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Increased caloric intake after a high-fat preload: Relation to circulating triglycerides and orexigenic peptides

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Abstract

To investigate mechanisms that mediate the greater food intake induced by a fat-rich diet, the present study tested an acute "preload-to-test meal" paradigm in normal-weight rats. In this paradigm, the rats were given a small high-fat (HF) compared to low-fat (LF) preload and, after an intermeal interval, allowed to consume freely on a subsequent test meal. Modified versions of this paradigm were tested to determine the robustness of the greater caloric intake induced by the HF preload while standardizing the test protocol. A HF preload of 10–15 kcals, compared to an equicaloric LF preload, significantly increased food intake by 40–50% in the subsequent test meal. This effect, a 4–6 kcal increase, was observed with HF preloads equal in energy density and palatability to the LF preloads. It was evident with preloads or test meals that were liquid or solid, preloads that were injected, test meals that had variable fat content, and natural intermeal intervals of 60–120 min. This overeating after a HF preload was invariably associated with a 2- to 3-fold increase in circulating levels of triglycerides (TG), with no change in leptin or insulin. It was also accompanied by increased expression of the orexigenic peptides, galanin in the paraventricular nucleus and orexin in the perifornical lateral hypothalamus. Moreover, if given repeatedly over several days, the HF compared to equicaloric LF preload significantly increased 24-h food intake. These results establish a protocol for studying the phenomenon of increased feeding on a HF diet under controlled conditions and suggest possible underlying mechanisms involving circulating lipids and orexigenic peptides.

Keywords: Galanin; Orexin; Triglycerides; Fat-induced feeding

1. Introduction

There is extensive evidence showing overeating to occur in animals allowed to feed on a high-fat (HF) diet with greater than 40% fat compared to a low-fat (LF) diet with less than 20% fat. With diets available *ad libitum*, this increased consumption of a HF diet relative to a LF diet has been described under both chronic and acute conditions and in humans as well as rodents [1–5]. Rats maintained chronically on a HF diet, whether solid, liquid or semisolid, consume more daily calories then on a LF diet [5–8], and this HF-induced overeating is observed even when a nutritionally complete food, standard lab chow, is present [7,9]. With acute dietary manipulations, *ad libitum* consumption of a liquid or solid HF diet over a 1–3 h period produces a larger meal than with the LF diet, and this effect can be seen even when the animal itself infuses the liquid HF meal intragastrically [1,10–13]. This greater caloric intake occurs independently of the energy density of the meal, and it can be revealed in the form of a larger meal size as well as greater meal frequency [9,12]. Similar results have been obtained in human studies, which show that dietary fat increases meal size as well as daily caloric intake [14].

Behavioral studies in rats and humans, under both chronic and acute conditions with *ad libitum* feeding, have focused on specific properties of a HF diet, most notably its greater palatability, greasy texture and energy density, as being important in the stimulation of eating behavior [2,3,15–17]. Dietary fat enhances the flavor of the other macronutrients in the mouth [18], and rats show a preference for a chow diet made greasy with a non-caloric mineral oil [17]. However, these properties alone do not increase food intake, as shown by the evidence that rats fail to overeat a greasy, mineral oil diet when given *ad libitum* [17]. Moreover, in studies that bypass the ingestion

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process, a HF solution continuously infused intragastrically or intravenously similarly increases caloric intake as compared to a chronically infused, equicaloric glucose solution [19,20]. Whereas the higher energy density of a HF compared to LF diet may have some impact on caloric intake, the importance of this factor is also in question, since overeating can occur even when the diets are equal in caloric density [7,12,21,22]. This evidence has led investigators to consider post-ingestive and post-absorptive factors in producing overeating, although information on such mechanisms has proven difficult to obtain using the *ad libitum* feeding paradigms. Free feeding on a chronic or acute HF diet increases body weight and body fat as well as daily caloric intake and meal size [2,5–7,12,13,16,17]. Thus, any physiological and neurochemical changes observed in association with this greater caloric intake may be more a consequence of these factors than an indication of mechanisms causally related to the overeating on a fat-rich diet.

An acute feeding model, referred to as a "preload-to-test meal" paradigm, directly addresses this issue and therefore may help to elucidate mechanisms underlying the greater caloric intake associated with a HF relative to LF diet. This paradigm, which involves a HF or LF preload meal followed after a short interval by a test meal, is particularly useful in controlling for variables related to palatability, energy density and caloric intake, as well as body weight and adiposity. With these controls, one can investigate signals produced by a HF meal that, in addition to shortening the post-meal interval, promote overeating in a subsequent test meal [13,22,23]. In animal studies, there are reports with pure macronutrients that show a preload of corn oil to be less satiating than an equicaloric carbohydrate or protein preload and to be followed after 90 min by a larger test meal [22]. This HF-induced increase in caloric intake can be seen when the HF and LF preload diets are equal in energy density as well as caloric content [11,24,25], and it is evident across a range of preload volumes and intermeal intervals [11]. Moreover, it is observed when the preloads are equally palatable [11] or infused intragastrically [26], supporting the idea that palatability is not a key factor. In some [23,27] but not all [22,28] investigations in humans, food ingestion or gastric infusions demonstrate a similar pattern of reduced satiety and increased eating after a HF preload. Thus, the acute preload-to-test meal paradigm, with greater control of palatability, energy density and caloric intake compared to the *ad libitum* feeding paradigms, should be a useful tool in revealing mechanisms that precede and possibly contribute to the phenomenon of overeating in normal-weight animals.

The present report focused on this preload-to-test meal model. Building on the work of Warwick et al. [7,9,11,12,24], the first series of experiments set out to validate the increase in feeding effect in tests involving a variety of small preload diets (10–15 kcals), test meal diets, intermeal intervals and preload injections that bypass the ingestion process. With these tests standardizing a paradigm that consistently reveals greater food intake after a small HF preload, the next series of experiments examined in this preload-to-test meal model whether endocrine and neurochemical changes known to occur on a chronic HF diet can actually be detected prior to the increase in feeding. Studies to date with *ad libitum* consumption of a solid HF diet indicate that the overeating of fat attenuates responsiveness to the actions of gut peptides that normally inhibit feeding and slow gastric emptying [10,29,30] and to the satiety-producing effects of leptin and insulin [31–34] and the melanocortin peptides [35]. It also increases circulating levels of triglycerides (TG) and stimulates the hypothalamic orexigenic peptides, galanin (GAL) and orexin (ORX), in close association with the rise in lipids [5,36]. Since these peptides themselves stimulate a stronger feeding response on a HF compared to LF diet, they may provide the basis for a positive feedback loop whereby intake of a HF diet produces peptide changes that promote further consumption of this diet [33,37–39]. Using the acute preload-to-test meal paradigm, the present experiments examined whether a single HF preload (10–15 kcals), relative to a LF preload equal in calories, energy density and palatability, is sufficient to produce an increase in TG levels and also to stimulate expression of these orexigenic peptides subsequent to the preload and prior to the increase in feeding. In the final experiment, these single HF preloads were given daily over a period of several days. This tested whether the acute changes in food intake, circulating TG and orexigenic peptide seen immediately after the HF preload can have long-term consequences, impacting perhaps on daily food consumption and body weight gain.

