

Dietary fat stimulates endogenous enkephalin and dynorphin in the paraventricular nucleus: role of circulating triglycerides

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Chang GQ, Karatayev O, Ahsan R, Gaysinskaya V, Marwil Z, Leibowitz SF. Dietary fat stimulates endogenous enkephalin and dynorphin in the paraventricular nucleus: role of circulating triglycerides. *Am J Physiol Endocrinol Metab* 292: E561–E570, 2007; doi:10.1152/ajpendo.00087.2006.—The opioid peptides enkephalin (ENK) and dynorphin (DYN), when injected into the hypothalamus, are known to stimulate feeding behavior and preferentially increase the ingestion of a high-fat diet. Studies of another peptide, galanin (GAL), with similar effects on feeding demonstrate that a high-fat diet, in turn, can stimulate the expression of this peptide in the hypothalamus. The present study tested different diets and variable periods of high- vs. low-fat diet consumption to determine whether the opioid peptides respond in a similar manner as GAL. In six experiments, the effects of dietary fat on ENK and DYN were examined in three hypothalamic areas: the paraventricular nucleus (PVN), perifornical hypothalamus (PFH), and arcuate nucleus (ARC). The results demonstrated that the ingestion of a high-fat diet increases gene expression and peptide levels of both ENK and DYN in the hypothalamus. The strongest and most consistent effect is seen in the PVN. In this nucleus, ENK and DYN are increased by 50–100% after 1 wk, 1 day, 60 min, and even 15 min of high-fat diet consumption. While showing some effect in the PFH, these peptides in the ARC are considerably less responsive, exhibiting no change in response to the briefer periods of diet intake. This effect of dietary fat on PVN opioids can be observed with diets equal in caloric density and palatability and without a change in caloric intake, body weight, fat pad weight, or levels of insulin or leptin. The data reveal a strong and consistent association between these peptides and a rise in circulating levels of triglycerides, supporting a role for these lipids in the fat-induced stimulation of opioid peptides in the PVN, similar to GAL.

galanin; opioid peptides; arcuate nucleus; feeding behavior; lipids

RECENT EVIDENCE has demonstrated a close relationship between the orexigenic peptide galanin (GAL) and the consumption of a fat-rich diet. When injected into the paraventricular nucleus (PVN), this peptide stimulates feeding behavior in rats, and while having no specific effect on macronutrient preferences, GAL produces its strongest response in animals that prefer fat or are maintained on a fat-rich diet (34, 41, 65). Moreover, measurements of endogenous GAL reveal a stimulatory effect of acute or chronic high-fat diet consumption on gene expression and peptide levels specifically in the PVN (13, 36, 52). This supports the existence of a positive feedback loop, whereby a peptide stimulates intake of a diet that, in turn, increases production of this same peptide.

The focus of this study is on the endogenous opioid peptides, enkephalin (ENK) and dynorphin (DYN). These peptides, like GAL, are known to increase feeding in a dose-dependent manner, and this response is observed with injection into the

PVN and also into forebrain sites, such as the nucleus accumbens (NAc) (7, 26, 46, 66, 74). These opioid peptides stimulate intake of a high-fat diet more than a low-fat diet and in some studies preferentially increase the ingestion of pure fat more than carbohydrate, independent of baseline preference (33, 74). A role for endogenous opioids in the feeding process is supported by evidence that feeding of a fat-rich diet and a preferential increase in fat intake are reduced by peripheral injection of opiate antagonists (7, 74) and also by central injection of selective antagonists of μ - and κ - but not δ -opioid receptors (5, 28).

The question to be investigated in this report is whether the endogenous opioids in the hypothalamus are similar to GAL in their response to dietary fat. There is limited evidence on this question, with published reports yielding inconsistent results. A recent study from this laboratory (13) showed that ENK mRNA in the PVN is stimulated by acute 4 h of exposure to a high-fat compared with low-fat diet and also by peripheral injection of the lipid emulsion Intralipid. However, this effect in the PVN is not apparent after chronic ingestion of a fat-rich diet (71), and in the arcuate nucleus (ARC) there is no change in ENK with either chronic diet consumption or Intralipid injection (13, 71). Also, in the NAc, a very different result is obtained, with ENK mRNA suppressed by chronic high-fat diet consumption but unaffected by acute diet intake (30). Although there are no studies of DYN in the NAc, measurements of this peptide in the hypothalamus with chronic diet manipulations show an increase in gene expression in the PVN and ARC in rats maintained on a high-fat diet (71) and a reduction in rats switched from a fat-rich diet to low-fat chow (4, 39). These results indicate that these hypothalamic opioids are responsive to dietary fat, although the precise nature and timing of this response still remain to be established.

