Differential expression of hypothalamic neuropeptides in the early phase of diet-induced obesity in mice

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Ziotopoulou, Mary, Christos S. Mantzoros, Stanley M. Hileman, and Jeffrey S. Flier. Differential expression of hypothalamic neuropeptides in the early phase of diet-induced obesity in mice. Am J Physiol Endocrinol Metab 279: E838–E845, 2000.—Exposure to high-fat diets for prolonged periods results in positive energy balance and obesity, but little is known about the initial physiological and neuroendocrine response of obesity-susceptible strains to high-fat feeding. To assess responses of C57BL/6J mice to high- and low-fat diets, we quantitated the hypothalamic expression of neuropeptides implicated in weight regulation and neuroendocrine function over a 2-wk period. Exposure to high-fat diet increased food consumption over a 2-day period during which leptin levels were increased when assessed by a frequent sampling protocol [area under the curve (AUC): 134.6 ± 10.3 vs. 100 ± 12.3, P = 0.03 during first day and 126.5 ± 8.2 vs. 100 ± 5.2, P = 0.02 during second day]. During this period, hypothalamic expression of neuropeptide Y (NPY) and agouti-related protein (AgRP) decreased by ∼30 and 50%, respectively (P < 0.001). After 1 wk, both caloric intake and hypothalamic expression of NPY and AgRP returned toward baseline. After 2 wk, cumulative caloric intake was again higher in the high-fat group, and now proopiomelanocortin (POMC) was elevated by 76% (P = 0.01). This study demonstrates that high-fat feeding induces hyperphagia, hyperleptinemia, and transient suppression of orexigenic neuropeptides during the first 2 days of diet. The subsequent induction of POMC may be a second defense against obesity. Attempts to understand the hypothalamic response to high-fat feeding must examine the changes as they develop over time.

MATERIALS AND METHODS

Animals

Three-week old male C57BL/6J control mice were obtained from Jackson Laboratories (Bar Harbor, ME). All animals (wt/wt), were maintained at 25°C, with a 12:12-h light-dark cycle (lights on from 6:30 AM to 6:30 PM), for at least 1 wk before the experiment to acclimate to the new research environment. All animals had free access to water and chow (Purina Rodent Chow #5008: 6.5% fat, 49% carbohydrate, and 23% protein, wt/wt, 3.5 kcal/g) for the acclimation period. After the acclimatization period, animals were weighed and divided into groups of approximately equal mean body weight for the experiments outlined in Experimental Procedures. For subsequent experiments, animals

Obesity is the most common nutritional disorder in Western populations and is characterized by a chronic imbalance between energy intake and expenditure (29). During periods of positive energy balance, the ability to limit obesity depends on the ability of the organism to increase energy expenditure and to decrease subsequent energy intake. Our understanding of the factors regulating energy homeostasis has been greatly advanced by the discovery of leptin (42), a hormone that communicates information about energy stores to the brain (14). The hypothalamus is a major site of leptin action (13). Numerous hypothalamic neuropeptides that are involved in the control of feeding behavior are direct or indirect targets of leptin action (11, 13, 24).

Exposure to high-fat diets for a prolonged period results in positive energy balance and obesity in certain strains of mice. Despite the potential importance of this nutritional model for our understanding of human obesity, detailed information on the response of leptin and leptin-regulated neuropeptides to high-fat feeding is limited. Previous studies have examined the effect of caloric intake and diet composition on leptin levels and on the expression of neuropeptides involved in energy homeostasis. However, these studies have generally focused on single specific time points and examined only a limited number of neuropeptides (5–7, 15, 19, 34, 39). The effect of high- vs. low-fat diets on the expression of hypothalamic neuropeptides and neuroendocrine function has not been examined in a longitudinal manner. To gain further insight into the early physiological response of normal mice to diets that are capable of inducing obesity, we studied the hypothalamic expression of neuropeptides implicated in weight regulation and the neuroendocrine system in the obesity-susceptible C57BL/6J strain of mice fed high- or low-fat diets longitudinally, over a period of 2 wk.
were fed a choice of two high-fat diets or a low-fat diet as indicated below. The high-fat diets used in these experiments were from either Harlan Teklan (#TD 88137; 42.16% of calories from fat, 42.81% carbohydrate, and 15.02% protein (wt/wt), 4.53 kcal/g, as previously described; Ref. 17), and will be referred to as H-T diet, or from Research Diets (#D12451: 44.9% fat, 35.1% carbohydrate and 20.0% protein (wt/wt), 4.73 kcal/g, as previously described; Ref. 37) and will be referred to as R.D. The low-fat diet was from Research Diets (#D12450B: 10.0% fat, 70.0% carbohydrate and 20.0% protein (wt/wt), 3.85 kcal/g, as previously described; Ref. 37). The high-fat R.D diet had the same source of fat as the low-fat diet, and the amounts of protein and fiber were also identical.

