the mice fed on the 5% protein diet with either 60% fat or 20% fat were slightly higher than the mice fed on diets with higher protein contents, while there was no difference between the other protein levels (Figures 2A and 2B). At both levels of fat in the diet, total energy intake over the final week of measurement did not differ significantly in relation to protein content from 10% to 30%, with only a significant difference between 5% protein group and other protein groups ( $p = 1.40 \times 10^{-6}$  for 60% fat and  $p = 2.52 \times 10^{-5}$  for 20% fat) (Figures 2A and 2B). The consequence was that protein intake was strongly, linearly, and directly related to the protein content in the diet ( $p = 2.80 \times 10^{-28}$  for 60% fat and  $p = 5.43 \times 10^{-62}$  for



(legend on next page)

20% fat) (Figures 2A and 2B). These relationships had intercepts that did not differ significantly (at  $p < 0.01$ ) from zero ( $p = 0.103$  for 60% fat and  $p = 0.045$  for 20% fat). The percentage protein leverage was at most 3.5% to 4% (not significantly different to zero). The same patterns were observed if we used the average food intake over the entire 12 weeks rather than intake over the final 10 days (Figures S1A and S1B).

### Body Adiposity Increased with Increasing Dietary Protein Content

On the 60% fat diets with variable protein contents, body weight, lean mass, and adiposity all *increased* with increasing protein content in the diets at the end of the experimental period ( $p = 1.92 \times 10^{-5}$  for body weight,  $p = 2.30 \times 10^{-4}$  for fat mass, and  $p = 2.10 \times 10^{-4}$  for lean mass) (Figure 2C), the opposite of the predictions of the protein leverage hypothesis. On the 20% fat diets, body weight, body adiposity, and lean mass also increased as protein content increased from 5% to 20% protein, and then decreased when protein increased from 20% to 30% ( $p = 0.024$  for body weight,  $p = 0.005$  for fat mass, and  $p = 0.002$  for lean mass) (Figure 2D). Changes in the overall adiposity (percent fatness) showed the same trends as fat mass (Figures S2A and S2B). The patterns of overall adiposity were also reflected in the changes in size of the individual fat depots (Figures S2E and S2F).

Mice may theoretically burn off excess energy intake by becoming more physically active. However, the physical activity of the mice did not follow any consistent trend in the mice fed on diets with different protein contents, and there were no significant differences between the different protein groups ( $p = 0.712$  for 60% fat and  $p = 0.452$  for 20% fat). Daily energy expenditure (DEE) ( $p = 0.603$  for 60% fat and  $p = 0.128$  for 20% fat) and resting energy expenditure (REE) ( $p = 0.874$  for 60% fat and  $p = 0.214$  for 20% fat) also followed the same pattern under either high-fat or low-fat conditions, with no significant changes between groups fed on diets with different protein levels (Figures 2E and 2F). Respiratory exchange ratio (RER) was independent of protein content of the diets, except for a significant difference between 5% and 30% protein at 60% fat ( $p = 0.016$  and  $p = 0.108$  for 20% fat). The estimated energy expenditure of mice over the entire duration of the manipulation calculated using the software of Guo and Hall (2009) (Table 2) showed the same trends as the point estimates of metabolism.

The activity of aminopeptidase-N in the gut increased with increasing protein content at 20% fat (Figures 2G and 2H) ( $p = 0.002$  for 20% fat and a trend  $p = 0.071$  for 60% fat). Small leveraging of total energy intake by protein may still in theory cause adiposity if maintained over protracted periods. There were significant differences in body weight and fat mass between the 5% protein group and other protein groups with either 60% fat ( $p = 4.35 \times 10^{-31}$  for body weight and  $p = 2.30 \times 10^{-4}$  for

fat mass) or 20% fat ( $p = 9.29 \times 10^{-31}$  for body weight and  $p = 0.005$  for fat mass) during the last week of measurement (Figures 2C and 2D).

### Responses of Four Other Mouse Strains on Diets with Variable Protein Content Were Consistent with C57BL/6 Mice

Mice from four other strains (BALB/c, C3H, DBA/2, and FVB) were used to validate the core results with respect to protein on C57BL/6 mice, by feeding them diets containing variable protein and constant high fat (60% fat). In all four strains, we repeated the observation in the C57BL/6 strain that there was a linear decrease in absolute protein intake with declining protein content in the diet ( $p = 5.67 \times 10^{-7}$  for BALB/c,  $p = 9.00 \times 10^{-20}$  for C3H,  $p = 7.12 \times 10^{-34}$  for DBA/2, and  $p = 2.74 \times 10^{-21}$  for FVB) (Figure 3). Energy intake of BALB/c mice and FVB mice showed a slight but significant increase as protein content decreased, with no differences when protein content was over 10% ( $p = 2.08 \times 10^{-5}$  for BALB/c and  $p = 0.045$  for FVB) (Figures 3A and 3G). Total energy intake was independent of the protein content in C3H mice ( $p = 0.517$ ) and DBA/2 ( $p = 0.132$ ) (Figures 3E and 3G). The same patterns were observed if we used the average food intake over the entire experiment period rather than intake over the final 10 days (Figures S3A, S3C, S3E, and S3G). The calculated percentage protein leverage was 12.2% in BALB/c mice ( $p = 0.047$ ), 6.1% in C3H mice ( $p = 0.019$ ), 1.3% in DBA/2 mice ( $p = 0.479$ ), and 4.65% in FVB mice ( $p = 0.089$ ). In spite of these slight trends for increased energy intake at lower protein levels in two of the strains, body weight, fat, and lean mass of BALB/c and C3H mice were both *increased* when the protein content in the diets increased from 5% to 20%. Fat mass then decreased when the protein content was increased from 20% to 30% (BALB/c,  $p = 4.73 \times 10^{-8}$  for fat mass and  $p = 0.008$  for lean mass; C3H,  $p = 1.17 \times 10^{-5}$  for fat mass and  $p = 0.009$  for lean mass) (Figures 3B and 3D). In the other two strains, there were no significant body composition differences between different protein groups (Figures 3F and 3H) (DBA/2,  $p = 0.669$  for fat mass and  $p = 0.301$  for lean mass; FVB,  $p = 0.843$  for fat mass and  $p = 0.505$  for lean mass). Changes in the overall adiposity (percent fatness) showed the same trends as fat mass (Figures S4A, S4C, S4E, and S4G). Overall, the responses of the five different strains were consistent that altering the protein content of the diet across a large range produced only minimal impacts on energy intake, but enormous differences in protein intake. These changes had no significant impact on adiposity.

### Dietary Fat Content Drives Energy Intake and Body Adiposity of Mice

Another two series of six diets with fixed protein content (10% or 25%) and variable fat content were designed and fed to C57BL/6

**Figure 2. Changes of Energy Intake, Body Composition, Energy Expenditure, and Activity of Aminopeptidase-N of Mice Following Exposure to Diets with Variable Protein Content and Fixed Fat (60% and 20%)**

Values are represented as mean  $\pm$  SD.

(A and B) Energy intake averaged over the last 10 days of measurement.

(C and D) Body composition.

(E and F) Energy expenditure and physical activity.

(G and H) Activity of aminopeptidase-N. Groups with a same letter were not significantly different ( $p > 0.05$ ). A total of 120 mice were used with 20 mice per diet. See also Figures S1, S2, S6, and S8.

**Table 2. Averaged Energy Expenditure and Averaged Energy Intake of Five Mouse Strains over 12 Weeks of Experimental Period**