The mechanisms underlying a high-fat diet effect on endogenous opioids also remain to be determined. The opioid peptides are generally believed to mediate the palatability and hedonistic impact of energy-dense foods, with evidence showing injection of morphine and other opioid agonists to enhance taste palatability and preferentially stimulate intake of preferred diets rich in fat or sugar (10, 22, 23, 25, 54). Thus the effect of a fat-rich diet on endogenous opioids may reflect the greater palatability or higher caloric intake characteristic of this diet (59, 62, 63). Although the NAc is believed to play an important role in mediating opioid-induced increase in palatable food intake (29, 30), it is not clear that palatability or calories ingested are major factors in hypothalamic peptide

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control of feeding. In fact, recent evidence (36) demonstrates that ENK in the PVN is stimulated by a high-fat compared with a low-fat diet even when the number of calories consumed is the same for the two diets. Also, they are similarly increased by injection of a fat emulsion, Intralipid, that bypasses the ingestion process and thus avoids stimuli related to palatability (13). Although taste stimuli or caloric intake may have some impact on hypothalamic peptides, these studies suggest that other, perhaps postingestive, factors such as circulating lipids may be important in determining the expression level and functions of these peptides in the PVN.

The purpose of this article was to systematically study both ENK and DYN in the hypothalamus as they relate to dietary fat. Using a variety of feeding paradigms, this report examined 1) the effect of different periods of diet exposure, ranging from a single 15-min meal to periods of 60 min, 1 day, and 1 wk; 2) changes in peptides that occur in the absence of changes in factors such as caloric intake, body weight, adiposity, and circulating hormones like insulin and leptin; 3) both ENK and DYN in the same experiment and different hypothalamic nuclei, namely the PVN, ARC, and perifornical hypothalamus (PFH); 4) levels of both mRNA and peptide; 5) diets that control for palatability and energy density; and 6) circulating levels of triglycerides (TG) to determine whether they are positively related to the opioid peptides, as recently shown for GAL (13, 36). With these different feeding paradigms and measures, the results demonstrate a significant change in endogenous ENK and DYN, most consistently in the PVN, that is invariably accompanied by and possibly attributed to a rise specifically in circulating levels of TG.

METHODS

Animals. Adult male Sprague-Dawley rats (275–300 g; Charles River Breeding Labs, Kingston, NY) were individually housed (22°C, with lights off at 3:30 PM for 12 h) in a fully accredited American Association for the Accreditation of Laboratory Animal Care facility according to institutionally approved protocols as specified in the National Institutes of Health Guide for the Use and Care of Animals and also with the approval of the Rockefeller University Animal Care Committee. All animals were given 1 wk to acclimate to lab conditions, during which time they were maintained ad libitum on laboratory chow and water. All protocols fully conformed to the Guiding Principles for Research Involving Animals and Human Beings (1).

Diets. Different low-fat and high-fat diets were used. *Experiments 1–3* tested solid mixed diets. The constituents of the low-fat diet were fat with 10% vegetable oil, carbohydrate with 30% dextrin, 30% cornstarch (ICN Pharmaceuticals), and 40% sucrose (Domino), and protein with casein (Bioserv, Frenchtown, NJ) and 0.03% L-cysteine hydrochloride (ICN Pharmaceuticals), and the constituents of the high-fat diet were fat with 75% lard (Armour) and 25% vegetable oil (Crisco), carbohydrate with 30% dextrin, 30% cornstarch (ICN Pharmaceuticals), and 40% sucrose (Domino), and protein with casein (Bioserv) and 0.03% L-cysteine hydrochloride (ICN Pharmaceuticals). These solid diets, given to the rats for periods of 1 day to 1 wk, were supplemented with minerals (USP XIV Salt Mixture Briggs; ICN Pharmaceuticals) and vitamins (Vitamin Diet Fortification Mixture; ICN Pharmaceuticals), as described previously (19, 36). The macronutrient composition of these diets was calculated as percentage of total kcal, with the low-fat diet containing 10% fat, 65% carbohydrate, and 25% protein (3.8 kcal/g) and the high-fat diet containing 50% fat, 25% carbohydrate, and 25% protein (5.2 kcal/g). *Experiments 4–6* tested liquid mixed diets presented in 100 ml plastic cylinders. In *experiments 4* and *5*, the low-fat diet consisted of one part Half & Half

cream (Tuscan) and 10 parts skim milk (0.45 kcal/g, 25% fat), and the high-fat diet consisted of two parts Half & Half cream and one part skim milk (0.99 kcal/g, 80% fat). The liquid diets used in *experiment 6* were the low-fat (17% fat) and high-fat (60% fat) diets described in detail by Warwick (69), which are equal in caloric density (2.3 kcal/g) and are equally palatable.