All animals were handled in accordance with the principles and guidelines established by the National Institutes of Health, and the protocol was approved by the Institutional Review Board, Beth Israel Hospital, Boston, MA, as previously described (26).

**Experimental Procedures**

In all experiments, animals were studied in the fed state, because study in the fasted state would result in altered neuropeptide levels due to fasting.

**First study.** In two experiments, we evaluated the effect of two high-fat diets with a similar percentage of calories derived from fat (42.0 and 44.9%, respectively) vs. a low-fat diet (10% of calories from fat) on the expression of hypothalamic neuropeptides after 2 days and 1 wk on the diet. Mice (n = 8/group) were killed between 8:00 and 9:30 AM; hypothalami were collected for the analysis of neuropeptide gene expression, and sera were collected for the analysis of leptin, insulin, corticosterone, and free fatty acids (FFA).

**Second study.** Based on the results of feeding the high-fat diet for 2 days and 1 wk, we performed a second study to examine the effects of a shorter (1 day) and longer (2 wk) duration of high-fat feeding on hypothalamic expression of mRNA encoding neuropeptides implicated in weight regulation and neuroendocrine function. Because the two high-fat diets gave identical results for the initially studied time points, for this experiment, only the H-T high-fat diet was compared with the low-fat diet. Mice (n = 8/group for the 1-day time point and n = 6/group for the 2-wk time point) were again killed between between 8:00 and 9:30 AM. Hypothalami and sera were also collected at this time.

**Frequent sampling study.** Results from the above described studies showed that neuropeptide Y (NPY) and agouti-related protein (AgRP) mRNA levels were altered by 48 h of high-fat feeding, even though leptin levels were similar. However, plasma leptin levels display a significant ultradian and circadian variation with a nocturnal peak, which is partially nutrition dependent. Thus changes in NPY and AgRP gene expression seen after 2 days of high-fat feeding might have been due to changes of leptin, insulin, or corticosterone release that would not be evident from baseline blood sampling. To investigate this possibility, 144 C57Bl/6J male mice were divided into nine cohorts. Each cohort included two groups of mice, one fed the high-fat diet and the other the low-fat diet (n = 8/group). The first cohort of mice was killed at 0 h (8:00 AM) to obtain baseline hormone levels, and the other cohorts were killed after 6 h (2:00 PM), 12 h (8:00 PM), 18 h (2:00 PM), 24 h (8:00 AM), 30 h (2:00 PM), 36 h (8:00 PM), 42 h (2:00 AM), and 48 h (8:00 AM) after exposure to these respective diets.

**Physiological Characterization**

Body and food weights were obtained using an analytical balance, at the beginning and at the end of the experiment for the 1-day, 2-day, and 1-wk experiments. Body weights and food intake were measured daily from the day before the experiment was begun, through the entire experimental period for the 2-wk experiment. Food was weighed at the beginning of each day. The differences were assumed to represent the amount of food eaten per day. Careful inspection of the cages revealed no detectable spillage of food.

**Tissues**

After death, the brain was dissected from the skull as previously described (25). A region bordered dorsally by the thalamus, rostrally by the optic chiasm, and caudally by the mamillary bodies was excised (25). Hypothalami removed were immediately frozen in liquid nitrogen and stored at −80°C.

**Measurements**

After death and decapitation, truncal blood was collected for measurement of hormones. Serum was immediately isolated and stored at −80°C until assayed for leptin, corticosterone, insulin, and FFA. Leptin, corticosterone, and insulin concentrations were assessed by radioimmunoassay (mouse leptin and rat insulin kit: Linco Research Institute, St. Louis, MO; mouse corticosterone kit: ICN, Costa Mesa, CA) as previously described (4, 25). Nonesterified FFA were measured by an in vitro enzymatic colorimetric method (Wako Chemicals, Richmond, VA).

**Carcass Analysis**

Body composition was assessed by whole carcass analysis, which was performed as described earlier (32). Briefly, eviscerated carcasses were digested by alcoholic potassium hydroxide hydrolysis at 60°C over 2 days with saponification of all fats, neutralization, and then enzymatic determination of glycerol. Body lipid was determined by multiplying moles of glycerol per mouse and the average relative molecular mass of a triglyceride molecule (860 g/mol).