		Protein Content (%)						p Value			
		5	10	15	20	25	30				
C57BL/6	60% fat	EE (kJ/day)	77.78 ± 5.53 <sup>a</sup>	70.17 ± 9.98 <sup>b</sup>	67.38 ± 6.57 <sup>b</sup>	68.66 ± 6.85 <sup>b</sup>	66.21 ± 8.04 <sup>b</sup>	65.09 ± 8.30 <sup>b</sup>	4.32 × 10 <sup>-6</sup>		
		EI (kJ/day)	81.87 ± 5.92 <sup>a</sup>	75.04 ± 9.17 <sup>ab</sup>	72.86 ± 6.32 <sup>b</sup>	72.21 ± 6.69 <sup>b</sup>	73.48 ± 12.10 <sup>b</sup>	70.24 ± 6.88 <sup>b</sup>	2.49 × 10 <sup>-4</sup>		
	20% fat	EE (kJ/day)	67.85 ± 4.87 <sup>a</sup>	58.96 ± 4.39 <sup>b</sup>	59.20 ± 5.95 <sup>b</sup>	57.25 ± 4.28 <sup>b</sup>	58.97 ± 4.04 <sup>b</sup>	56.37 ± 4.74 <sup>b</sup>	1.56 × 10 <sup>-11</sup>		
		EI (kJ/day)	67.90 ± 5.27 <sup>a</sup>	60.52 ± 4.15 <sup>b</sup>	60.58 ± 6.21 <sup>b</sup>	58.84 ± 3.99 <sup>b</sup>	60.55 ± 4.12 <sup>b</sup>	57.62 ± 4.75 <sup>b</sup>	7.62 × 10 <sup>-9</sup>		
		Fat Content (%)						p Value			
		10	20	30	40	50	60	80			
10% protein	EE (kJ/day)	58.32 ± 4.33 <sup>a</sup>	58.96 ± 4.39 <sup>a</sup>	63.41 ± 6.44 <sup>ac</sup>	65.95 ± 8.90 <sup>ab</sup>	72.32 ± 11.58 <sup>b</sup>	70.17 ± 9.98 <sup>bc</sup>	65.92 ± 9.41 <sup>ab</sup>	64.37 ± 8.21 <sup>ab</sup>	2.60 × 10 <sup>-7</sup>	
		EI (kJ/day)	59.26 ± 4.45 <sup>a</sup>	60.52 ± 4.15 <sup>a</sup>	66.31 ± 5.79 <sup>ab</sup>	70.7 ± 8.50 <sup>b</sup>	78.87 ± 13.04 <sup>b</sup>	75.04 ± 9.17 <sup>b</sup>	71.86 ± 11.44 <sup>b</sup>	72.06 ± 13.01 <sup>b</sup>	5.06 × 10 <sup>-11</sup>
	25% protein	EE (kJ/day)	55.72 ± 3.89 <sup>a</sup>	58.95 ± 4.14 <sup>ac</sup>	57.65 ± 3.84 <sup>ac</sup>	57.81 ± 3.8 <sup>ac</sup>	65.50 ± 6.51 <sup>b</sup>	65.92 ± 7.00 <sup>b</sup>	66.27 ± 8.23 <sup>b</sup>	63.10 ± 5.57 <sup>bc</sup>	7.36 × 10 <sup>-12</sup>
		EI (kJ/day)	56.55 ± 3.68 <sup>a</sup>	60.55 ± 4.12 <sup>a</sup>	59.14 ± 3.92 <sup>a</sup>	61.03 ± 3.87 <sup>a</sup>	69.60 ± 6.46 <sup>b</sup>	71.26 ± 6.86 <sup>b</sup>	73.48 ± 12.10 <sup>b</sup>	67.85 ± 5.82 <sup>b</sup>	2.31 × 10 <sup>-18</sup>
		Protein Content (%)						p Value			
		5	10	15	20	25	30				
BALB/c	60% fat	EE (kJ/day)	92.56 ± 3.36 <sup>a</sup>	76.44 ± 7.07 <sup>bc</sup>	84.97 ± 9.04 <sup>ab</sup>	74.24 ± 7.59 <sup>c</sup>	70.15 ± 4.82 <sup>cd</sup>	63.72 ± 2.11 <sup>d</sup>	6.31 × 10 <sup>-11</sup>		
		EI (kJ/day)	95.37 ± 3.94 <sup>a</sup>	79.48 ± 7.66 <sup>bc</sup>	84.92 ± 11.55 <sup>ab</sup>	77.53 ± 8.36 <sup>bc</sup>	72.36 ± 6.35 <sup>cd</sup>	65.27 ± 2.52 <sup>d</sup>	8.75 × 10 <sup>-9</sup>		
	10% protein	Fat Content (%)						p Value			
		EE (kJ/day)	62.66 ± 2.75 <sup>a</sup>	66.93 ± 2.95 <sup>ab</sup>	73.71 ± 3.89 <sup>bd</sup>	86.83 ± 8.44 <sup>c</sup>	76.44 ± 7.07 <sup>d</sup>	79.47 ± 7.00 <sup>cd</sup>	71.66 ± 3.48 <sup>bd</sup>	2.03 × 10 <sup>-10</sup>	
		EI (kJ/day)	63.26 ± 2.67 <sup>a</sup>	67.67 ± 3.28 <sup>ac</sup>	75.21 ± 3.47 <sup>bc</sup>	92.09 ± 10.15 <sup>d</sup>	79.48 ± 7.66 <sup>b</sup>	81.36 ± 6.92 <sup>b</sup>	73.24 ± 3.66 <sup>d</sup>	1.28 × 10 <sup>-11</sup>	
		Protein Content (%)						p Value			
		5	10	15	20	25	30				
C3H	60% fat	EE (kJ/day)	114.51 ± 19.78 <sup>a</sup>	99.76 ± 21.20 <sup>ab</sup>	97.83 ± 14.17 <sup>ab</sup>	94.24 ± 8.33 <sup>ab</sup>	93.86 ± 8.67 <sup>ab</sup>	83.86 ± 9.99 <sup>b</sup>	0.007		
		EI (kJ/day)	114.03 ± 14.95 <sup>a</sup>	94.83 ± 13.24 <sup>ab</sup>	100.91 ± 13.92 <sup>ab</sup>	98.69 ± 8.49 <sup>ab</sup>	97.35 ± 8.49 <sup>ab</sup>	87.43 ± 9.60 <sup>b</sup>	0.006		
	10% protein	Fat Content (%)						p Value			
		EE (kJ/day)	74.81 ± 3.01 <sup>a</sup>	76.56 ± 2.66 <sup>ac</sup>	90.83 ± 12.70 <sup>ace</sup>	112.01 ± 16.70 <sup>b</sup>	99.76 ± 21.20 <sup>be</sup>	94.15 ± 8.55 <sup>bcd</sup>	78.95 ± 3.07 <sup>ad</sup>	1.47 × 10 <sup>-7</sup>	
		EI (kJ/day)	73.15 ± 3.76 <sup>a</sup>	78.8 ± 3.49 <sup>ab</sup>	92.51 ± 12.71 <sup>b</sup>	112.72 ± 14.81 <sup>c</sup>	94.83 ± 13.24 <sup>a</sup>	98.14 ± 9.93 <sup>bc</sup>	82.46 ± 3.42 <sup>ab</sup>	1.89 × 10 <sup>-9</sup>	
		Protein Content (%)						p Value			
		5	10	15	20	25	30				
DBA/2	60% fat	EE (kJ/day)	83.35 ± 4.65 <sup>a</sup>	75.67 ± 7.82 <sup>ab</sup>	73.48 ± 5.21 <sup>b</sup>	79.91 ± 7.83 <sup>ab</sup>	76.15 ± 5.55 <sup>ab</sup>	74.27 ± 4.80 <sup>ab</sup>	0.02		
		EI (kJ/day)	86.89 ± 4.39 <sup>a</sup>	79.47 ± 9.11 <sup>b</sup>	78.39 ± 6.47 <sup>b</sup>	84.71 ± 8.91 <sup>b</sup>	80.16 ± 5.97 <sup>b</sup>	78.29 ± 6.38 <sup>b</sup>	0.084		
	10% protein	Fat Content (%)						p Value			
		EE (kJ/day)	65.15 ± 3.53 <sup>a</sup>	66.84 ± 4.23 <sup>ac</sup>	73.46 ± 4.41 <sup>ab</sup>	77.97 ± 8.21 <sup>b</sup>	75.67 ± 7.82 <sup>bc</sup>	78.62 ± 6.51 <sup>b</sup>	76.93 ± 2.52 <sup>b</sup>	1.39 × 10 <sup>-5</sup>	
		EI (kJ/day)	64.80 ± 3.85 <sup>a</sup>	68.36 ± 4.03 <sup>ab</sup>	76.39 ± 4.25 <sup>bc</sup>	82.35 ± 8.89 <sup>c</sup>	79.47 ± 9.11 <sup>c</sup>	80.82 ± 4.35 <sup>bc</sup>	81.61 ± 3.49 <sup>c</sup>	6.33 × 10 <sup>-8</sup>	

(Continued on next page)

**Table 2. Continued**

		Protein Content (%)				p Value	
		5	10	15	20	25	30
FVB	60% fat	EE (kJ/day)	94.89 ± 6.21 <sup>a</sup>	75.52 ± 6.79 <sup>b</sup>	76.06 ± 4.82 <sup>b</sup>	75.32 ± 9.48 <sup>b</sup>	73.99 ± 3.70 <sup>b</sup>
		EI (kJ/day)	98.02 ± 7.89 <sup>a</sup>	78.55 ± 7.06 <sup>b</sup>	77.92 ± 3.97 <sup>b</sup>	77.69 ± 9.70 <sup>b</sup>	78.06 ± 6.06 <sup>b</sup>
10% protein		Fat Content (%)				p Value	
		10	30	40	50	60	70
10% protein	EE (kJ/day)	66.26 ± 4.35 <sup>a</sup>	68.81 ± 4.80 <sup>ac</sup>	77.02 ± 8.16 <sup>ab</sup>	77.29 ± 8.19 <sup>bc</sup>	75.52 ± 6.79 <sup>ab</sup>	80.22 ± 10.03 <sup>b</sup>
		EI (kJ/day)	68.26 ± 4.9 <sup>a</sup>	70.08 ± 5.27 <sup>abd</sup>	71.94 ± 4.32 <sup>abd</sup>	80.9 ± 9.75 <sup>bc</sup>	78.55 ± 7.06 <sup>abc</sup>

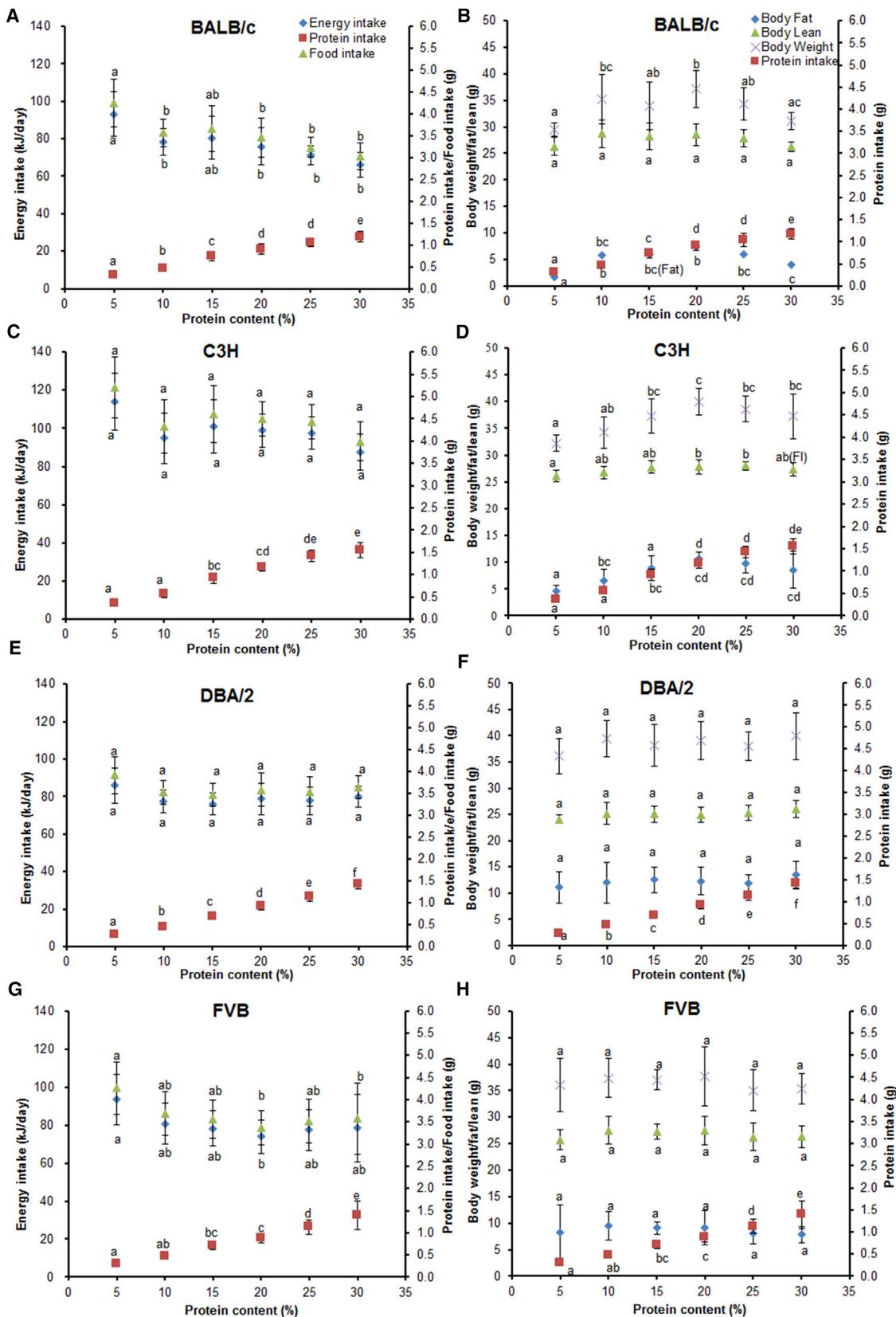
Averaged energy expenditure was calculated using the software of Guo and Hall (2009). Groups with the same letter were not significantly different ( $p > 0.05$ ). EE, energy expenditure; EI, energy intake.