2. Materials and methods

2.1. Subjects

Adult, male Sprague–Dawley rats (220–240 g) (Charles River Breeding Labs, Kingson, NY) were individually housed in plastic cages, in a fully accredited AAALAC facility (22 °C, with a 12:12-h light–dark cycle with lights off at 2 pm), according to institutionally approved protocols as specified in the NIH Guide to the Use and Care of Animals and also with the approval of the Rockefeller University Animal Care Committee. All animals were given 1 week to acclimate to the lab conditions before the start of the experiments, and their body weights were measured weekly. The experiments, including the period of adaptation to the feeding paradigm, lasted approximately 6–7 weeks, with the rats weighing 250–270 g at the start of the adaptation, 310–330 g at the start of the testing, and 460–490 g by the end of the experiment. Standard laboratory chow and water were available *ad libitum*, except for brief periods in the test paradigm when there was no food or when only the test diet was available (see below). The tests were conducted in the home cages 3.5 h before dark onset, with lab chow removed 90 min before the test.

2.2. Diets

The diets used in the experiments (Table 1) were either liquid (Experiments 1–3) or solid (Experiments 4, 5 and 7) for the preload and either liquid (Experiments 1–2) or lab chow (Experiments 3–7) for the test meal. The liquid high-fat (HF) and low-fat (LF) preload diets, of equal caloric density (2.3 kcals/ml), were prepared as previously described (11, 24)

Table 1
Liquid and solid preload diets

	Liquid[a]		Solid[b]	
	LF	HF	LF	HF
Evaporated milk (ml)	540	540	–	–
Corn oil emulsion (ml)	–	250	–	–
Fat (g)				
Land	–	–	0	230
Vegetable oil	–	–	45	50
Carbohydrate (g)				
Dextrin	–	–	95	97
Cornstarch	–	–	95	98
Sucrose	390	140	445	130
Protein (g)				
Casein	–	–	250	325
Vitamin Mix	–	–	30	30
Mineral Mix	–	–	40	40
Water (ml)	Bring volume to 1000 ml		–	–
Total weight (g)	1000	1000	1000	1000
Energy density	2.3 kcal/ml	2.3	3.93 kcal/g	5.15
% Macronutrient				
Fat	16.7	59.9	10	50
Carbohydrates	77.5	33.3	65	25
Protein	6.8	6.8	25	25

[a] Liquid diet: evaporated milk (Carnation); sugar (Domino); corn oil (Mazola) emulsified in tap water with 0.6% sodium steroyl lactylate (Sigma).

[b] Solid diet: lard (Armour); vegetable oil (Crisco); dextrin, cornstarch (I.C.N. Pharmaceuticals); sucrose (Domino); casein (Bioserv, Frenchtown,N.J. mixed with 0.003% l-cystine hydrochloride (I.C.N. Pharmaceuticals); Vitamin mix (Vitamin Diet Mixture, I.C.N. Pharmaceuticals); Mineral mix(USP XIV Salt Mixture Briggs, I.C.N. Pharmaceuticals).

and presented in 50 cc plastic tubes (PETCO). The liquid HF diet (60% fat) consisted of evaporated milk (Carnation), sucrose (Domino), and corn oil emulsion (44% wt/vol), while the liquid LF diet (17% fat) consisted of only evaporated milk and sucrose. The solid HF and LF diets were presented in metal round jars, with the HF diet containing 50% fat, 25% carbohydrate and 25% protein (5.15 kcals/g) and the LF diet containing 10% fat, 65% carbohydrate and 25% protein (3.93 kcals/g). The solid HF diet consisted of fat, with 82% lard and 18% vegetable oil, and carbohydrate, with 30% dextrin, 30% cornstarch and 40% sucrose, while the solid LF diet had fat with 100% vegetable oil and carbohydrate with 15% dextrin, 15% cornstarch and 70% sucrose. The diet composition for both solid and liquid diets was calculated as percent of total energy. For the test meals, the diets were either a diluted evaporated milk solution with 50% fat (one part milk, one part water) and caloric density of 0.67 kcal/ml (Experiment 1), an undiluted evaporated milk with 50% fat and caloric density of 1.34 kcal/ml (Experiment 2), or solid lab chow with 12% fat (3.3 kcals/g) (Experiments 3–7). The suppliers of the diet ingredients are listed in the legend of Table 1.

2.3. Test procedures

Using the diets and procedures outlined in Tables 1 and 2, seven experiments were conducted to test the effects of a HF preload in rats (n=20–24/experiment) on different measures of caloric intake, hormones, metabolites and hypothalamic peptides. For these experiments, the preloads were given in the form of a liquid diet (Experiments 1–3), solid diet (Experiments 4, 5 and 7), or intraperitoneal (i.p.) injection (Experiment 6). A 2-week period of adaptation to the diets and test paradigm preceded the actual test, with the rats generally receiving daily exposures for 4–5 days to both the HF and LF preload diets. The preload-to-test meal protocol involved a 15-min preload at 3.5 h before dark onset, followed by a 60-min and/or 120-min intermeal interval (IMI) with no food and then a test meal of at least 15 min in duration shortly before the start of the natural feeding cycle. After receiving these daily exposures to each of the preload diets (in counterbalanced order) and then the test meal diet, the rats learned to consume approximately 6 ml (15 kcals) of the preload within 15 min and at least 1 kcal of the test meal within 15 min. The few rats (<4% of total group) that

Table 2
Experimental design

Experiment #		Preload *	IMI (min)	Test meal	Measures	Time (min)
1	Liquid	HF (60%) vs LF (17%)	60	Milk (50% fat, 0.67 kcal/ml)	Kcals (test meal)	15, 30, 60, 60, 120
2	Liquid	HF (60%) vs LF (17%)	60, 120	Milk (50% fat, 1.34 kcal/ml)	Kcals (test meal)	15, 30
3	Liquid	HF (60%) vs LF (17%)	60, 120	Chow (12% fat, 3.3 kcal/g)	Kcals (test meal) Triglycerides Leptin, Insulin	15
4	Solid	HF (50%) vs LF (10%)	60, 120	Chow (12% fat, 3.3 kcal/ml)	Kcals test meal Triglycerides	15
5	Solid	HF (50%) vs LF (10%)	60, 120	Chow (12% fat, 3.3 kcal/ml)	Kcals test meal Triglycerides Leptin, insulin Galanin	15
6	Injection	Intralipid vs Glucose	60, 120	Chow (12% fat, 3.3 kcal/ml)	Kcals test meal Triglycerides Leptin, insulin Galanin, orerxin	15
7	Liquid	HF (50%) vs LF (10%)	60	Chow (12% fat, 3.3 kcal/ml)	Kcals (test meal)	15, 24 h