**Preparation and Quantification of NPY, AgRP, POMC, Orexin, and Suppressor of Cytokine Signaling-3 Protein mRNA**

RT-PCR analysis was used to assess the effect of diets on the hypothalamic mRNA expression of NPY, AgRP, POMC, orexin, and suppressor of cytokine signaling protein-3 (SOCS-3). Total hypothalamic RNA was purified using the RNA STAT-60 method from TEL-TEST “B” INC (TEL-TEST, Friendswoods, TX). Quantity and purity were determined by absorption at 260 and 280 nm. cDNA was synthesized in parallel from 1 µg of total RNA from all hypothalamic samples at each time point using the Advantage RT-PCR kit from Clontech (Clontech, Palo Alto, CA). The final volume of cDNA was 100 µL. 141-bp NPY cDNA was amplified using the following primers: upstream primers 5'-GCTTGAAAGACCTCTTCCATGTGGTGTG-3', downstream primers 5'-GGCGGAGTCGACGCCATGCTG-3'; 251-bp AgRP cDNA was amplified using the following primers: upstream primers 5'-GAAGGGCTCGACCAGGCTCTGTTCC-3', downstream primers 5'-AAAGGCTATTGAGAAGGCGCAG-3'; 200-bp orexin cDNA was amplified using the following primers: upstream primers 5'-CTTGGTATTTGGACAGCAGCAG-3', downstream primers 5'-ACGTCTTCTGGCCGACAGCAG-3'; 301-bp POMC cDNA was amplified...
amplification was linear for a number of cycles: for NPY, AGRP, POMC, and orexin; and to 25 cycles for SOCS-3: denaturation at 95°C for 1 min, annealing at 60°C for 1 min, and extension at 72°C for 45 sec (8). Then 10 μl of the reaction were combined with 5 μl of sequencing stop solution (Amersham International, Buckinghamshire, UK) and heated to 85°C for 5 min before 4 μl were loaded onto a 4% urea-acrylamide gel (38 × 31 × 0.3 cm). Electrophoresis was carried out at 60 W of constant power for 1.45 h, before the gels were transferred to filter paper, dried, and finally subjected to 32P-quantitation by PhosphorImager analysis (Molecular Dynamics, Sunnyvale, CA; Ref. 8).

Table 1. Effects of high- and low-fat feeding of C57Bl/6J mice on body weight, cumulative food intake, and carcass lipid

<table>
<thead>
<tr>
<th>Groups</th>
<th>Initial Weight, g</th>
<th>Final Weight, g</th>
<th>Cumulative Food Intake, g</th>
<th>Cumulative Caloric Intake, g</th>
<th>Total Lipid, g</th>
</tr>
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<tbody>
<tr>
<td>1 day</td>
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<tr>
<td>High fat (H-T)</td>
<td>20.27 ± 0.59</td>
<td>20.54 ± 0.68</td>
<td>4.24 ± 0.31</td>
<td>19.20 ± 1.39</td>
<td>ND</td>
</tr>
<tr>
<td>Low fat</td>
<td>20.18 ± 0.42</td>
<td>20.42 ± 0.39</td>
<td>3.94 ± 0.36</td>
<td>15.16 ± 1.40</td>
<td>ND</td>
</tr>
<tr>
<td>2 days</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>High fat (H-T)</td>
<td>20.81 ± 0.14</td>
<td>21.28 ± 0.10</td>
<td>7.43 ± 0.26</td>
<td>33.64 ± 1.16</td>
<td>1.95 ± 0.15</td>
</tr>
<tr>
<td>High fat (R.D)</td>
<td>20.84 ± 0.16</td>
<td>21.26 ± 0.25</td>
<td>7.00 ± 1.28</td>
<td>31.74 ± 1.62</td>
<td>1.92 ± 0.11</td>
</tr>
<tr>
<td>Low fat</td>
<td>20.81 ± 0.13</td>
<td>20.63 ± 0.31</td>
<td>6.34 ± 0.41</td>
<td>24.40 ± 1.58</td>
<td>1.66 ± 0.14</td>
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<tr>
<td>1 week</td>
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<tr>
<td>High fat (H-T)</td>
<td>20.78 ± 0.25</td>
<td>21.65 ± 0.45</td>
<td>21.48 ± 0.36</td>
<td>97.28 ± 1.61</td>
<td>2.40 ± 0.40</td>
</tr>
<tr>
<td>High fat (R.D)</td>
<td>20.76 ± 0.23</td>
<td>21.08 ± 0.24</td>
<td>20.05 ± 0.34</td>
<td>94.84 ± 1.59</td>
<td>2.45 ± 0.29</td>
</tr>
<tr>
<td>Low fat</td>
<td>20.73 ± 0.18</td>
<td>21.31 ± 0.35</td>
<td>24.50 ± 0.47</td>
<td>94.37 ± 1.83</td>
<td>2.45 ± 0.34</td>
</tr>
<tr>
<td>2 weeks</td>
<td></td>
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</tr>
<tr>
<td>High fat (H-T)</td>
<td>20.64 ± 0.31</td>
<td>23.97 ± 0.32</td>
<td>52.80 ± 0.90</td>
<td>239.18 ± 4.08</td>
<td>2.46 ± 0.26</td>
</tr>
<tr>
<td>Low fat</td>
<td>20.66 ± 0.27</td>
<td>22.95 ± 0.28</td>
<td>55.99 ± 0.15</td>
<td>215.55 ± 4.45</td>
<td>2.19 ± 0.23</td>
</tr>
</tbody>
</table>