mice to investigate the effect of dietary fat content on body adiposity. Fat intake (during the last 10 days) increased linearly and significantly with increasing fat content in the diet either with 10% protein ( $p = 2.18 \times 10^{-45}$ ) or 25% protein ( $p = 4.27 \times 10^{-68}$ ) (Figures 4A and 4B). Increased fat intake was related to higher energy intake of the mice fed on diets with either 10% protein ( $p = 3.24 \times 10^{-12}$ ) or 25% protein ( $p = 6.26 \times 10^{-12}$ ), even though the mice reduced their food intake when the fat content was increased in the diet ( $p = 8.71 \times 10^{-17}$  for 10% protein and  $p = 5.36 \times 10^{-8}$  for 25% protein) (Figures 4A and 4B). Consequently, the increased energy intake caused the increase in body weight ( $p = 3.45 \times 10^{-7}$  for 10% protein and  $p = 1.68 \times 10^{-28}$  for 25% protein) and body fat mass ( $p = 1.51 \times 10^{-9}$  for 10% protein and  $p = 3.02 \times 10^{-24}$  for 25% protein), when dietary fat content was lower than 60% (Figures 4C and 4D). The same patterns were observed if we used the average intake over the 12 weeks of study rather than intake in the last 10 days (Figures S1C and S1D). Body weight and fat mass of the mice were slightly decreased because of significantly reduced food intake, when fat content in the diet was higher than 60% (Figures 4C and 4D). Only slightly higher lean mass was observed in the mice fed on diets with 50%–60% fat either with 10% protein ( $p = 1.66 \times 10^{-4}$ ) or 25% protein ( $p = 0.003$ ). Trends in adiposity (percent fatness) and the sizes of individual fat depots mirrored the pattern of change in fat mass (Figures S2C, S2D, S2G, and S2H). Therefore, increasing dietary fat content up to 60% fat leads to increased energy intake and causes adiposity in mice; however, further increase in the fat content led to a slight decrease in the energy intake via reduction in the absolute weight of food intake, and as a consequence, body weight and fat mass decreased.

The activity of aminopeptidase-N showed a trend similar to body weight and body fatness in either 10% protein or 25% protein groups; however, there were no significant differences ( $p = 0.079$  for 10% protein and  $p = 0.057$  for 25% protein) (Figures 4G and 4H). There was no significant trend in the change of physical activity. Significantly higher physical activity was observed in the mice fed on diet with 10% fat, in comparison with mice fed on diets with higher fat content when protein was fixed at 10% ( $p = 2.71 \times 10^{-5}$ ), with no differences between other diet groups (Figure 4E). When protein was fixed at 25%, there were no significant differences in physical activity between mice fed on diets with variable fat content, except significantly higher physical activity in mice fed with diets with 25% fat (Figure 4F). No significant differences were observed in DEE ( $p = 0.060$  for 10% protein and  $p = 0.737$  for 25% protein) and REE ( $p = 0.017$  for 10% protein and  $p = 0.421$  for 25% protein) with either 10% or 25% protein (Figures 4E and 4F). Significantly higher RERs were observed in the mice fed on diets with fat content lower than 30% with either 10% or 25% protein, with no differences between mice fed on diets with fat content over 30% regardless of protein content ( $p = 1.78 \times 10^{-8}$  for 10% protein and  $p = 5.23 \times 10^{-9}$  for 25% protein) (Figures 4E and 4F).

### The Responses of Four Other Strains on Diets with Variable Fat Content Confirmed that Dietary Fat Regulates Energy Intake and Adiposity Even in Strains Regarded as “Obesity Resistant”

The effect of dietary fat content was also replicated in the other four mouse strains (Figure 5). Energy intake of BALB/c and C3H



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mice increased when fat content in the diet increased to 50% fat, which was then decreased when fat content increased further to 80% ( $p = 3.33 \times 10^{-7}$  for BALB/c mice and  $p = 3.92 \times 10^{-4}$  for C3H mice) (Figures 5A and 5C). The weight of food ingested by these two mouse strains decreased gradually when fat content increased from 10% to 80% ( $p = 8.06 \times 10^{-12}$  for BALB/c mice and  $p = 2.06 \times 10^{-7}$  for C3H mice) (Figures 5A and 5C). Energy intake of DBA/2 and FVB mice increased gradually with increasing fat content in the diet ( $p = 2.56 \times 10^{-4}$  for DBA/2 mice and  $p = 0.019$  for FVB mice), even though the mice decreased the weight of food intake ( $p = 8.10 \times 10^{-7}$  for DBA/2 mice and  $p = 0.002$  for FVB mice) (Figures 5E and 5G). Body weight ( $p = 2.08 \times 10^{-4}$  for BALB/c,  $p = 4.63 \times 10^{-7}$  for C3H,  $p = 1.48 \times 10^{-8}$  for DBA/2, and  $p = 3.004 \times 10^{-6}$  for FVB) and body fatness ( $p = 2.05 \times 10^{-4}$  for BALB/c,  $p = 3.88 \times 10^{-8}$  for C3H,  $p = 1.67 \times 10^{-8}$  for DBA/2, and  $p = 4.17 \times 10^{-7}$  for FVB) were increased significantly in all four stains with similar trends, while there were no significant differences in body lean mass in all four strains ( $p = 0.093$  for BALB/c,  $p = 0.001$  for C3H,  $p = 0.020$  for DBA/2, and  $p = 0.023$  for FVB) (Figures 5B, 5D, 5F, and 5H).

### Sucrose Content in the Diet Does Not Drive Energy Intake and Affect Adiposity in Mice

In all the above diets dietary sucrose was fixed at 5% by energy. The effect of sucrose on body adiposity was investigated by fixing protein content at 25% and fat content at 41.7%, while varying the contribution of the sucrose in the carbohydrate fraction from 5% to 30% of total energy (see Table S1 for diet details). Sucrose intake also increased linearly in relation to sucrose content in the diet ( $p = 8.0 \times 10^{-39}$ ) (Figure S5A). Energy intake over the last 10 days, however, remained constant in the mice fed on diets with variable sucrose content, with no significant differences between different sucrose groups ( $p = 0.320$ ). Energy intake over the entire experimental period also showed the same trend (Figure S5B). Body weight ( $p = 0.855$ ), fat mass ( $p = 0.620$ ), and lean mass ( $p = 0.902$ ) also did not differ significantly in relation to the sucrose content (Figure S5C). No significant differences were observed in DEE ( $p = 0.435$ ), REE ( $p = 0.350$ ), RER ( $p = 0.473$ ), and physical activity ( $p = 0.994$ ) (Figure S5D).

### Hypothalamic Hunger Signaling Pathways and Adipose Tissue Browning-Related Signaling Pathways of Mice Fed on Diets with Variable Protein and Fat Content

To investigate if protein and fat contents of the diets had effects on energy balance via the canonical hunger signaling pathways in the hypothalamus, RNA sequencing (RNA-seq) was performed on RNA extracted from the hypothalami of a subset of

the mice exposed to the different diets, followed by alignment using standard tools, and pathway analysis using the Ingenuity Pathway Analysis (IPA) software. The responses of this subset of mice to the dietary manipulations did not differ from the total sample (Figure S6). We then explored the impacts of fat and protein on gene expression profiles across all diets using generalized linear modeling (GLM), with gene expression as the dependent variables and dietary levels and interactions of the two macronutrients as the independent variables. There were no significant associations (GLM,  $p \geq 0.05$ ) between the four primary hypothalamic genes that drive hunger (*Pomc*, *Cartpt*, *Agrp*, and *Npy*) and the level of protein in the diet (Figure 6; Table S2). There were also no significant associations between protein contents of the diets and gene expression of components of the melanocortin signaling, dopamine, or opioid receptor systems (Figure 6; Table S2). However, dietary protein content was associated significantly with elevated gene expression levels of three serotonin (5-HT) receptors, *Htr1a*, *Htr4*, and *Htr5a* (Figure 6; Table S2). Consistent with the small, non-significant calculated impacts of protein contents on overall intake, these data indicate that enormous 6-fold changes in dietary protein (from 5% to 30%) did not have any significant impacts on hunger signaling pathways in the hypothalamus. There were only seven significant changes in hypothalamic gene expression in the FGF signaling pathway (7/76 genes), including a positive correlation between the expression of *Fgf2* and protein content (Figure S7B; Table S3). Changes in the expression levels of 16/152 genes in the mechanistic target of rapamycin (mTOR) signaling pathway were significantly associated with dietary protein levels, most (12/16) of which were negatively related to elevated protein content of the diet (Figure S7A; Table S4).