* High-fat (HF) and low-fat (LF) diets were given on alternate days for each experiment except Experiment 7 when the rats were exposed daily to the same HF or LF diet, as described in the Materials and methods and Test procedures.
Intermeal interval (IM) indicates the time between the beginning of the preload and the test meal.

consumed less than 13.5 kcals during the 15-min preload and/or less than 1.0 kcal during the 15-min test meal were eliminated from the study. The actual tests with each IMI were conducted in counterbalanced order over a period of 4 consecutive days, with 2 days on each of the preload diets. After completion of these behavioral tests with the preload-to-test meal paradigm, some additional tests were performed. These included preference tests in Experiments 1 and 4, to determine the palatability of the liquid or solid preloads. For these tests, the HF and LF diets were given *ad libitum* for 30 min either separately in randomized order over 2 days (1-bottle/jar test) or together in the same test with their positions in the cage alternated over the 2 test days (2 bottle/jar test). Further tests were performed in Experiments 3–6, which involved blood collections via tail vein puncture (see below) at 60 or 120 min after the HF vs LF preloads, to measure circulating triglyceride levels, or trunk blood collections and brain dissections after rapid decapitation at 120 min after preload, to measure circulating hormones and metabolites as well as hypothalamic peptides via real-time quantitative PCR.

The specific procedures and rationale for each experiment were the following (see Table 2). In Experiment 1, the effect of a liquid preload diet on the intake of a subsequent liquid test meal was tested. After being adapted to the diets and experimental paradigm, the rats ($n=20$) were given two tests each with the 15-kcal liquid HF (60% fat) and LF (17% fat) preloads, which were equal energy density, and this was followed by a 60-min IMI with no food and then a test meal with a diluted, low caloric density (0.67 kcal/ml) liquid diet containing 50% fat. Measurements were taken at 15, 30, 60, 90 and 120 min and averaged across the two tests. In Experiment 2, an additional set of rats ($n=22$) was examined with the same 15-kcal HF (60% fat) vs LF (17% fat) liquid preloads but specific modifications to the experimental paradigm. A liquid test meal with a fat content of 50% consisting of undiluted calorie-dense milk was used, and a 120-min as well as 60-min IMI was tested, to determine whether the length of this period is a critical factor. In the third set of rats ($n=20$), Experiment 3 tested whether the increased caloric intake induced by the equicaloric liquid preloads (15 kcals) can be seen with a solid test meal diet, specifically standard laboratory chow, which has only 12% fat. To examine possible physiological mechanisms that may contribute to the overeating, this experiment also performed blood collections and took measurements of circulating TG, which are known to stimulate orexigenic peptides (see Introduction), and of insulin and leptin, which rise in relation to body fat (5, 8). Blood collections were performed via tail vein puncture at the end of the behavioral tests. To assess whether the type of preload is critical for enhancing intake and elevating TG levels, Experiment 4 used a solid mixed diet for the preload with a similar test paradigm to that in Experiments 1–3. The rats ($n=20$) were given a solid HF or LF preload (15 kcals), and intake of the lab chow test meal was measured after the 60- and 120-min IMI. Blood was then collected via tail vein puncture in the final preload test, to measure TG levels after the 120-min IMI. As in Experiment 1 with the liquid preload diets, these rats were further tested to determine the palatability of these solid preload diets in 1-jar and 2-jar preference tests, with *ad libitum* access for 30 min. Using procedures similar to Experiment 4, Experiment 5 examined the rats ($n=20$) behaviorally after the solid preload diets (15 kcals), and then after the final preload test, they were sacrificed by decapitation for measurements of TG, leptin and insulin in trunk blood and GAL in hypothalamic nuclei. Experiment 6 differed in that the preloads were injected rather than ingested. In 3 groups of rats ($n=7$/group), the effects of i.p. injection of Intralipid (20%, 10 kcals) were tested in comparison to i.p. injection of an equicaloric glucose solution (50%, 10 kcals) or non-caloric saline. As in Experiment 5, the behavioral tests were conducted first, with 15-min chow intake measured at 60 and 120 min after injection, and the blood collection tests were conducted next via tail vein puncture, with TG levels also measured at 60 and 120 min after injection. At the end of these tests, rats were sacrificed at 120 min after injection, trunk blood was collected for measurements of TG and hormones, and the brains were dissected for measurement of GAL and ORX expression in the hypothalamus. In contrast to the previous experiments, the rats in Experiment 7 ($n=20$) were adapted to and tested with only one of the two liquid preload diets. At 3.5 h before dark onset, they were given daily access over a 4-day period to 15 kcals of either the HF or the LF preload. After a 60-min IMI, daily measurements were then taken of lab chow intake, at 15 min and then 24 h after the preload, and also of body weight.

2.4. Blood sampling procedures

In Experiments 3, 4 and 6, blood was collected during testing using a tail vein puncture technique, for measurements of TG levels. For this procedure, the rats received their appropriate preloads, and blood was collected at 60 min (Experiments 3 and 6) or 120 min (Experiments 3, 4 and 6) after the preload. The blood was collected in a cross-over design, with two tail veins performed 1 day apart for each IMI. The rats were gently placed in a plastic restrainer (Harvard Apparatus), and their tails were wrapped with a warm towel for about 10 s to facilitate blood flow. The tail vein was punctured with a 21G1 needle, and the blood (approximately 100 μl) was allowed to drip from the other, cut-off end into a 5 ml glass tube for a brief period of approximately 45 s. In Experiments 3, 5 and 6, trunk blood was collected at sacrifice, 120 min after the preload, for measurements of TG, leptin and insulin levels.

2.5. Hormone and metabolite determinations

Plasma from tail vein or trunk blood was assayed for insulin and leptin using RIA kits from Linco Research Inc, MO. Triglycerides (TG) were measured with an E-Max Microplate Reader using a TG Assay Kit (Sigma).