Values are means ± SE (n = 8/group for 1 day; 2-day, and 1-wk time points; n = 6/group for 2-wk time point). Comparison of effect of high- and low-fat feeding of C57Bl/6J mice on final weight, cumulative food intake, total caloric intake, and total lipid for 1-day, 2-day, 1-wk, and 2-wk time points. H-T, Harlan Teklen diet; R.D, high-fat Research Diets (see MATERIALS AND METHODS for diet compositions; ND, not determined. *P < 0.05; *P < 0.01; †P < 0.001 vs. the high-fat group by ANOVA with post hoc tests. ‡P < 0.05, §P < 0.01 vs. the high-fat H-T group by ANOVA with post hoc tests; †P < 0.05, §P < 0.01 vs. the low-fat group by unpaired t-test.

Calculations and Statistical Analysis

All results are means ± SE of the number of values indicated (see Fig. 1 and Tables 1 and 2). This study was designed to have >80% power at the conventional a = 0.05 level to detect a significant difference in mean levels that would be ≥1.1 times the respective SD. Statistical significance was assessed by unpaired two-tailed Student’s t-test and with ANOVA using the Statview program (Abacus). To confirm that equality of variances did not interfere with data interpretation, data were also analyzed using the SPSS program (SPSS, Chicago, IL), which provides P values after taking into consideration whether the respective variances are equal or unequal (Levene’s test). Differences were considered significant at the two-tailed P < 0.05 value.

RESULTS

Effects of High- and Low-fat Diets of Various Durations on Body Weight, Caloric Intake, and Body Composition

One-day experiment. Changes in body weight, food intake, and caloric intake were assessed in response to 1 day of high- and low-fat feeding. The groups were weight-matched at baseline. After 1 day on the diet, mean body weights did not differ between the high-fat (H-T) and the low-fat fed groups (Table 1). Although the high-fat group tended to ingest more total kilocalories than did the low-fat group, these differences did not reach statistical significance (Table 1).

Two-day experiment. After 2 days on the diets, the final weights did not differ among the two high-fat- and the low-fat-fed groups (Table 1). Both high-fat groups had significantly greater cumulative caloric intake over the 2-day period than did the low-fat group. Total carcass lipid did not differ among the three groups (Table 1).

One-week experiment. After 1 wk of exposure to the two high-fat and the low-fat diets, the final weights did
not differ among the three groups (Table 1). There was also no difference in the cumulative caloric intake among the three groups after 1 wk (Table 1). Given the increase in cumulative caloric intake in high-fat groups after 2 days, this indicates that a compensatory fall occurred in caloric intake in the period between 2 and 7 days. The total carcass lipid was not statistically different among the three groups (Table 1).

**Two-week experiment.** The 2-wk time point was the first time point at which significant changes in body weight were observed, with final weight being higher in the high-fat group. The total caloric intake was also significantly higher in the high-fat-fed group. Total carcass lipid was not different between the two groups (Table 1).

**Hypothalamic Neuropeptide mRNA Expression in Response to High-Fat and Low-Fat Feeding of C57BL/6J Mice**

To determine the effect of diet on the expression of hypothalamic neuropeptides in C57BL/6J mice, hypothalami of male mice were obtained and hypothalamic mRNA was isolated.

**NPY mRNA expression.** NPY has been implicated in upregulation of food intake and reduction of sympathetic nervous system activation and energy expenditure and in the activation of several neuroendocrine axes (40). After 1 day, hypothalamic NPY mRNA did not differ between the high- and the low-fat-fed groups (Table 2). After 2 days of high-fat feeding, NPY mRNA levels were significantly decreased in both high-fat groups compared with the low-fat-fed group (Table 2). After 1 wk, the expression of NPY mRNA returned to baseline and remained similar in the high-fat and low-fat groups at 2 wk (Table 2).