A suggested mechanism by which animals may avoid obesity is by burning off excess energy via upregulation of white adipose tissue (WAT) browning. To investigate if protein content of the diet had effects on energy balance via the browning-related signaling pathways in the WAT, RNA-seq was performed on RNA extracted from the sWAT and eWAT of a subset of mice exposed to the different diets, followed by alignment using standard tools and pathway analysis using the IPA software. As for the hypothalamus sample, the responses of this subset of mice to the dietary manipulations did not differ significantly from the total sample (Figure S8). *Ucp1*, *Cpt1b*, *Acaca*, *Acacb*, *Pnpla2*, *Fabp4*, *Acsl1*, *Adrb1*, *Adrb2*, *Adrb3*, *Ppargc1a*, *Pparg*, *Fgf21*, *Fgfr1*, *Fgfr2*, *Fgfr3*, *Fgfr4*, *Fgfr1l*, and *Cidea* are general thermogenic-related genes. There were significant negative associations between the protein contents in the diet and the expression of *Ucp1*, *Acaca*, *Fgf21*, and *Cidea* (Figure S9B; Table S5) in WAT. *Prdm16*, *Tgfb1*, *Bmp7*, *Ebf2*, *Tbx1*, *Tnfrsf9*, *Tmem26*, *Slc27a1*, *Hoxc9*, *Mtus1*, and *Kcnk3* are genes

**Figure 3. Energy Intake, Body Weight, and Body Composition Changes in Four Mouse Strains after Feeding on Diets with Variable Protein Contents and 60% Fat**

Values are represented as mean  $\pm$  SD.

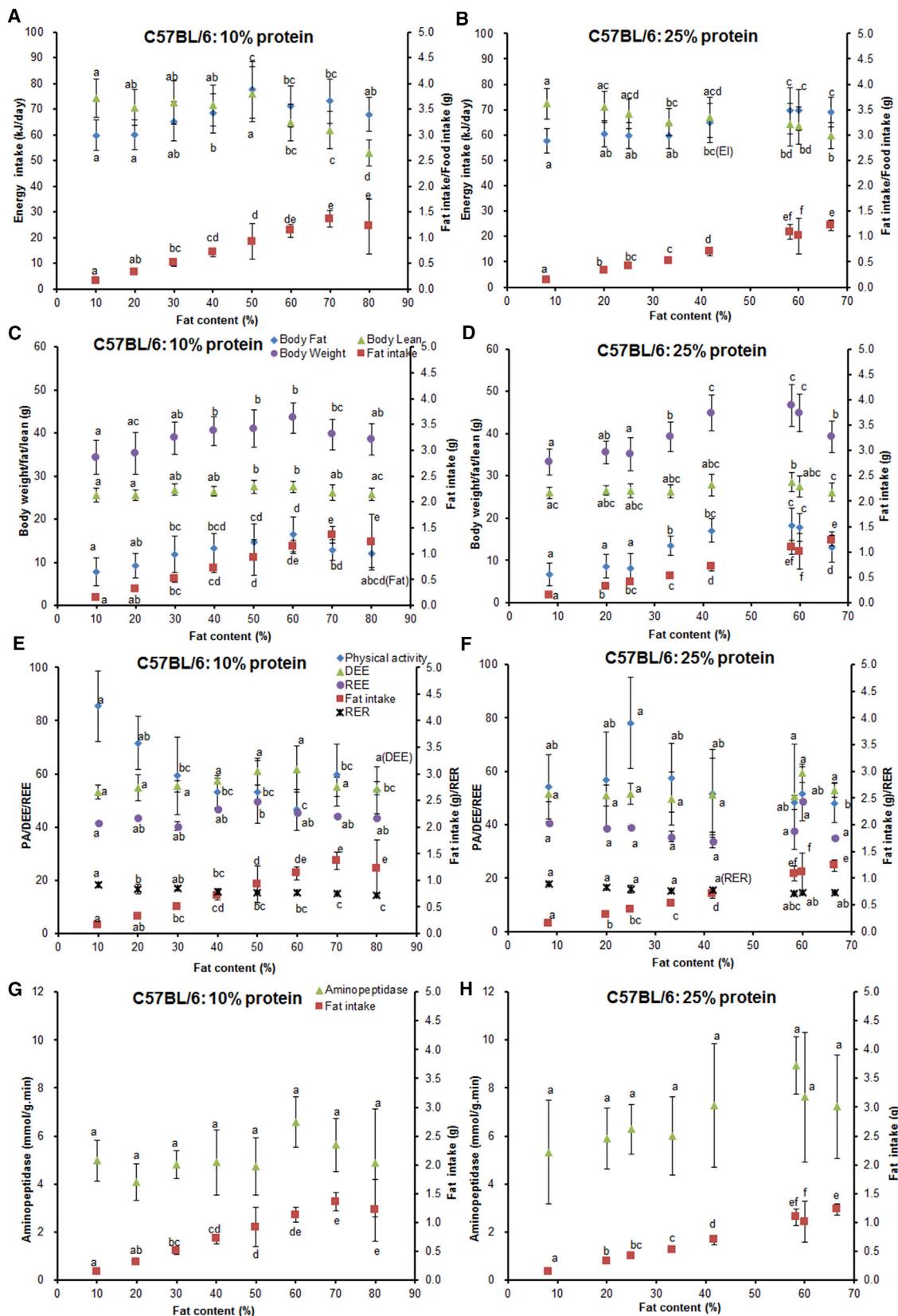
(A and B) DBA/2 mice.

(C and D) BALB/c mice.

(E and F) FVB mice.

(G and H) C3H mice. Groups with a same letter were not significantly different ( $p > 0.05$ ). Energy intake was averaged over the last 10 days of dietary manipulation. A total of 60 mice of each strain was used (10 per diet).

See also Figures S3 and S4.



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specifically related to WAT browning. *Bmp7* and *Tnfrsf9* showed significant negative associations to the dietary protein contents, but the other “browning genes” were unrelated (Figure S9B; Table S5). *Sirt1*, *Mtor*, *Cyp26b1*, *Eya2*, *Hspb7*, *Pdk4*, *Rnf34*, *Mindy2*, *Egln3*, *Stac2*, *Tns2*, *Fgf1*, and *Fgf10* are also involved in the browning signaling pathways. There were also no significant regressions between protein content of the diet and these genes (Figure S9B; Table S5). In WAT, only 5/76 genes involved in FGF signaling showed changes in expression, including a negative correlation between protein content of the diet and *Fgf21* gene expression itself (Figure S9C; Table S6). In WAT, only 4/152 genes in the mTOR signaling pathway were correlated with dietary protein levels, and expression of *Mtor* itself was not significantly changed (Figure S9A; Table S7).

Significantly positive associations were evident between the fat levels of the diet and the main hedonic signaling systems linked to food intake, i.e., dopamine (*Drd1* and *Drd5*) and opioid receptor (*Oprk1*, *Oprd1*, and *Oprm1*) systems (Figure 7; Table S2). There was no significant association between fat contents and components of the melanocortin signaling pathway (Figure 7; Table S2). However, surprisingly there were strong significant negative associations between fat content and the two primary hypothalamic genes that drive hunger (*Npy* and *Agrp*; Figure 7). This was not paralleled by associations between dietary fat content and the primary hunger-suppressing genes (*Pomc* and *Cartpt*) (Figure 7; Table S2). Nevertheless positive associations between fat content and elements of the serotonin (5-HT) receptor (*Htr2a*, *Htr2c*, *Htr1a*, *Htr1b*, *Htr5a*, and *Htr4*) signaling, upregulation of which is also generally considered inhibitory of intake, were significant. These changes were consistent with the system attempting to compensate the enhanced hedonic signals and were mirrored by the reduced weight of food ingested as the fat content increased. In addition, significantly positive associations were observed between the dietary fat levels and insulin signaling components (*Insr*, *Stat3*, and *Jak2*), IGF signaling (*Igf1* and *Igf1r*), and the growth hormone receptor (Figure 7; Table S2). In hypothalamic FGF signaling, 46/76 genes showed significant changes in gene expression in relation to dietary fat changes, 35 (including changes in *Fgf1*, 2, 9, 10, 12, 13, 14, and 18) of which were positively associated to fat content of the diet, and the other 11 (including change in *Fgfr1*) were negative (Figure S7D; Table S3). In hypothalamic mTOR signaling, 110/152 genes showed significant changes in gene expression, 42 of which were positively correlated to dietary fat while the other 68 were negatively correlated (Figure S7C; Table S4).

We also investigated the impact of dietary fat levels on browning-related signaling pathways in the WAT. There were significant negative relationships between the levels of fat in the diet and gene expressions of *Ucp1*, *Acaca*, *Acacb*, *Fgfr2*, *Fgfr3*, *Fgfr4*, and *Cidea* (Figure S9E; Table S5). *Bmp7*, *Egln3*, and

*Tns2* showed significant negative associations to the dietary fat content while *Tgfb1*, *Pdk4*, and *Fgf1* were positive (Figure S9E; Table S5). With respect to WAT FGF signaling, there were 30/76 significant correlations to dietary fat content in gene expression in components of the FGF pathway, 18 of which were positive while the others were negative (Figure S9F; Table S6). Expressions of *Fgf1*, 13, and 18 were positively correlated with dietary fat, while gene expression of *Fgf2*, 3, 4, 11, and 12 were negatively correlated to dietary fat contents (Figure S9F; Table S6). In the WAT, 46/152 genes in the mTOR signaling pathway correlated significantly to dietary fat content, 29 of which were positive while the others were negative, and there was no significant change in expression of *Mtor* itself (Figure S9D; Table S7).