2.6. Real-time quantitative PCR

Immediately after sacrifice, the brains removed for peptide measurements using real-time quantitative PCR were placed in a matrix with the ventral surface facing up, and three 1.0 nm coronal sections were made, with the middle optic chiasma as the anterior boundary. The sections were placed on a glass slide, and

three hypothalamic areas, the paraventricular nucleus (PVN, Bregma − 1.3 to 2.1 mm), the perifornical lateral hypothalamus (PFH, Bregma − 2.8 to −3.6 mm), and the arcuate nucleus (ARC, Bregma − 2.56 to −3.3 mm) were rapidly microdissected under a microscope, using the fornix and third ventricle as landmarks. The PVN was dissected as a reversed isosceles triangle, 1.0 mm bilateral to the ventricle and between the fornix structures. For the PFH, the dissection was taken from the area surrounding the fornix, within a range of 0.2 mm medial and ventral to the fornix, 0.3 mm dorsal and 0.1 mm lateral. For the ARC, the area adjacent to the bottom of the third ventricle was dissected parallel to the border of the ventricle, with the width of 0.1 mm at the top gradually widening to 0.3 mm at the bottom. These dissections were immediately frozen in liquid nitrogen and stored at −80 °C until processed. As previously described [36], total RNA from pooled microdissected PVN, PFH or ARC samples was extracted with TRIzol reagent. RNA was treated with RNase-free DNase I before RT. For quantitative PCR, cDNA and minus RT were synthesized using an oligo-dT primer with or without SuperScript II Reverse Transcriptase. The SYBR Green PCR core reagents kit (Applied Biosystems, CA) was used, with β-actin as endogenous control. PCR was performed in MicroAmp Optic 96-well Reaction Plates (Applied Biosystems) on an ABI PRISM 7700 Sequence Detection system (Applied Biosystems), with the condition of 2 min at 50 °C, 10 min at 95 °C, then 40 cycles of 15 s at 95 °C and 1 min at 60 °C. Each study consisted of 4 independent runs of PCR in triplicate, and each run included a standard curve, non-template control, and negative RT control. The levels of GAL or ORX gene expression were quantified relative to the level of β-actin, using standard curve method. The primers, designed with ABI Primer Express V.1.5a software based on published sequences, were: 1) β-actin: 5′-GGCCAACCGTGAAAAGATGA-3′ (forward) and 5′-CACAGCCTGGATGGCTACGT-3′ (reverse); 2) GAL: 5′-TTCCCACCACTGCTCAAGATG-3′ (forward) and 5′-TGGCTGACAGGGTTGCAA-3′ (reverse) and 3) ORX: 5′-AGATACCATCTCTCCGGATTGC-3′ (forward) and 5′-CCAGGGAACCTTTGTAGAAGGA-3′(reverse). The concentrations of primers were 100 to 200 nM, and all reagents, unless indicated, were from Invitrogen.

2.7. Data analysis

All values are expressed as mean±SEM. Statistical analyses of the different measures for the groups in each experiment were performed using a one-way ANOVA followed by post-hoc tests for multiple comparisons between groups, or using an unpaired *t*-test where appropriate. The probability values given in the text or legends to the figures and tables reflect the results of these tests.

3. Results

3.1. Experiment 1: food intake with liquid preload and diluted fat liquid test meal paradigm

This first experiment tested the effect of a liquid preload diet (50% fat, 0.67 kcal/ml) on intake of a subsequent liquid test meal. Cumulative measurements of the subsequent liquid meal, taken at 15, 30, 60, 90 and 120 min and averaged across the two test days, revealed significantly more food intake after the HF preload relative to the equicaloric LF preload (Fig. 1). This effect was strongest during the first 15 min of the test meal, when the HF preload was followed by a 45% increase (+5.7 kcals) in the amount consumed. It was also evident in the cumulative 30- and 60-min measurement periods, with diet intake at the 90-min and 120-min intervals still elevated but not significantly (Fig. 1). The preference tests conducted in these rats (see Test procedures) showed that the liquid HF preload diet was equally palatable to the LF preload (Table 3). With *ad libitum* access for 30 min, the rats ingested similar amounts of both diets, whether given separately on different days (1-bottle test) or together in the same test (2-bottle test). These results, obtained with preload diets equal in both palatability and energy density, suggest that these two factors are not essential for the greater food intake seen after a 15-kcal HF relative to LF preload.

3.2. Experiment 2: food intake with liquid preload and undiluted high-fat liquid test meal paradigm

To test the robustness under different conditions of the increased feeding effect seen after a liquid HF preload, the procedures were varied to include a test meal of higher caloric density (50% fat, 1.34 kcal/ml) and a larger 120-min IMI, with the same liquid preload as that tested in Experiment 1.With these modifications and the same liquid preload as used in Experiment 1, the HF compared to LF preload was followed by 45% greater caloric intake (+6.0 kcals) during the subsequent test meal (Table 4). As in Experiment 1, this effect was similarly evident in both the 15-min and 30-min measurement periods, although most of the food intake occurred during the first

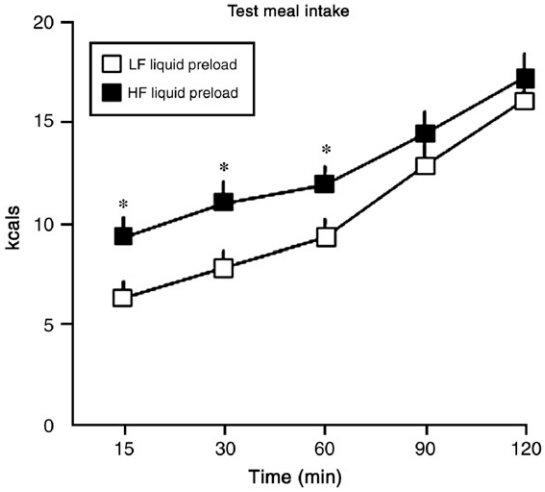

Fig. 1. Effects of a liquid high-fat (HF) vs equicaloric low-fat (LF) preload (15 kcals) on intake of a diluted high-fat test meal after a 60-min post-preload interval (Experiment 1). The data (mean±SEM) reveal a significant increase in kcal intake after the HF vs LF preload ($F(1,90)=13.12$, $p<0.001$). * $p<0.05$ for comparisons of the effect of HF vs LF preloads on cumulative intake of the test meal at 15, 30 and 60 min.

Table 3
Preference tests on liquid and solid diets (Experiments 1 and 4)

Diet	Preference test	Low-fat diet (kcals in 30 min)	High-fat diet (kcals in 30 min)
Liquid	1-bottle	34.0±15	34.3±13
	2-bottle	20.3±3.7	20.5±2.4
Solid	1-jar	17.7±2.8	27.5±2.7*
	2-jar	3.1±0.71	25.3±2.4*

* $p<$ for comparisons between the high-fat and low-fat diets.

15 min of the test. It was robust enough to be seen even after a 120-min as well as 60-min IMI and with a test meal high in fat content closer to that of the HF preload (Table 4). Thus, these changes in the length of the IMI or fat composition of the test meal had no apparent effect on the nature or magnitude of the feeding response.