**AgRP mRNA expression.** Most NPY neurons in the arcuate nucleus co-express AgRP, an orexigenic neuropeptide that is an endogenous antagonist to α-melanocyte-stimulating hormone at the melanocortin MC3 and MC4 receptor level (11). AgRP expression is increased in low leptin states and is reduced by leptin, and its ectopic expression in transgenic mice causes obesity (28). Similar to NPY, 1 day of high-fat feeding had no effect on the expression of AgRP mRNA, but 2 days of high-fat feeding caused a significant reduction of ~50% (Table 2). After 1 wk, the expression of AgRP mRNA in the high-fat-fed groups returned toward baseline and continued to remain similar to that in the low-fat group at 2 wk (Table 2).

**Orexin mRNA.** Hypothalamic orexin mRNA expression was similar in the high and low-fat fed animals at all the time points studied (Table 2). Thus neither high nor low-fat feeding alters hypothalamic orexin expression over a 2-wk period.

**POMC mRNA expression.** We observed no significant difference in POMC mRNA between the two diet groups at the 1-day, 2-day, or 1-wk time points. A significant increase in POMC expression was observed in the high-fat-fed group only after 2 wk on the high-fat diet (Table 2).

**SOCS-3 mRNA expression.** Hypothalamic SOCS-3 mRNA expression in high-fat fed mice was not significantly different from that of low-fat fed mice at all the time points studied (Table 2).

**Effects of High or Low-Fat Feeding on Circulating Hormone Concentrations and FFA**

**Leptin.** Mice fed high-fat diets for 1 day, 2 days, 1 wk, and 2 wk had basal leptin levels similar to the low-fat group (data not shown).

**Corticosterone.** For all the time points analyzed, corticosterone levels showed no significant difference between the high- and the low-fat-fed groups (data not shown).

**Insulin.** Insulin levels did not differ between the studied groups at any of the time points analyzed (data not shown).

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**Table 2. Effects of high- and low-fat feeding on C57Bl/6J mice on hypothalamic expression of neuropeptides**

<table>
<thead>
<tr>
<th>Groups/Diets</th>
<th>NPY mRNA</th>
<th>AgRP mRNA</th>
<th>POMC mRNA</th>
<th>Orexin mRNA</th>
<th>SOCS-3 mRNA</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 day</td>
<td></td>
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</tr>
<tr>
<td>High fat (H-T)</td>
<td>85.8 ± 4.9</td>
<td>107.1 ± 10.4</td>
<td>110.0 ± 11.8</td>
<td>80.2 ± 5.3</td>
<td>89.4 ± 9.6</td>
</tr>
<tr>
<td>Low fat</td>
<td>100.0 ± 9.6</td>
<td>100.0 ± 12.2</td>
<td>100.0 ± 10.3</td>
<td>100.0 ± 12.9</td>
<td>100.0 ± 18.8</td>
</tr>
<tr>
<td>2 days</td>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>High fat (H-T)</td>
<td>71.1 ± 4.6*</td>
<td>47.7 ± 3.2†</td>
<td>88.5 ± 8.9</td>
<td>103.1 ± 14.1</td>
<td></td>
</tr>
<tr>
<td>High fat (R.D)</td>
<td>74.5 ± 5.2†</td>
<td>48.4 ± 4.8†</td>
<td>86.6 ± 8.9</td>
<td>106.7 ± 7.6</td>
<td></td>
</tr>
<tr>
<td>Low fat</td>
<td>100.0 ± 8.0</td>
<td>100.0 ± 11.2</td>
<td>100.0 ± 10.9</td>
<td>100.0 ± 14.2</td>
<td>100.0 ± 9.9</td>
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<tr>
<td>1 week</td>
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<tr>
<td>High fat (H-T)</td>
<td>119.6 ± 7.2</td>
<td>91.9 ± 11.5</td>
<td>114.6 ± 6.4</td>
<td>129.0 ± 18.1</td>
<td>118.7 ± 15.0</td>
</tr>
<tr>
<td>High fat (R.D)</td>
<td>104.7 ± 9.8</td>
<td>82.3 ± 8.9</td>
<td>104.3 ± 12.1</td>
<td>136.7 ± 13.8</td>
<td>88.7 ± 12.1</td>
</tr>
<tr>
<td>Low fat</td>
<td>100.0 ± 8.7</td>
<td>100.0 ± 12.8</td>
<td>100.0 ± 13.4</td>
<td>100.0 ± 8.1</td>
<td>100.0 ± 11.8</td>
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<tr>
<td>2 weeks</td>
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</tr>
<tr>
<td>High fat (H-T)</td>
<td>104.9 ± 9.6</td>
<td>107.5 ± 18.8</td>
<td>176.0 ± 20.3</td>
<td>100.5 ± 9.7</td>
<td>119.3 ± 7.3</td>
</tr>
<tr>
<td>Low fat</td>
<td>100.0 ± 9.1</td>
<td>100.0 ± 7.9</td>
<td>100.0 ± 7.6</td>
<td>100.0 ± 10.8</td>
<td>100.0 ± 5.1</td>
</tr>
</tbody>
</table>