## DISCUSSION

Alternative ideas about food intake regulation include the protein leverage hypothesis, the homeostatic energy regulation model, and the hedonic overdrive model (Figure 1). It is clear from the responses to the diets where protein varied, but fat contents were kept constant, that the responses of these mice conformed most closely to the pattern illustrated in Figure 1B: regulating energy rather than protein intake. The percentage level of protein leverage was not significantly different to zero in all five strains (using  $p = 0.05$  and Bonferroni correction for multiple testing). We therefore found little evidence to support the notion that as protein content declined the mice were stimulated to consume more food to compensate. In 4/5 strains there was a non-significant trend in the direction of elevated intake, but in BALB/c mice it reached significance. This absence of a strong effect was mirrored by the lack of stimulation of the main hunger pathway in the hypothalami of C57BL/6 mice in response to declining dietary protein, and there was no impact on FGF signaling. Nevertheless, small increases in energy intake over protracted durations such as used in the current experiment could in theory lead to elevated fat deposition. However, the trends we observed were insufficient to generate elevated adiposity in any of the strains. The pattern of change in the aminopeptidase enzyme levels mirrored the protein levels in the diet. Mice did not upregulate this enzyme to facilitate greater uptake of protein as its levels in the diet declined, but rather its expression mirrored the amount of protein that had to be handled as a by-product of taking in a fixed quantity of food energy that varied in its protein content. These data suggest that in mice food intake is primarily regulated by energy requirements (energy homeostatic model), and that protein does not leverage intake to cause adiposity.

Our conclusions contrast several previous studies of the protein leverage hypothesis across a wide range of species from

**Figure 4. Changes of Energy Intake, Body Composition, Energy Expenditure, and Activity of Aminopeptidase-N of C57BL/6 Mice Following Exposure to Diets with Variable Fat Content and Fixed Protein (10% and 25%)**

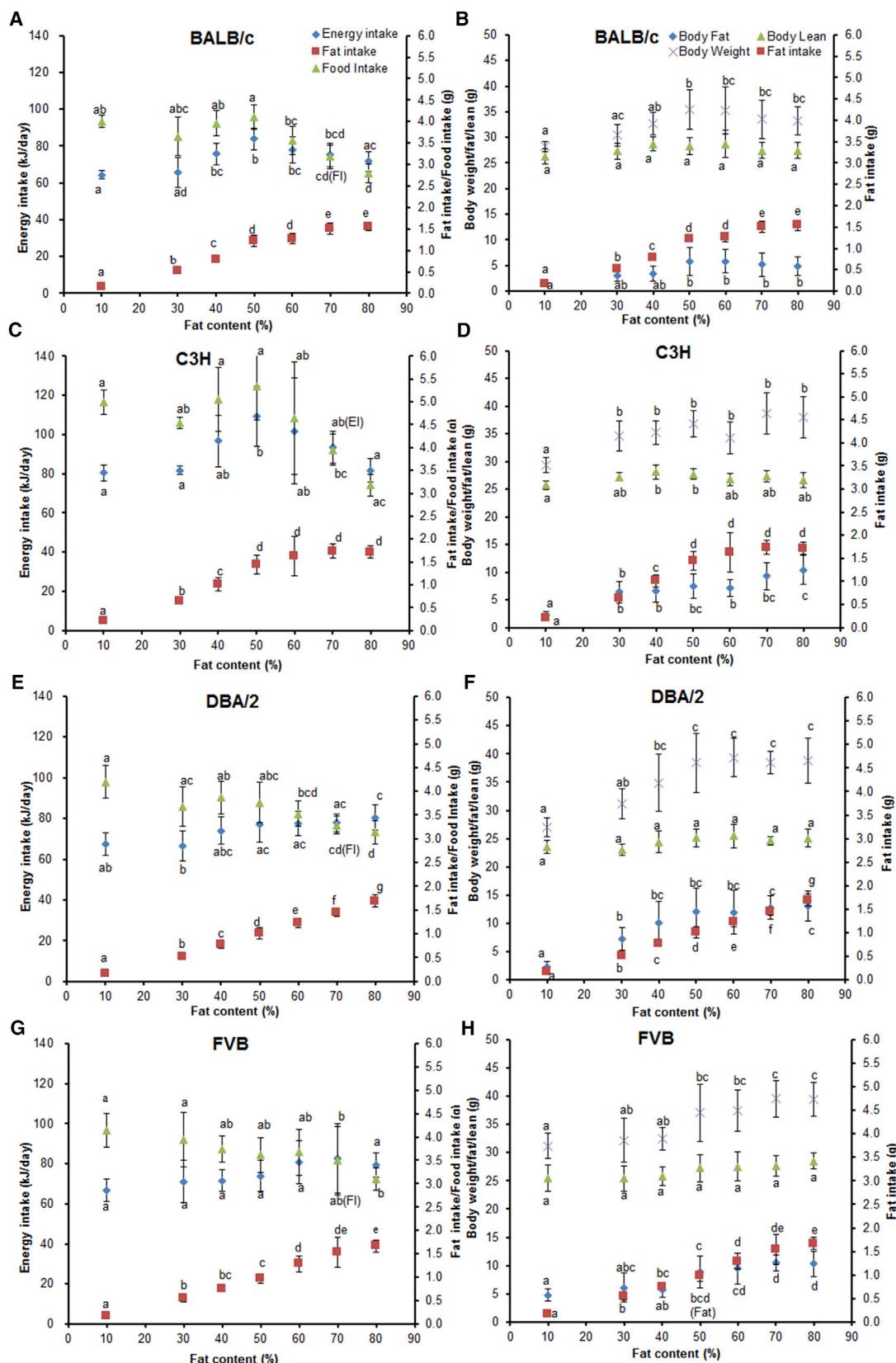
Values are represented as mean  $\pm$  SD.

(A and B) Energy intake averaged over the last 10 days of measurement.

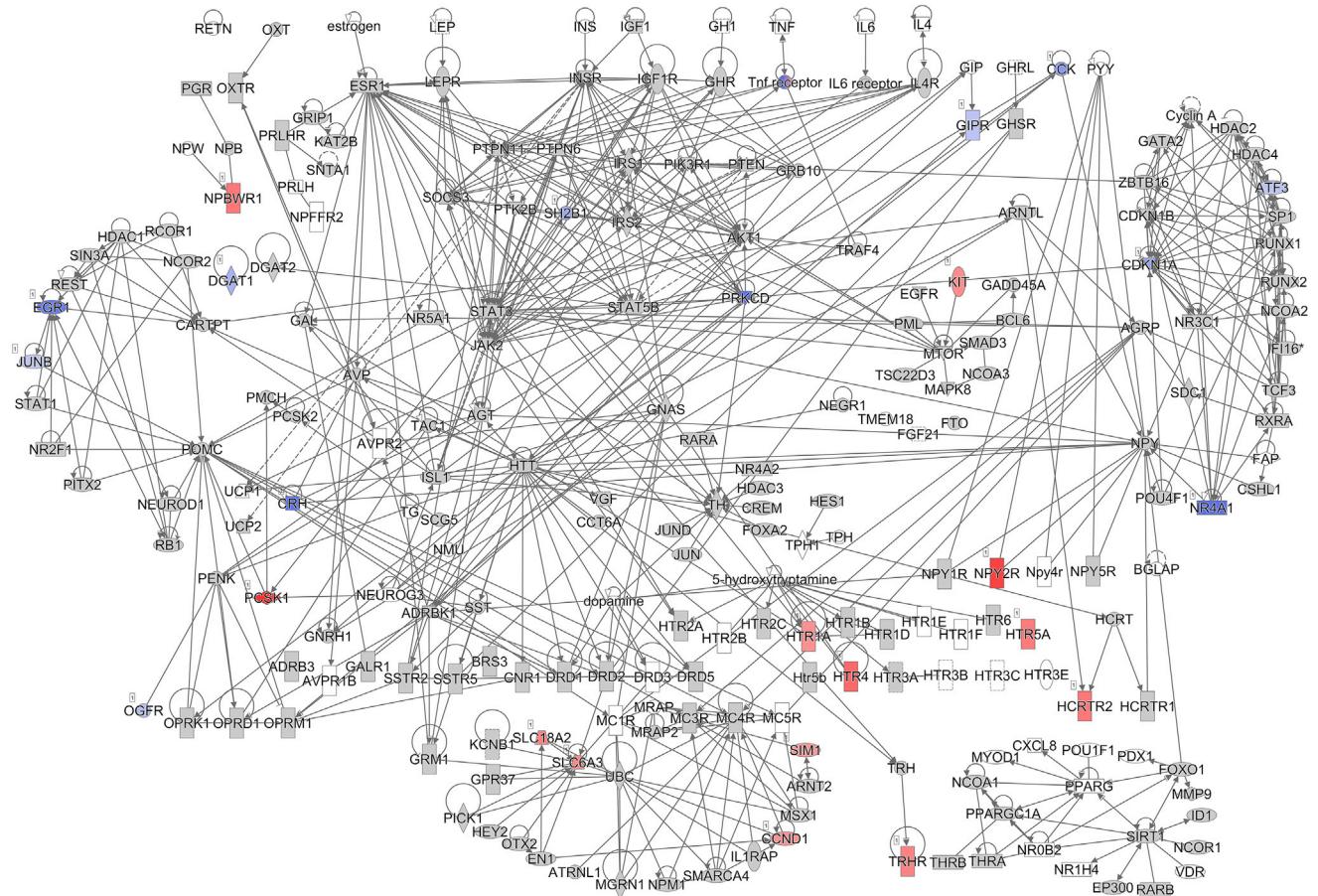
(C and D) Body composition.

(E and F) Energy expenditure and physical activity.

(G and H) Activity of aminopeptidase-N. Groups with a same letter were not significantly different ( $p > 0.05$ ). A total of 120 mice were used with 20 mice per diet. See also Figures S1, S2, S6, and S8.



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**Figure 6. Hunger Pathway Diagram Showing GLM Regression of Gene Expression against Dietary Protein Contents in Key Hunger- and Feeding Behavior-Related Genes in the Hypothalamus of C57BL/6 Mice**

Red indicates the positive and blue indicates the negative regressions with the protein levels in the diets ( $p < 0.05$ ). Intensity of the color is related to the absolute values of  $\log_{10}(p)$  value. Gray indicates no significance. A total of 48 pooled samples were used in the analysis across the different protein levels, each sample being pooled from four animals.