3.3. Experiment 3: food intake and TG measures with liquid preload and low-fat solid test meal paradigm

To examine possible physiological mechanisms that may contribute to the HF-induced greater food intake, rats receiving a solid low-fat test meal after the HF preload consumed significantly more lab chow, a 56% and 53% increase (+4.1 and 4.7 kcals), during the first 15 min of the test meal, and this effect occurred after both the 60- and 120-min IMI (Fig. 2, left panel). Although total intake on this dry LF test meal was generally lower, the rats showed very similar patterns to those described in Experiments 1 and 2 with diluted or undiluted liquid test meals. Thus, in addition to the palatability and energy density of the preload, the nature and fat content of the test meal are not critical variables, leading us to use lab chow in all subsequent experiments of HF-induced overeating. The greater caloric intake after the HF compared to LF meal was found to be accompanied by a significant increase in lipids. The 15-kcal liquid HF preload compared to LF preload produced a marked (100–150%) rise in serum levels of TG (Fig. 2, right panel). As with the higher food intake, this rise in lipids was evident after both the 60- and 120-min IMI with no food. This effect was confirmed with analyses of trunk blood. When sacrificed at 120 min after the HF (vs LF) preload, the rats had significantly elevated TG (349±48 vs 191±19 mg/dl, $p<0.01$). They showed no change, however, in their serum levels of leptin (5.1±1.5 vs 5.1±1.4 ng/ml) or insulin (1.8±0.3 vs 2.0±0.3 ng/ml).

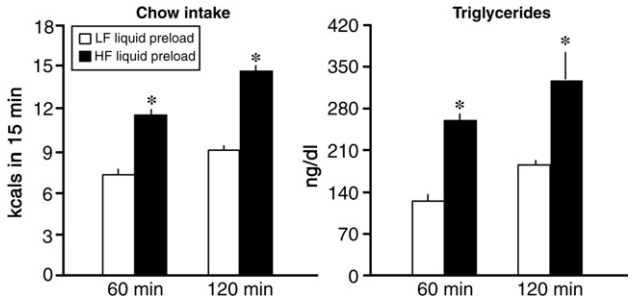

Fig. 2. Effects of a liquid high-fat (HF) vs equicaloric low-fat (LF) preload (15 kcals), at the 60-min and 120-min post-preload intervals, on chow intake (15 min) and triglyceride levels (Experiment 3). Values are mean±SEM. * $p<0.05$ for comparisons between the HF and LF preloads.

3.4. Experiment 4: food intake and TG measures with solid preload and lab chow test meal paradigm

This experiment showed that solid preloads have the same effect as the liquid preloads, with the HF compared to LF preload followed by greater caloric intake (4–5 kcals) in 15 min after both the 60- and 120-min IMI (Table 5). The solid HF preload also raised circulating levels of TG (+150%) at the 120-min interval, confirming its close relation to the increase in feeding (Table 5). In contrast to the equally palatable liquid preloads, the solid preloads were different in their palatability, as revealed by significantly greater intake of the HF preload whether presented separately or together with the LF preload (Table 3). With the solid and liquid HF preloads having very similar effects on subsequent food intake, however, this differential palatability would appear to have little significance in the phenomenon of increased feeding after a HF preload.

3.5. Experiment 5: food intake, TG and GAL measures with solid preload and test meal paradigm

Building on the results obtained with measurements of TG, this experiment took an initial step towards determining whether the orexigenic peptide GAL, which is known to be highly responsive to dietary fat and circulating TG [5,36], is altered by the single 15-kcal HF preload used in this acute preload-to-test meal paradigm. This experiment yielded similar behavioral results to the previous experiments, with the solid HF compared to LF preload after a 120-min IMI producing a 50% increase in lab chow intake during the 15-min test meal

Table 4
HF-induced hyperphagia on a liquid test meal (50% fat) after a liquid preload (Experiment 2)

Intermeal interval	Low-fat preload (kcals)	High-fat preload (kcals)
60 min		
15 min	13.0±1.3	19.0±1.1*
30 min	15.1±1.2	21.6±0.9*
120 min		
15 min	23.5±1.5	31.0±1.8*
30 min	26.7±1.8	36.6±2.2*

* $p<0.05$ for comparisons between the high-fat and low-fat preloads.

Table 5
HF-induced hyperphagia on a chow test meal (12% fat) after a solid preload (Experiment 4)

Intermeal interval	Low-fat preload (kcals)	High-fat preload (kcals)
60 min		
Kcal intake	5.2±1.0	9.0±1.4*
120 min		
Kcal intake	9.2±1.6	14.3±1.2*
Triglycerides	102±12	251±21*

* $p<0.05$ for comparisons between the high-fat and low-fat preloads.

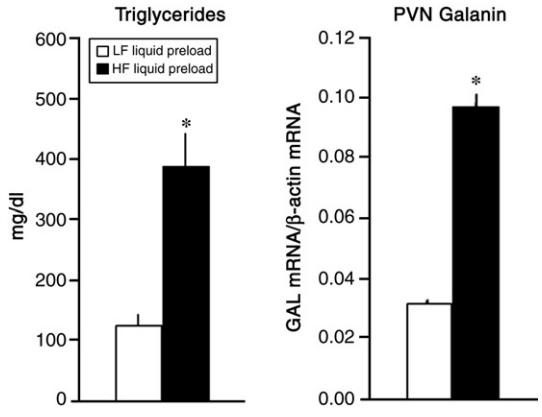

Fig. 3. Effects of a solid HF vs LF preload (15 kcals), after a 120-min post-preload interval, on triglyceride levels and galanin mRNA in the PVN (Experiment 5). Values are mean±SEM. *$p<0.05$ for comparisons between the HF and LF preloads.

(14.3±1.2 vs 9.2±1.6, $p<0.01$). Analyses of serum from trunk blood revealed a marked increase in TG levels after the HF preload (Fig. 3, left panel), with no change in levels of leptin (5.7±1.9 vs 4.8±0.6 ng/ml) or insulin (2.2±1.1 vs 3.0±1.0 ng/ml). As revealed by real-time quantitative PCR, this HF-induced rise in TG at 120 min was accompanied by a marked increase in expression of GAL in the PVN, as indicated by the ratio of GAL mRNA to β-actin mRNA (Fig. 3, right panel). This effect of the HF preload was specific to the PVN, with measurements in the ARC showing no change in this ratio (0.051±0.012 vs 0.063±0.016). Thus, a HF preload equal in calories to a LF preload (15 kcals) produced a significant increase in TG and PVN GAL at the same time when the greater food intake is known to occur.