*Data are means ± SE (n = 8/group for 1-day, 2-day, and 1-wk time points; n = 6/group for 2-wk time point). Data are mean values (from arbitrary PhosphorImager units) of each group corrected for β-actin values and then normalized to mRNA expression of low-fat fed group, which is considered to be 100. Comparison of the effect of high- and low-fat feeding of C57Bl/6J mice for 1-day, 2-day, 1-wk, and 2-wk time points on the hypothalamic expression of mRNA of neuropeptide Y (NPY), agouti-related protein (AgRP), proopiomelanocortin (POMC), orexin, and suppressor of cytokine signaling-3 (SOCS-3). *P < 0.01; †P < 0.001 vs. low-fat diet group by ANOVA with post hoc tests; ‡P = 0.01 vs. the low-fat group by unpaired t-test.*
FFA. At the 1-day, 2-day, and 1-wk time points, there was no significant difference between the high and low-fat-fed groups (data not shown). A significant difference was observed at 2 wk, corresponding to the first time point at which a significant increase in body weight was observed at the high-fat fed group (1.97 ± 0.15 vs. 1.18 ± 0.24 meq/l, for the high- vs. the low-fat-fed group, respectively, \( P < 0.05 \)).

**Frequent Sampling Experiment: Effects of High or Low-Fat Feeding on Circulating Hormone Concentrations Throughout a 2-day Period**

To determine whether the changes of NPY and AgRP gene expression seen after 2 days of high-fat feeding might have been due to changes of leptin, insulin, or corticosterone that were not evident from baseline blood sampling, we killed mice fed high- or low-fat diets every 6 h for a 2-day period and collected plasma for the analysis of several hormones.

**Leptin.** We confirmed that leptin has a diurnal rhythm. Its levels are higher at night and start to increase after the onset of the light-off period, when the rodents increase their eating behavior (2, 31). During the first day of high-fat feeding, the area under the curve (AUC) for leptin was significantly higher compared with the AUC for the low-fat fed group (134.6 ± 10.3 vs. 100.0 ± 12.3, \( P = 0.028 \)). During the second day of high-fat feeding, the same pattern was observed, with the high-fat-fed group having significantly higher AUC than the low-fat group (126.5 ± 8.2 vs. 100.0 ± 5.2, \( P = 0.016 \)) (Fig. 1). Thus use of this frequent sampling approach, induction of a state of positive energy balance after high-fat feeding, increases leptin levels during the first 48 h of diet exposure.

**Insulin.** During both the 1st and 2nd days, the AUC for insulin for the high-fat-fed group was not statistically different from the low-fat group. Thus insulin levels appear not to change significantly during 2 days of high-fat feeding compared with the low-fat group. As expected, insulin secretion is stimulated in response to feeding, with highest levels between 8.00 PM and 2.00 AM (data not shown).

**Corticosterone.** During both days, corticosterone levels increased in the dark cycle and decreased to the lowest levels at the beginning of the light cycle. The AUC during the first day of high-fat feeding was significantly lower than in the low-fat group (66.0 ± 6.9 vs. 100.0 ± 12.6, \( P = 0.03 \)), and the second day it did not differ from the AUC for the low-fat fed group (103.7 ± 17.1 vs. 100.0 ± 7.2, \( P = 0.84 \)).

**DISCUSSION**

Exposure to a high-fat diet has the capacity to induce positive energy balance and eventually obesity, but the central mechanisms that accompany and underlie this transition have not been fully elucidated. Previous studies have examined the physiological regulation exerted by nutritional factors (caloric intake/diet composition) on the hypothalamic neuropeptides involved in energy homeostasis, but these studies generally focused on only limited time points and specific neuropeptides, most commonly NPY (5–7, 15, 19, 34, 39). We therefore studied the responses of several hypothalamic neuropeptides (NPY, AgRP, POMC, and orexin) and a potential regulatory molecule of leptin action (SOCS-3) in C57Bl/6J mice exposed to continuous high-fat vs. low-fat feeding for a period of 2 wk. The sensitivity of mice to high-fat diet-induced obesity varies considerably between mouse strains, and C57Bl/6J mice are among the more sensitive to this effect (35, 38).