See also Table S2 and Figure S7.

insects to humans. These also include two studies of mice (Huang et al., 2013; Sorensen et al., 2008). Several differences between our study and these previous studies may explain the different conclusions drawn. First, the mice in the previous studies were younger when the experimental manipulations started. In one case (Sorensen et al., 2008), the mice were only 5 weeks old and hence still growing. For growing animals, protein intake may be more important to facilitate growth and hence may play a greater role in driving intake. Second, the diets covered a different range of fat and protein contents. In particular, their diets had lower fat content (ca. 15% by energy)

compared with 20% and 60% by energy used here. Moreover, the range of protein contents was also different: from 10% to 50% by energy compared with 5%–30% by energy used here. The biggest difference, however, is how the effects of protein on intake were quantified and described. Hence, while energy intake increased as the protein content declined, and hence the data were claimed to support the protein leverage hypothesis, the effect was modest (from 1780 to 2,100 kJ). Using the same approach for quantification used here, the extent of protein leverage was only 4.6% in NMRI mice (Sorensen et al., 2008) and 4.9% in C57BL/6 mice (Huang et al., 2013), similar to the

**Figure 5. Energy Intake, Body Weight, and Body Composition Changes in Four Mouse Strains after Feeding on Diets with Variable Fat Contents and 10% Protein**

Values are represented as mean  $\pm$  SD.

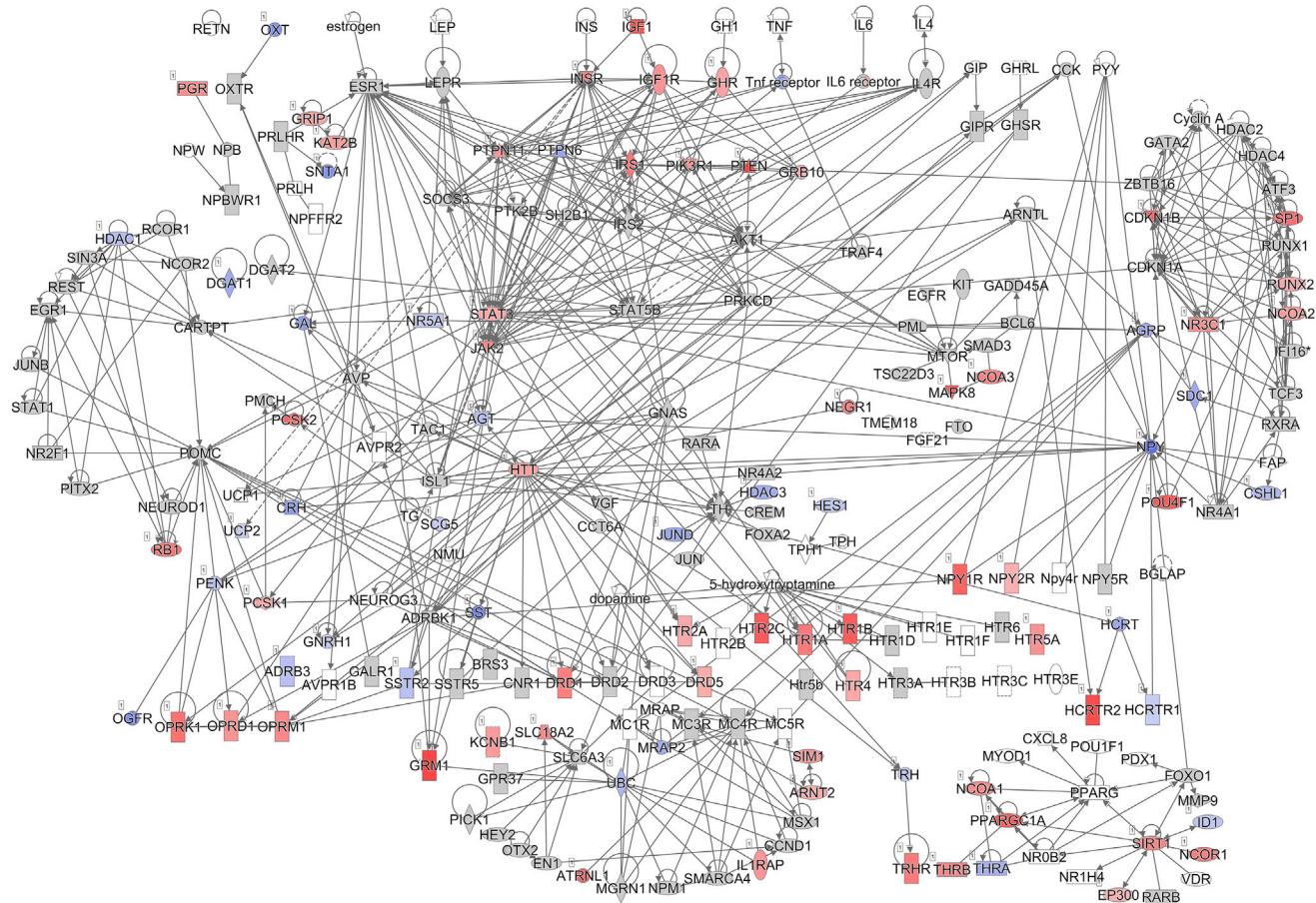
(A and B) DBA/2 mice.

(C and D) BALB/c mice.

(E and F) FVB mice.

(G and H) C3H mice. Groups with a same letter were not significantly different ( $p < 0.05$ ). Energy intake was averaged over the last 10 days of measurement. A total of 60 mice per strain was used at 10 mice per diet.

See also Figures S3 and S4.



**Figure 7. Hunger Pathway Diagram Showing GLM Regression of Gene Expression against Dietary Fat Contents in Key Hunger- and Feeding Behavior-Related Genes in the Hypothalamus of C57BL/6 Mice**

Red indicates the positive and blue indicates the negative regressions with the fat levels in the diets ( $p < 0.05$ ). Intensity of the color is related to the absolute values of  $\log_{10}(p)$  value. Gray indicates no significance. A total of 48 pooled samples were used in the analysis across the different fat levels, each sample being pooled from 4 animals.

See also Table S2 and Figure S7.

observations here. In our case, this was not significantly different to zero and insufficient to drive elevated adiposity.

When we allowed the fat content of the diets to vary but held the protein content constant, there was a clear stimulatory effect of the fat content on total energy intake. At low fat contents (<40% fat) the mice ate very similar weights of food, but because the energy density was greater as the fat content increased, their energy intakes increased. Above 50% fat in the diet the mice reduced the weight they consumed, but still continued to eat more energy than when feeding on the low-fat diets. This elevated consumption of energy led to increased adiposity that was highest at dietary fat levels of 50%–60%. The normal homeostatic regulation of energy intake revealed when we varied protein content was therefore perturbed by elevated levels of fat and hence more similar to the hedonic overdrive model (Figure 1D). This elevated energy intake was associated with hypothalamic stimulation of the dopamine, opioid, and 5-HT receptor systems, all pointing to increased reward when ingesting higher levels of fat. These data indicate that overconsumption of energy occurs in mice primarily because dietary fat stimulates hypothalamic he-

donic systems that over-ride the homeostatic control (Figure 1D; see also Berthoud and Morrison, 2008). These stimulatory changes in the hypothalamic hedonic system appeared to be counter-regulated by reductions in the primary hypothalamic drivers of hunger (*Npy* and *Agrp*) and increases in elements of the 5-HT signaling system. This pattern was associated with reductions in the actual weight of food that was ingested as fat content increased, but until fat contents were elevated above 60% this effect was insufficient to blunt energy intake. The responses of the mice to fat intake therefore departed from the simple linear models introduced above (Figure 1). Since we only measured gene expression in the hypothalamus, we can only make inferences with respect to the hedonic system located in this brain region. Clearly other brain regions mediate reward and may be involved in the impact of fat on dietary intake. We found very limited evidence of stimulated WAT browning as dietary fat content increased, not changes in physical activity levels, and these were insufficient to have any impact on whole-body metabolic rates. When dietary fat levels are fixed, variation in other macronutrients appears largely irrelevant for weight and adiposity

regulation. Interestingly, given the strong focus in public health messages about refined sugar intake (e.g., the [WHO, 2015] guidelines that sugar intake should be limited to 10% of energy), we found no stimulation of energy intake or elevated adiposity by varying levels of sucrose in the diet (up to 30% by calories) independent of other macronutrient changes.

These data have some important translational implications. Studies of humans have suggested that they may be susceptible to both protein leverage (Gosby et al., 2011; Martens et al., 2013; Martinez-Cordero et al., 2012; Simpson et al., 2003) and dietary sucrose (Fortino et al., 2007; Kuhnle et al., 2015), and such studies have guided public health policies in terms of recommendations of what we should eat. One interpretation of why we did not find these effects in mice might be because mice have different food intake and macronutrient regulation systems from humans, and that such systems are not strongly conserved across species. However, the interpretations of how macronutrients affect human body composition are generally based on epidemiological correlational data, or on short-duration feeding trials, lasting maximally several weeks. Hence, another possibility is that the differences arise because it is simply not possible to perform in humans the type of prolonged controlled experiment we have done in mice. For example, the equivalent protocol in humans to that performed here would require subjects to be confined with access to only a single food source for approximately 9 years (equivalent in lifespan terms to 90 days in mice). Clearly such a protocol is never going to be applied to human subjects, but such trials are perhaps necessary to reveal the true nature of how macronutrients relate to body composition. A human feeding trial lasting several weeks is equivalent in lifetime terms to exposing mice to a diet change for just a couple of days. In our experiment, the responses of mice over the first few days of dietary change differ markedly from those reported over the entire experiment. For example, the immediate response of the mice to elevated sucrose was to increase intake for several days, but this then returned to the baseline levels, with no long-term impact on intake or body composition. In this case our study provides a useful insight into the relationships between macronutrients and body composition that would be impossible to achieve in human experimentation.