3.6. Experiment 6: food intake, TG, GAL and ORX measures with Intralipid injection paradigm

If the elevated TG and orexigenic peptide after a HF preload are more critical to the overeating than signals from the mouth or gut, peripheral injection of a lipid emulsion, which bypasses the ingestion process and orosensory stimulation while still increasing TG levels as well as GAL [36], should be equally effective in promoting feeding. Building on this report which compared Intralipid to a non-caloric saline vehicle [36], we found that as with the ingested HF vs LF preloads, i.p. injection of Intralipid compared to the equicaloric glucose solution significantly increased intake of the chow test meal, after both the 60- and 120-min intervals with no food (Fig. 4, left panel). Further, when compared to the saline injection scores, the 10 kcals of the glucose solution markedly reduced subsequent chow intake by approximately 50% at both post-injection intervals, while 10 kcals of Intralipid produced no change in intake. Thus, calories from the fat emulsion, in addition to increasing subsequent food intake relative to that seen after the equicaloric glucose solution, failed to suppress food intake relative to that seen after the non-caloric saline solution. When the preload calories (10 kcals) were added to the test meal calories, there was a significant rise ($p<0.05$) in the total number of calories in the test with Intralipid compared to both glucose and saline. As with the consumption of fat, increased food intake induced by the injection of fat compared to saline was accompanied by a significant increase in TG levels in blood collected via tail vein puncture at 60 and 120 min after injection, with no change seen after injection of glucose (Fig. 4, right panel). Similar results were obtained with analyses of trunk blood collected in the final test after a 120-min IMI (Fig. 5, left panel). Compared to the saline baseline and glucose, the rats injected with Intralipid showed a 190% increase in TG levels, with no change observed after injection of glucose. Hormone measurements at 120 min after injection of Intralipid vs glucose or saline revealed no change in leptin (3.0±0.3 vs 2.6±0.8 vs 3.3±0.7 ng/ml) or insulin (2.1±0.3 vs 1.8±0.5 vs 1.6±0.4 ng/ml), as previously reported for Intralipid vs saline [36]. This rise in TG after Intralipid was accompanied by a significant increase in expression of GAL in the PVN and ORX in the PFH compared to saline and glucose, with no significant change after glucose as compared to saline injection (Fig. 5, right panel). These results obtained with injections of the diet underscore the close

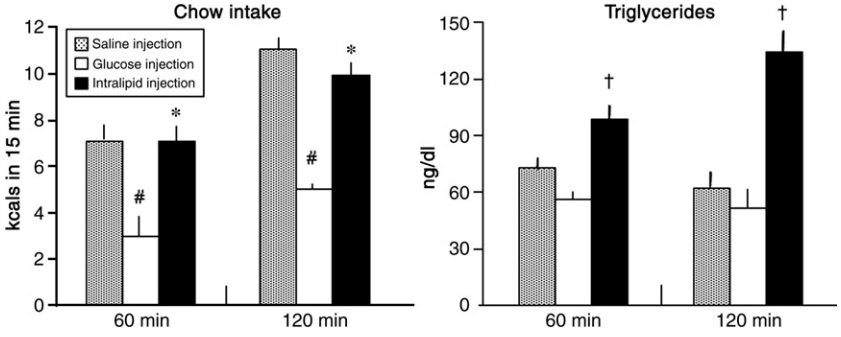

Fig. 4. Effects of injection of the lipid emulsion, Intralipid (20%, 10 kcals) compared with an equicaloric glucose solution (50%, 10 kcals) or saline, after 60 min and 120 min, on chow intake (15 min) and triglycerides in serum collected from tail vein puncture (Experiment 6). The data (mean±SEM) for chow intake at 60 min ($F(2,45)=3.26$, $p<0.05$) and 120 min ($F(2,29)=4.04$, $p<0.03$) show a significant difference in kcals following injection of Intralipid compared with glucose (*) and of glucose compared with saline or Intralipid (#). The measures of triglycerides at 60 min ($F(2,26)=6.38$, $p<0.01$) and 120 min ($F(2,16)=7.76$, $p<0.01$) show a significant difference after injection of Intralipid compared with glucose and saline (†).

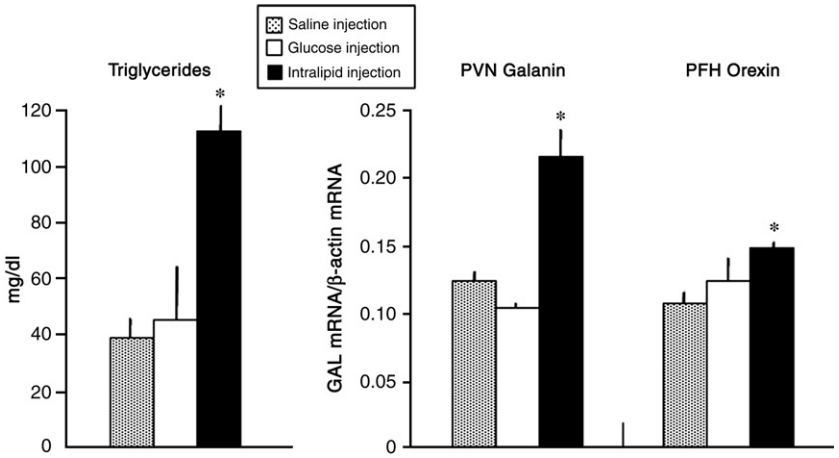

Fig. 5. Effects of injection of the lipid emulsion, Intralipid (20%, 10 kcals) compared with an equicaloric glucose solution (50%, 10 kcals) or saline, after 120 min, on triglycerides (in serum from trunk blood), PVN galanin mRNA, and PFH orexin mRNA (Experiment 6). The data (mean±SEM) reveal a significant change in TG levels ($F(2,23)=4.00$, $p<0.03$), PVN GAL ($F(2,11)=29.9$, $p<0.001$), and PFH orexin ($F(2,11)=32.52$, $p<0.01$), with each measure increased after Intralipid compared with glucose or saline injection (*).

relationship of elevated TG and peptide expression, rather than the ingestion process and diet palatability, to the stimulatory effect of a HF preload on subsequent food intake.

3.7. Experiment 7: daily food intake and body weight measures with standard preload-to-test meal paradigm

With Experiments 1–6 showing a single HF meal or injection to consistently increase food intake during a subsequent 15-min test meal, this experiment questioned whether several consecutive days of this small HF preload compared to an equicaloric LF preload can actually affect total daily food intake and possibly even weight gain. As in the other experiments, the HF liquid preload compared to the LF preload was followed by a significant increase in chow intake during the 15-min test meal (Fig. 6, left panel). This effect, an increase of approximately 5 kcals, was evident on each of the 4 test days, including on day 2 when there was an unexplained dip in the caloric intake of both groups. Interestingly, with this 15-min intake measure included in the 24-h intake measure, a significant increase in daily intake (+7 kcals) was still evident after the HF preload on test days 2, 3 and 4 (Fig. 6, middle panel). This indicates that there was no significant compensatory reduction in intake throughout the light or dark periods following the initial HF-induced overeating. This 10% increase in daily chow intake was accompanied by a small increase (+7%) in body weight, although this change failed to reach statistical significance (Fig. 6, right panel).