We found that 1 day on high- or low-fat diets was not sufficient to alter the hormonal levels and the hypothalamic expression of the neuropeptides studied. After 2 days on diets, when cumulative caloric intake was significantly higher in the high-fat-fed groups compared with the low-fat-fed group, NPY mRNA expression was reduced in the hypothalamism of the high-fat-fed mice. Levels of AgRP mRNA, which is coexpressed with NPY in certain neurons in the arcuate nucleus, were also decreased. This decrease in NPY and AgRP mRNA levels in the high-fat-fed group was temporally associated with the induction of a state of positive energy balance and hyperphagia in the high-fat-fed group. Thus hyperphagia in the early period after high-fat feeding is not mediated by induction of these orexigenic neuropeptides. Rather, these changes appear to be a compensatory response to the hyperphagia and positive energy balance.

Although it is now accepted that food deprivation increases NPY and AgRP mRNA expression (3, 27), these data are the first to show a decrease of NPY and AgRP mRNA in the early response to a high-fat diet. To elucidate the mechanisms underlying this decrease in AgRP and NPY mRNA in the high-fat-fed group, we examined several potential hormonal regulators of NPY and AgRP expression. Leptin, a circulating adipocyte-derived hormone, the levels of which reflect the amount of fat stores (23), inhibits food intake and
stimulates energy expenditure (9) at least in part by altering the expression of hypothalamic neuropeptides (9). Leptin levels measured as a single morning specimen, as is typical in studies of this kind, were not different between the two groups at that early time point. Because leptin has a diurnal rhythm (21), we decided to perform a frequent sampling experiment to evaluate the leptin axis more carefully. This allowed us to confirm that leptin has a diurnal rhythm and that leptin levels were higher at night, increasing after the onset of the light-off period (2, 31). Moreover, this allowed us to determine that leptin AUC for the high-fat-fed group was significantly higher than that of the low-fat-fed group on both the first and second days. These changes in leptin levels, although modest, may underlie the decreased NPY and AgRP mRNA expression observed after 2 days on a high-fat diet. We recently showed that NPY expression falls in response to modest elevations of plasma leptin above the normal fed range in normal rats (1). The suppression of NPY and AgRP expression could also be influenced by factors other than leptin.

Basal levels of other hormones implicated in the regulation of energy homeostasis and NPY expression, i.e., corticosterone and insulin, were not different between the two groups. Corticosterone levels displayed a diurnal rhythm as reported previously (2, 41). The AUC for corticosterone was lower for the high-fat-fed group during the first day but did not differ between the two groups during the second day. Thus our data provide no evidence that either insulin or corticosterone is responsible for the observed changes in hypothalamic neuropeptides.

After 1 wk, the hyperphagia observed after 2 days on high-fat feeding was no longer present, and caloric intake was reduced in the period between 2 and 7 days. Interestingly, the expression of the hypothalamic neuropeptides was similar at the 1-wk time point. It has been previously reported that rats fed a highly palatable, intermediate high-fat diet (34.34% calories from fat) for 7 days did not have altered arcuate nucleus NPY mRNA levels compared with rats fed a bland cornstarch-based diet (5% calories from fat), whether they were allowed to overeat or were pair fed to the control group (19). Similarly, Giraudo et al. (15) showed that intermediate high-fat feeding (59.0% from fat) of rats for 1 wk, which did not produce weight gain or alter caloric intake, did not alter the expression of NPY mRNA in the arcuate nucleus. Our findings regarding NPY mRNA in mice fed a 42% high-fat diet are consistent with these studies. In contrast, in the study of Giraudo et al., another group of rats fed a higher-fat diet (77% of total calories from fat) for 1 wk had decreased NPY mRNA levels in the arcuate nucleus, despite equivalent caloric intake. Thus the expression of NPY may be influenced by the fat content of the diet in a manner that reflects both the duration of the diet and the precise percentage of dietary fat. Although caloric intake was higher in the high-fat-fed group after 2 days, by 1 wk these mice had diminished their food intake to maintain a stable body weight. This may be a result of the decreased expression of the two orexigenic neuropeptides observed after 2 days.

The 2-wk time point was the first at which significant changes in body weight were observed and total caloric intake was significantly higher in the high-fat-fed group. In a previous study (37), when rats were fed either a high-carbohydrate diet (10% of energy from fat), a high-fat diet (68% energy from fat), or a control well-balanced diet (30% of energy from fat), the total caloric intake was significantly higher in rats fed the high-fat diet, and high-fat-fed rats gained more weight than the two other groups. In addition, NPY mRNA was decreased in the lateral hypothalamus of the high-fat-fed group but was unchanged in other hypothalamic sites, including arcuate, dorsomedial, ventromedial, or suprachiasmatic nuclei. Similarly, Seeley et al. (33) have shown that forced overfeeding for a similar period of time fails to alter hypothalamic NPY gene expression in rats. It has also been reported (20) that feeding diet-prone or diet-resistant rats a diet high in energy as in our study (4.47 kcal/g, 31% of energy from fat) for 2 wk did not alter NPY gene expression in the arcuate nucleus. Our findings are consistent with the results from these studies, because 1 wk after high-fat feeding we observed no change in the expression of either NPY or AGRP mRNA.