### Limitations of Study

Although we used over 1,000 mice from five different strains and compiled over 100,000 daily measurements of food intake and body weight, our work has several limitations. We were only able to address the impact of dietary macronutrients on one sex at one time point in their lives (early adulthood) for a period equivalent in humans to about a decade. It is hence quite possible that using females at other times of life and over longer durations of exposure might result in different macronutrient impacts on weight regulation. Moreover, the nutritional space is so complex and multi-dimensional that, despite using 30 different carefully controlled diets, we were only able to scratch the surface of potential variations that might be important. For example, we only used a single source of protein, yet some studies suggest that adiposity may depend on an interaction between protein source and high dietary fat content (McManus et al., 2015). We only used a single combination of saturated, monounsaturated, and poly-unsaturated fats, and again variation in

these components has also been suggested to be important for weight gain (Yang et al., 2017; Piers et al., 2003; Liao et al., 2010). Finally, we used only one type of refined carbohydrate (sucrose) and delivered this in the food. Yet other studies suggest that mode of delivery may impact the effects of sucrose on adiposity, with sucrose presented in the drinking water having a greater effect (Rattigan and Clark, 1984; Berkey et al., 2004; Kawasaki et al., 2005). Lastly, none of the diets we used were sufficiently low in carbohydrate to drive the individuals into ketogenesis, which is another factor suggested to affect weight regulation (Sumithran and Proietto, 2008; Wilder and Winter, 1922).

### STAR★METHODS

Detailed methods are provided in the online version of this paper and include the following:

- KEY RESOURCES TABLE
- CONTACT FOR REAGENT AND RESOURCE SHARING
- EXPERIMENTAL MODEL AND SUBJECT DETAILS
  - Ethical Statement
  - Registration
  - Mice
- METHOD DETAILS
  - Experimental Diets
  - Food Intake, Body Weight and Composition Measurement
  - Energy Expenditure and Physical Activity Measurement
  - The Activity of Aminopeptidase-N Assay
  - RNA Isolation and Transcriptome Analysis
- QUANTIFICATION AND STATISTICAL ANALYSIS
- DATA AND SOFTWARE AVAILABILITY

### SUPPLEMENTAL INFORMATION

Supplemental Information includes nine figures and seven tables and can be found with this article online at <https://doi.org/10.1016/j.cmet.2018.06.010>.

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### AUTHOR CONTRIBUTIONS

S.H. analyzed the data and co-wrote the manuscript. D.Y. and L. Li managed the overall day-to-day experimental process and contributed to the data collection. L.W. and A.D. performed the RNA extractions and the RNA-seq and IPA-related analysis. Q.L., L. Luo, and S.W. performed the analysis of the activity of aminopeptidase-N in the jejunum. J.T., Y.W., B.L., M.L., G.W., X.Z., C.N., J.L., Y.X., E.C., A.W.-D., and M.M. were involved in the initial experimental design and performed body weight, food intake, and body composition measurements. J.R.S. directed the project, conceived and designed the experiments, contributed to the analysis, and co-wrote the paper. All authors approved the final version prior to submission for publication.

## DECLARATION OF INTERESTS

The authors declare no competing interests.

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**STAR★METHODS****KEY RESOURCES TABLE**

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Chemicals, Peptides, and Recombinant Proteins		
NaCl	Guangzhou Chemical Reagent Factory	N/A
L-alanine- <i>p</i> -nitroanilide	Sigma-Aldrich	CAS: 31796-55-1
<i>p</i> -nitroanilide	Sigma-Aldrich	CAS: 100-01-6
NaH <sub>2</sub> PO <sub>4</sub>	Tianjin Fuchen Chemical Reagents Factory	N/A
Na <sub>2</sub> HPO <sub>4</sub>	Chendu Kelong Chemical Reagent Factory	N/A
Acetic acid	Guangzhou Zhongnan Reagent Industry CO.LTD	N/A
Critical Commercial Assays		
RNeasy Mini Kit	Qiagen	74104
Deposited Data		
Data for Figure 2	Mendeley	<a href="https://doi.org/10.17632/7x3nv4gytv.1">https://doi.org/10.17632/7x3nv4gytv.1</a>
Data for Figure 3	Mendeley	<a href="https://doi.org/10.17632/kvh99yn2v.1">https://doi.org/10.17632/kvh99yn2v.1</a>
Data for Figure 4	Mendeley	<a href="https://doi.org/10.17632/ythsb23n5.1">https://doi.org/10.17632/ythsb23n5.1</a>
Data for Figure 5	Mendeley	<a href="https://doi.org/10.17632/pk8b95srz7.1">https://doi.org/10.17632/pk8b95srz7.1</a>
Data for Figure S1	Mendeley	<a href="https://doi.org/10.17632/jfpccxx2fbp.1">https://doi.org/10.17632/jfpccxx2fbp.1</a>
Data for Figure S2	Mendeley	<a href="https://doi.org/10.17632/94kh9k7gyk.1">https://doi.org/10.17632/94kh9k7gyk.1</a>
Data for Figure S3	Mendeley	<a href="https://doi.org/10.17632/j5zn6hx2xy.1">https://doi.org/10.17632/j5zn6hx2xy.1</a>
Data for Figure S4	Mendeley	<a href="https://doi.org/10.17632/ht86j7hbt.1">https://doi.org/10.17632/ht86j7hbt.1</a>
Data for Figure S5	Mendeley	<a href="https://doi.org/10.17632/xgrmj4fv8d.1">https://doi.org/10.17632/xgrmj4fv8d.1</a>
Data for Figure S6	Mendeley	<a href="https://doi.org/10.17632/kvfyc4hjb4.1">https://doi.org/10.17632/kvfyc4hjb4.1</a>
Data for Figure S8	Mendeley	<a href="https://doi.org/10.17632/zftg44986j.1">https://doi.org/10.17632/zftg44986j.1</a>
Transcriptomics data for Figures 6, 7, S7, and S9	GEO	GEO: GSE114960
Experimental Models: Organisms/Strains		
Mouse: C57BL/6	Charles River Laboratories	RRID: IMSR_CRL:27
Mouse: BALB/c	Charles River Laboratories	RRID: IMSR_CRL:28
Mouse: C3H	Charles River Laboratories	RRID: IMSR_CRL:25
Mouse: DBA/2	Charles River Laboratories	RRID: IMSR_CRL:26
Mouse: FVB	Charles River Laboratories	RRID: IMSR_CRL:207
Software and Algorithms		
Microsoft Excel	Microsoft	Version 2013
SPSS	IBM	Version 20
R platform	R Core Team	Version 3.2.2
IPA	Ingenuity Systems	<a href="http://www.ingenuity.com/">http://www.ingenuity.com/</a>
GraphPad Prism	GraphPad Software	Version 6.0

**CONTACT FOR REAGENT AND RESOURCE SHARING**

Further information and requests for resources and reagents may be directed to and will be fulfilled by the Lead Contact, John R. Speakman ([j.speakman@abdn.ac.uk](mailto:j.speakman@abdn.ac.uk)).

## EXPERIMENTAL MODEL AND SUBJECT DETAILS

### Ethical Statement

All procedures were reviewed and approved by the Institutional Review Board, Institute of Genetics and Developmental Biology, Chinese Academy of Sciences: approval numbers AP2014011 and AP2015004, pertaining to the study of C57BL/6 mice and the 4 additional strains study, respectively.

### Registration

This project has been registered at the open science framework (<https://doi.org/10.17605/OSF.IO/YH9GZ>).

### Mice

C57BL/6, DBA/2, BALB/c, FVB and C3H mouse strains were used. All the mice were male and purchased at age 8 weeks from Charles River and acclimated to the animal house for 2 weeks. We exposed these mice to a panel of 29 different diets that varied in their fat, carbohydrate and protein contents (details below). The primary outcomes were food intake and body weight/adiposity. Based on the previously reported variation in the response of C57BL/6 mice to high fat diet (Zhang et al., 2012), a power analysis indicated that to detect an effect size of 0.3 g/day in mean food intake between groups with 80% power at alpha = 0.05 in a one-way ANOVA with 6 levels, a sample of 20 per group was necessary. A total of 480 male C57BL/6 mice were therefore used to investigate the effect of protein and fat content on adiposity, at 20 mice per diet. For the validation work on the other strains, we reduced the sample size to 8 per group. This increased the detectable effect size at 80% power to 0.48 g per day. Finally, we fixed the protein content at 25% and fat content at 41.7%, with only changing sucrose content (5% to 30%) in the diet, to investigate if changing sucrose content affect body fatness in mice. C57BL/6 mice were used at 10 mice per diet. In total, the number of mice used across all experiments was 924.

All mice were housed individually (in a specific pathogen free facility) and maintained in environmentally controlled conditions (temperature 22–24°C, 12:12 LD cycle lights on at 0730h). Appropriate housing temperature for studies of mice and how best to mimic the situation in humans is disputed. Here we followed the suggestion in Speakman and Keiger (2012) that when provided with nesting and bedding materials standard room temperature (22–24°C) may not be too far from representative. They were provided *ad libitum* access to food and water and were monitored for health status daily. All mice were fed a standard diet with 10% fat and 20% protein (D12450B, Research Diets Ltd) for 2 weeks as the baseline period. Following 2 weeks of baseline monitoring (at age 12 weeks), all mice were randomly allocated to different groups and switched to the experimental diets for 10–12 weeks (12 weeks for C57BL/6 mice and 10 weeks for the other 4 strains). After 10–12 weeks all mice were sacrificed and dissected.

## METHOD DETAILS

### Experimental Diets

In total, mice were fed on 5 diet series, each series consisting of 6 different diets (total = 30 diets), full details of which are in Table S1. In the first two series (Series 1: D14071601 – D14071606 and series 2: D14071607 – D14071612) we fixed the level of fat at 60% (series 1) or 20% (series 2) by energy, and varied the protein content from 5% to 30% by energy. The protein source was casein. The balance was made up by carbohydrate (roughly equal mix of corn starch and maltodextrose). The source of fat was a mix of cocoa butter, coconut oil, menhaden oil, palm oil and sunflower oil. This mix was designed to match the balance of saturated, mono-unsaturated and polyunsaturated fats (ratio 47.5: 36.8: 15.8) and the n-6: n-3 ratio (14.7: 1) in the typical western diet. The proportions of the different fat constituents and hence fatty acid distributions did not change as the total fat content changed. Sucrose and cellulose were both fixed 5% by energy and weight respectively, and all diets were supplemented with a standard vitamin and mineral mix. In the second two series of diets (series 3: D14071613 – D14071618 and series 4: D14071619 – D14071624) we fixed the level of protein at 10% (series 3) or 25% (series 4) by energy and then allowed the fat content to vary. When the protein was at 10% the six fat contents were 10, 30, 40, 50, 70 and 80%. When the protein was 25% the six fat contents were 8.3, 25, 33.3, 41.7, 58.3 and 66.6%. Fat, protein and carbohydrate composition were the same as those in the first 2 series. In these diets the sucrose, cellulose and vitamin and mineral contents were the same as the diets in series 1 and 2. In a fifth series of diets we fixed the fat at 41.7%, and the protein at 25% and then allowed the sucrose to vary between 5% and 30% in 5% steps (diet codes D14071622, D16053101 to D16053105). All these diets can be ordered direct from research diets (<https://researchdiets.com/>) using the diet codes provided.