4. Discussion

4.1. Behavioral model of HF-induced increase in food intake

The first 4 experiments of this report performed a variety of tests with modified versions of the preload-to-test meal paradigm originally described by Warwick and colleagues [9,12,24]. The purpose was to assess the robustness of the HF-induced feeding phenomenon under different conditions and then

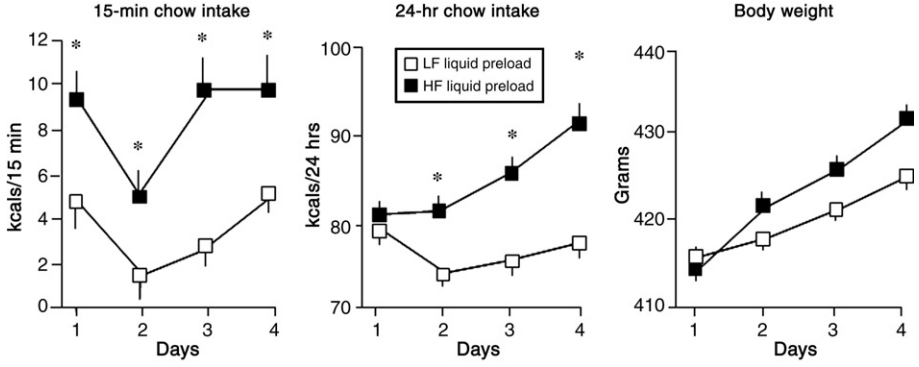

Fig. 6. Daily measurements of lab chow intake (15 min and 24 h) and body weight in rats given liquid high-fat (HF) vs low-fat (LF) preloads (15 kcals) on 4 consecutive days (Experiment 7). The data (mean±SEM) show a significant change in caloric intake at 15 min ($F(1,20)=7.06$, $p<0.02$) and 24 h ($F(1,20)=5.23$, $p<0.03$), with the intake measure significantly higher after the HF compared to LF preload (*). While somewhat higher across the 4 days, body weight in the HF preload rats was not significantly different from that in the LF preload rats.

standardize a test paradigm in our lab that yielded a consistent effect and was easiest to administer. The different tests with a HF relative to LF preload revealed a greater feeding response under a variety of conditions. The standard protocol involved tests with: 1) rats in their home cages and during the last few hours of the light cycle just prior to the start of spontaneous feeding at dark onset; 2) a 90-min, food-free interval prior to the start of the test, which minimizes random *ad libitum* feeding and thus variability in the intake scores during the test [40]; 3) 8–10 days of adaptation to the test paradigm and the preload and test meal diets, which allow the animals to reach the strict criteria of consuming between 13.5 and 15.5 kcals of either preload in 15 min and at least 1 kcal of the test meal diet; 4) a 60-min or 120-min post-preload period and 15-min test meal, which lie in the range of a natural IMI and meal duration typically seen in meal pattern analyses of rat eating behavior [13]; 5) a within-subject design, which tests all rats on both the HF and LF diets and yields results similar to a between-subject design; 6) a liquid oral preload of equal palatability, energy density and caloric content, which produces an increase in food intake similar to that of a solid oral preload or liquid injected preload; 7) complete macronutrient diets for both the preload and test meal, which avoid the flavor-preference conditioning often seen with pure macronutrient diets [41]; and 8) a test meal diet of standard lab chow, which reveals a feeding effect similar to that observed with liquid or solid test meal diets with higher fat content.

4.2. Behavioral and dietary determinants

As described above, the behavioral and dietary determinants of HF-induced increase in food intake have been extensively studied. Evidence with a chronic or acute HF diet given *ad libitum* generally suggests that they include the greater palatability and energy density of the diet [2,3,15–17]. Studies using the preload-to-test meal paradigm, however, demonstrate that these factors are not required for the phenomenon of increasing caloric intake. The greater feeding after a HF preload is evident when the HF and LF diets are equal in palatability and energy density [7,12,24] or infused directly into the stomach [26], and it is absent in sham-feeding paradigms when the sensory properties of the diet are retained but the post-ingestion processes eliminated [6,26]. The present experiments with HF vs LF preloads support these conclusions, revealing greater caloric intake after preloads that are equal (or unequal) in palatability, energy density and caloric content. While strongest during the first 15 min, this effect after a HF preload actually persisted for more than 60 min and could even be seen in the measurements of 24-hour intake. An increase in amount consumed, ranging from 45% to 150%, consistently occurred subsequent to a liquid or solid HF preload (50% fat) and different test meals that were either liquid or solid and unpalatable (lab chow with 12% fat) or palatable (mixed diet with 30–50% fat). It was also seen after peripheral injection of a lipid emulsion (Intralipid) compared to an equicaloric glucose solution, which bypasses the ingestion process and thus avoids the issues of palatability as well as energy density. Together, these results strengthen the idea that other factors besides orosensory cues, perhaps signals from the gut, circulation, adipose tissue or brain, are important in the HF-induced enhancement of food intake.

4.3. Gastric mechanisms

Although peptides released from the gastrointestinal tract after a meal are known to suppress feeding [42], there is evidence that dietary fat compared to carbohydrate or protein cause a resistance to the actions of these peptides. This is seen in rats chronically maintained on a HF diet, which compared to a LF diet reduces sensitivity to the feeding-inhibitory signals of cholecystokinin or bombesin [10,43], or intestinal infusion of oleate [44]. Intragastric infusion of lipids compared to glucose also has a smaller suppressive effect on subsequent food intake [19]. Thus, greater food intake after a HF preload may result, in part, from a lipid-induced reduction in the satiety-producing effects of gut peptides.