In contrast, we found POMC mRNA to be elevated after 2 wk of high-fat intake. Hagan et al. (16) reported that 11 days of forced overfeeding caused a significant elevation in POMC mRNA in the arcuate nucleus. Because POMC is the precursor of melanocyte-stimulating hormone, a potent inhibitor of food intake, the hypothalamic overexpression of POMC may in part mediate this effect of overfeeding and positive energy balance to resist the effect of high-fat diet to promote increased food intake in an effort to maintain energy homeostasis. The mechanism responsible for increased POMC expression remains to be elucidated, because altered hormonal levels at this time do not appear to be responsible for the observed changes in POMC expression. More specifically, basal corticosterone, insulin, and leptin levels were not different between the high-fat and the low-fat group, but circadian changes were not assessed.

In a long-term experiment, Bergen et al. (7) have shown that the A/J resistant to diet-induced obesity mice maintain reduced levels of NPY and increased levels of POMC after 14 wk on a high-fat diet. These compensatory hypothalamic changes may be associated with the resistance of the A/J mice to the effect of the high-fat diet. At the same time point, the C57Bl/6J obesity-prone mice failed to maintain these compensatory responses, and NPY and POMC levels in the high-fat-fed group were not different from the chow-fed group (7). From our data, it seems that the C57Bl/6J mice do show these compensatory changes at early time points, but they may not be able to maintain them, and this could contribute to their becoming obese after a prolonged period of high-fat diet.

Because body weight of these animals was increased at the 2-wk time point, we also assessed leptin levels.
and hypothalamic SOCS-3 mRNA expression. SOCS-3 expression is stimulated by leptin and provides an in vivo indicator of direct leptin stimulation of neurons expressing the long form of the leptin receptor (8). We show here that similar to leptin levels, SOCS-3 mRNA expression was not different between the two groups.

Orexin mRNA did not vary in response to high-fat or low-fat feeding in C57BL/6J mice at any of the study time points. Orexin A and B are recently described hypothalamic neuropeptides, which were originally proposed to act to promote feeding after intracerebroventricular administration (30). However, their effect is significantly weaker than that of NPY, and thus their physiological importance as appetite regulators remains doubtful (12). Others have concluded that injection of orexin A and B in the third ventricle is more likely to result in the control of energy metabolism than of food intake (22). Similarly, the results of Ida et al. (18) and findings from studying the orexin knockout mice (10) suggest that orexin might primarily regulate sleep/wakefulness states and other behavioral activities, rather than feeding. A recent study (36) has shown that the orexin A expression in several hypothalamic regions did not change significantly in response to fasting or high-fat diet or in hyperleptinemic and leptin-resistant states (obese Zucker rats). Our data are consistent with this study and indicate that high-fat feeding fails to alter orexin levels in normal mice, at early time points, when baseline leptin is not elevated, and at a later time point, when high-fat-fed mice have significantly higher baseline leptin levels.

In summary, it is apparent that the hypothalamic response to high-fat feeding is complex and involves temporal changes over a period of days and weeks. Although these observations provide important insights into the regulation of molecules influencing energy homeostasis during exposure to high-fat diet, there are a number of important unanswered questions arising from these results. Is the increase in leptin during the first 2 days responsible for the observed decrease in NPY and AgRP, and is this decrease of orexigenic neuropeptides responsible for the subsequent decrease in hyperphagic response? Is altered leptin circadian rhythm responsible entirely or in part for the late increase in POMC expression, and is this effect, like the decreased NPY and AGRP seen earlier, an important compensation for the attempted resistance to diet-induced obesity? Are other mediators of energy expenditure, like thyroid and growth hormones or peptides like cholecystokinin, gastrin-releasing peptide, and enterostatin (1) responsible in part for the observed changes in neuropeptides, and do they play a role in the development of diet-induced obesity? Future studies will be needed to clarify the underlying mechanisms through which high-fat diet affects the hypothalamic neuropeptides involved in the regulation of energy homeostasis and how these changes affect the variable sensitivity to diet-induced obesity among different strains of mice. In addition, future experiments will be needed to elucidate the specific hypothalamic areas where these changes occur.

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