### Food Intake, Body Weight and Composition Measurement

Over the 2 week baseline period, body weight (BW) and food intake (FI) were measured and recorded daily. Following this, all mice were exposed to the experimental diets for 12 weeks and BW and FI were also measured daily. Body composition including fat mass and lean mass of all mice were measured using an EchoMRI Body Composition Analyzer (Nixon et al., 2010) weekly over the 12 week experimental period. Canola oil was used as the standard for the measurements. Food intake was measured from the weight of food that went missing from the food hopper each day. Mice occasionally pulled pellets of food through the hopper bars and so a thorough search of the cage was made to return any uneaten food to the hopper before weighing. Very occasionally mice ground their food into small pieces and the large number of small fragments in the bedding could not be measured. We eliminated occasional days when the lab notes indicated this had been a problem. For the individuals that consistently ground their food we eliminated the entire track

of food intake data. The numbers of animals that ground food and were eliminated were as follows: n = 11 for C57BL/6 mice, n = 0 for BALB/c and DBA/2 mice, n = 7 for C3H mice, and n = 2 for FVB mice.

### Energy Expenditure and Physical Activity Measurement

Mice were moved to a TSE PhenoMaster/LabMaster system for 2 days after 10 weeks on the experimental diets. This duration is sufficient to obtain an accurate measure of energy metabolism (Speakman, 2013). After calibrating the system with the reference gases (20.950%/0.05% for O<sub>2</sub>/CO<sub>2</sub>), the oxygen (O<sub>2</sub>) consumption (mL/min), carbon dioxide (CO<sub>2</sub>) production (mL/min), respiratory exchange ratio (RER = VCO<sub>2</sub>/VO<sub>2</sub>) and locomotor activity (Counts), as well as food intake (g), were recorded. Measurements were taken at 1-min intervals for the whole period. Energy expenditure (EE) was calculated from O<sub>2</sub> consumption and CO<sub>2</sub> production according to the Weir Equation: EE (kJ/day) = ((3.9 x VO<sub>2</sub> (mL/min) + 1.1 x VCO<sub>2</sub> (mL/min)) x 1440 (min)/1000 x 4.184 (Weir, 1949). In addition to point estimates of energy expenditure we also used the software provided by Guo and Hall (2009)/Guo et al. (2009) to calculate the energy expenditure over the entire 12 week manipulation period from the data on body weight, body fat mass and food intake changes.

### The Activity of Aminopeptidase-N Assay

Aminopeptidase-N assays were performed according to a previously reported method (Liu and Wang, 2007; Liu et al., 2013). Immediately after sacrifice, the small intestines were opened and rinsed with cold 0.9% NaCl solution. Briefly, 0.8~1.0 cm pieces of jejunum (in mid-region of small intestines) were snap frozen in liquid nitrogen and then kept at -80°C for enzyme assays. Aminopeptidase-N assays were carried out using L-alanine-p-nitroanilide as a substrate (Maroux et al., 1973). Prior to enzyme assays, intestinal samples were thawed at 4°C and homogenized (30 s, using a homogenizer setting at 10 000 rpm) in 0.9% NaCl (1:10, w/v) in an ice-water bath. Ten µL tissue homogenate was mixed with 1 mL assay solution (2.04 mM L-alanine-p-nitroanilide in 0.2 M phosphate buffer (NaH<sub>2</sub>PO<sub>4</sub>/Na<sub>2</sub>HPO<sub>4</sub>, pH 7.0)) and incubated at 37°C for 10 min. The reaction was then terminated with 3 mL chilled 2 N acetic acid. The absorbance was measured at 384 nm. The activity was determined using a p-nitroanilide standard curve, and presented as activity per unit intestinal wet mass (µmol /min·g wet tissue).

### RNA Isolation and Transcriptome Analysis

From each diet group, the hypothalami of 8/20 individuals were collected. The left halves of two, and the right halves of another two, were pooled together as one sample, and the same was performed with the other 4 hypothalami, resulting in each diet group having 2 pooled samples of 4 hypothalami (n = 48 samples in total across 24 diets). From each diet group, the sWAT and eWAT of 12/20 individuals were also collected. A small piece from each of six sWAT collections were pooled together as one sample, and the same was performed with the other six eWAT collections. In this way each diet group had one pooled sWAT sample and one pooled eWAT sample (also n = 48 across 24 diets). The total RNA of the hypothalamus and white adipose tissues was isolated using the RNeasy Mini Kit (QIAGEN, 74104) according to manufacturer's protocol. All sequencing was carried out using the Illumina NextSeq 500 sequencer. RNA fragments were sequenced by 75 bp long reads from paired ends (PE 2 x 75 bp, 150 bp per fragment). FASTQ data files were analyzed using FASTQC (a quality control tool for high throughput sequence data; <http://www.bioinformatics.babraham.ac.uk/projects/fastqc/>). Paired-end reads were mapped to the *Mus musculus* genome (GRCm38) using Bowtie 2-2.1.0, Samtools-0.1.19, and TopHat-2.0.10; uniquely mapped reads for each gene were counted against the GTF file of GRCm38 provided by Ensembl (release 83) using HTSeq-0.6.1p1 using the strand = reverse; after obtaining the count data from the TopHat-HTSeq pipeline, genes with the counts per million (CPM) value ≥ 1 in at least one of the 24 diets group were retained (Anders et al., 2013).

### QUANTIFICATION AND STATISTICAL ANALYSIS

Statistical analysis were performed using the R platform (R Core Team, 2015), IBM SPSS 20, GraphPad Prism 6.0 and Microsoft Excel. All values are expressed as mean ± SD. Whole animal oxygen consumption and energy expenditures were evaluated using ANCOVA (Arch et al., 2006; Tschop et al., 2011). One-way ANOVA with Bonferroni post-testing were calculated using IBM SPSS 20. Differences were considered significant if p < 0.05. The transcriptome data counts were analyzed by using the R package 'edgeR' (version 3.12.0, R version 3.2.2) (Robinson and Smyth, 2007, 2008; Robinson et al., 2010; Robinson and Oshlack, 2010; Lund et al., 2012; McCarthy et al., 2012; Chen et al., 2014) to apply Generalized Linear Modelling (GLM) to analyse the simultaneous effects of both protein and fat content of diets. The protein or fat level changes were treated as continuous numeric covariates; Let  $\pi_{gi}$  be the fraction of all mapped reads in the  $i_{th}$  sample that originate from gene g. The total number of mapped reads in library i was denoted by  $N_i$  and the number that map to the gene g by  $y_{gi}$ . Then:

$$E(y_{gi}) = \mu_{gi} = N_i \pi_{gi}$$

$$\log(\mu_{gi}) = x_i \beta_g + \alpha_g + \log(N_i) \quad (\text{Bullard et al., 2010; Lu et al., 2005})$$

Here  $x_i$  is a vector of covariates that specifies the treatment conditions applied to RNA sample i,  $\beta_g$  is a vector of regression coefficients by which the covariate effects are mediated for gene g, and  $\alpha_g$  is a vector of intercepts. The use of the negative binomial distribution is equivalent to treating the  $\pi_{gi}$  as gamma distributed. The GLM model used here was:  $\sim p+f+p:f$ , which regresses gene

expression against the protein (p) and fat contents (f) of diets, as well as their interaction (p:f). However, when the effect of the interaction was not significant ( $p$  value  $\geq 0.05$ ), the interaction term was dropped and a revised model ( $\sim p+f$ ) was utilised. To make the comparisons we used the specific energy balance data that pertained to the individuals that had been selected for the tissue collections to perform RNAseq. In all cases these subsets of individuals did not differ from the entire group of individuals on each diet. The statistical significance was set at  $p$  value  $< 0.05$ . The  $p$  values and beta coefficients were loaded into the Ingenuity Pathway Analysis (IPA) program (Ingenuity Systems; <http://www.ingenuity.com/>) for core analysis. We used a custom built pathway for hunger signaling in the hypothalamus (Derous et al., 2016) to visualize the dietary impacts on hunger signaling, and the canonical pathways for FGF and mTOR signaling available in IPA.

## DATA AND SOFTWARE AVAILABILITY

Data for all individual figures have been uploaded to Mendeley and the relevant DOIs are listed as follows: <https://doi.org/10.17632/jfpcxx2fbp.1> for Figure 2, <https://doi.org/10.17632/kyvh99yn2v.1> for Figure 3, <https://doi.org/10.17632/ytthsb23n5.1> for Figure 4, <https://doi.org/10.17632/pk8b95srz7.1> for Figure 5, <https://doi.org/10.17632/jfpcxx2fbp.1> for Figure S1, <https://doi.org/10.17632/94kh9k7gyk.1> for Figure S2, <https://doi.org/10.17632/j5zn6hx2xy.1> for Figure S3, <https://doi.org/10.17632/ht86j7hbtc.1> for Figure S4, <https://doi.org/10.17632/xgrmj4fv8d.1> for Figure S5, <https://doi.org/10.17632/kvfyc4hjb4.1> for Figure S6, <https://doi.org/10.17632/zftg44986j.1> for Figure S8. The transcriptomics data for Figures 6, 7, S7, and S9 is now deposited at GEO (the accession number for the transcriptomics data is GEO: GSE114960).