4.4. Role of circulating lipids

The absorption of fat from the gut causes a rapid increase in circulating lipids, in direct proportion to the amount of fat consumed [5,45]. Using the preload-to-test meal paradigm standardized in Experiments 1–3, the present study tested whether the greater food intake seen after a single HF preload, which is equal in palatability and energy density to the LF preload, is associated with a rise in levels of TG in the blood. In all of the tests conducted, the HF preload in normal-weight rats produced a significant increase in circulating TG. This effect was consistently large and robust (100–150%), seen in each of the experiments with a preload of 10–15 kcals, similar to experiments with considerably larger meals [5,36,46]. It occurred at the same time as the increase in feeding response, whether induced by a liquid or solid preload diet and after a 60- or 120-min IMI interval. These results suggest a close, positive relationship between the circulating TG after a HF preload and a subsequent rise in food intake. This relationship is further supported by evidence obtained with injection of the lipid emulsion, Intralipid. Consistent with a prior study with chronic intravenous or intragastric infusions of Intralipid [19], the results obtained here with acute, i.p. injection of Intralipid (10 kcals) compared to an equicaloric glucose solution revealed a marked increase in TG levels as well as food intake after both the 60- and 120-min post-injection intervals, similar to the effects produced by the ingested preloads. While further studies are needed to determine whether circulating TG are causally related to the greater food intake after a HF preload, there is evidence suggesting a reasonable mechanism whereby TG contribute to increased caloric intake by reducing sensitivity to the satiety-producing effects of gut peptides. This is supported by the findings that a HF meal-induced rise in TG temporally overlaps the release of cholecystokinin [47], and attenuation of this TG rise by Orlistat suppresses fat-induced overeating [48].

4.5. Fat metabolism and leptin or insulin resistance

Along with the elevated TG levels, signals related to the metabolism of fat may also be involved in the increased feeding

induced by a HF diet. Evidence suggests that the oxidation along with consumption of fat is less tightly regulated than the oxidation and intake of carbohydrate. Dietary fat diverts metabolic fuels away from the oxidative pathway and into storage, thus making fat less satiating and possibly contributing to overeating of a HF diet [2,49]. This is supported by the finding that the ingestion of medium chain fatty acids, which are more readily oxidizable than long chain fatty acids, fail to produce overeating [2]. Furthermore, blockade of fat oxidation with mercaptoacetate attenuates the reinforcing effects and palatability of fat relative to a sucrose solution [50]. Thus, reduced fat oxidation along with elevated TG levels may contribute to the greater food intake seen during the 60–120 min period after a HF preload.

The hormones, insulin and leptin, are known to reduce food intake and specifically the size and duration of a meal [51,52]. While markedly elevating TG levels, the acute HF preloads (10–15 kcals) compared to LF preloads in the present study had little effect on circulating levels of these hormones. This suggests that they may not be directly involved in the increased feeding observed in this acute preload-to-test meal model. However, acute periods of fat consumption may cause a resistance to the peripheral and central actions of leptin and insulin [30–33] that blunts their satiety response [34,53]. Also, circulating TG may attenuate leptin transport across the blood brain barrier, thus removing an anorectic signal from the brain [54]. Thus, the rise in TG levels induced by a HF preload may contribute to the overeating by altering the transport and central or peripheral actions of these hormones.

4.6. Hypothalamic orexigenic peptides

The focus of the present study is on central mechanisms that may be involved in the greater food intake induced by dietary fat. Although a HF diet may attenuate the actions of brain neurochemicals that suppress feeding [9], recent studies have focused attention on specific peptides in the hypothalamus that stimulate food intake and are more closely related to HF diet consumption and circulating TG than to changes in total caloric intake, body fat accrual, or hormones related to adiposity [5,36,55]. These reports show that these orexigenic peptides, including GAL and ORX, are stimulated by chronic daily ingestion of a HF vs LF diet and *ad libitum* HF diet consumption over a 4-h period and also by peripheral injection of Intralipid as compared to the saline vehicle. The present study confirms and extends these findings. Tests with a single, solid HF preload (15 kcals consumed in 15 min) compared to an equicaloric LF preload was found to be sufficient in stimulating the expression of GAL in the PVN while increasing TG levels. A similar change in expression of both PVN GAL and ORX in the PFH along with circulating TG was also observed after peripheral injection of Intralipid (10 kcals), compared to an equicaloric glucose solution as well as a non-caloric, saline vehicle. These new findings demonstrate how responsive these hypothalamic peptides are to dietary fat, as well as to lipids in the blood which may then pass into the brain [7]. They show a significant change after a brief meal or acute injection of only 10–15 kcals, with an increase in circulating TG but no change in leptin or insulin. Thus, these orexigenic peptides in the PVN and PFH are likely candidates for contributing to the increased feeding induced by a HF preload.

4.7. Long-term impact of daily consumption of a HF preload

Prior studies have shown that constant intravenous or intragastric infusion of Intralipid [19] or chronic availability of a liquid HF solution [7] compared to a LF solution significantly increase total daily caloric intake of a lab chow diet. The results of the present study demonstrate for the first time that an acute HF meal of only 15 kcals can have long-term consequences. Over 4 consecutive days, rats given a daily 15-kcal preload of a HF liquid diet compared to an equicaloric and equally palatable LF liquid diet exhibited after a 60-min IMI a significant increase in their chow intake (+5 kcals) during a 15-min test meal before the onset of the dark cycle. This rise in food intake after the HF preload observed on each of the 4 test days is consistent with natural patterns seen with spontaneous meals, which show HF compared to equicaloric LF meals to be consistently followed by shorter intervals before the next meal [6]. This phenomenon may result from an increase in signals that stimulate feeding, as well as a reduction in the satiating effect of the HF meal. With the preload-to-test meal paradigm used here, this short-term effect of a HF preload was robust enough to produce a significant, 10% increase in food intake over the next 24 h. This indicates that the rats failed to compensate for the greater caloric intake after the extra HF preload, at least when presented during the few hours before onset of the nocturnal feeding cycle. This period, which normally has little spontaneous eating, precedes the first meal of the dark period, which is found to be the largest meal of the 24-h cycle [13].

This 10% increase in daily lab chow intake after the HF preload was accompanied by a somewhat more rapid weight gain compared to that after the LF preload (Fig. 6). While this effect failed to reach statistical significance after only 4 days, the steady divergence in body weights of the two groups suggests that a significant increase in weight gain might develop with a longer sequence of daily exposure to the HF preload and also a chronic, maintenance diet with higher fat content than the low-fat, lab chow diet. A 10% increase in daily caloric intake seen in rats on a chronic HF diet generally produces a significant increase body weight and body fat accrual after 7–10 days [5]. The present findings demonstrate that the behavioral, endocrine and neurochemical changes seen after chronic, *ad libitum* consumption of a fat-rich diet [5,46,55] are similarly detected *before* the increased feeding response associated with a brief, 10-kcal HF preload. This supports the idea that the increase in TG and expression of the orexigenic peptides, GAL and ORX, may in fact be causally related to the overeating commonly observed on a HF diet.

The phenomenon of increased caloric intake following consumption of a fatty preload is also observed in humans as shown in clinical studies [4,27]. These preloads are equivalent in size to snacks in everyday life, and periodic consumption of these

fatty preloads may lead to over-consumption of energy and subsequent weight gain. Our data suggest that just a 4-day exposure to high-fat preloads may lead to an increase in body weight in rats, indicating that humans may respond similarly with chronic eating habits that include fatty foods.

Acknowledgements

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