Test procedures. The purpose of this report was to examine the effect of different periods of diet exposure, ranging from 15 min to 1 wk, so that changes in circulating lipids and hypothalamic peptides can be examined in the absence of disturbances in caloric intake, body weight, adiposity, and hormones. Furthermore, different diets were used to control for palatability and energy density. A total of seven sets of rats ($n = 20$ /set) were employed in this study, with each set divided into two groups of equal body weight and daily food intake and assigned to either a low-fat or high-fat diet group. Prior to the start of the experiment, all rats were exposed on 3 consecutive days before the start of their respective diets, for 2 h at the onset of the nocturnal feeding cycle. Measures of 24-h food intake and body weight were taken on the 1–2 days prior to death. For the actual experiment, the diet was made available for either 1 wk (*experiments 1* and *3*), 1 day (*experiments 2* and *3*), 60 min at 2 h before dark onset (*experiments 4* and *5*), or 15 min at 2 h before dark onset (*experiment 6*). All rats were killed by rapid decapitation around dark onset, with food removed ≥ 30 min prior to death. The brains were rapidly removed, trunk blood was collected, and the unilateral retroperitoneal fat pad was dissected and weighed. As described in sections below, the PVN, PFH, and ARC were microdissected for measurements of ENK and DYN, using real-time quantitative PCR (*experiments 1, 2, 4, and 6*) and RIA (*experiments 3* and *4*). In *experiment 4*, the brains for one set of rats were placed in a 4% paraformaldehyde solution for peptide measurements using radiolabeled in situ hybridization histochemistry. All analyses were performed blind to the treatment condition.

Hormone and metabolite assays. Serum from trunk blood was assayed for insulin and leptin using RIA kits from Linco Research (St. Charles, MO). Serum TG levels were assayed using a Triglyceride Assay Kit (Sigma, St. Louis, MO) and E-Max Microplate Reader.

Brain dissection. Immediately after death, the brain was placed in a matrix with the ventral surface facing up, and three 1.0-mm coronal sections were made, with the middle optic chiasma as the anterior boundary. As previously described (13), the sections were placed on a glass slide, and three hypothalamic areas, the PVN (bregma -1.3 to -2.1 mm), PFH (bregma -2.8 to -3.3 mm), and ARC (bregma -2.8 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.

Real-time quantitative PCR. As previously described (13), total RNA from pooled microdissected hypothalamic samples was extracted with TRIzol reagent. RNA was treated with RNase-free DNase I before RT, and cDNA and minus RT were synthesized using an oligo(dT) primer with or without SuperScript II RT.

The real-time PCR was performed with Applied Biosystems' system (Foster City, CA). With Applied Biosystems Primer Express V1.5a software, primers were designed to have a melting temperature of 58 – 60°C and to produce an amplicon of 50 – 150 bp. The last five bases on the 3' end contained ≤ 2 G and/or C bases to reduce the possibility of nonspecific product formation. The primer pairs for preproenkephalin (ENK) (5'-GGA CTG CGC TAA ATG CAG CTA-3' and 5'-GTG TGC ATG CCA GGA AGT TG-3') (GenBank no. NM 017139) generate a 65-bp amplicon corresponding to the

nucleotide 411–474 of the sequence that crosses 2 exons spanning ~3,495 bases of intron. The primer pairs for preprodynorphin (DYN) (5'-CAG CCG ACT GCC TGT CC TT-3' and 5'-TCA GGG TGA GAA AAG ACC AAA AG-3') (GenBank no. NM019374) generate a 159-bp amplicon corresponding to nucleotide 217–375 of the sequence that crosses 2 exons. The primer pairs for β -actin (5'-GGC CAA CCG TGA AAA GAT GA-3' and 5'-CAC AGC CTG GAT GGC TAC GT-3') (GenBank no. NM031144) generate a 79 amplicon corresponding to the nucleotide 420–498 of the sequence that crosses exon 2 and exon 3.

The SYBR Green PCR core reagents kit (Applied Biosystems) was used, with β -actin as endogenous control. PCR was performed in Micro-Amp Optic 96-well reaction plates (Applied Biosystems) on an ABI PRISM 7900 Sequence Detection system (Applied Biosystems), with the condition of 2 min at 50°C, 10 min at 95°C, and then 40 cycles of 15 s at 95°C and 1 min at 60°C. Each study consisted of four independent runs of PCR in triplicate, and each run included a standard curve, nontemplate control, and negative RT control. The concentrations of primers were 100–200 nM, and all reagents, unless indicated, were from Invitrogen. The levels of target gene expression were quantified relative to the level of β -actin, using standard curve method. Although GAPDH and cyclophilin were also tested in some initial experiments and found to yield stable results with no response to a high-fat diet, β -actin was generally the least variable of these three housekeeping genes and thus used as the control in the present experiments.

The specificities of RT-PCR products were confirmed by both a single dissociation curve of the product and a single band with corresponding molecular weight revealed by agarose gel electrophoresis. In addition to the nontemplate control and negative RT control, an anatomical negative control was also performed by using the corpus callosum in the same brain to verify the specificity of the quantitative PCR. No signals above threshold of ENK and DYN were detected by quantitative PCR in any of these controls.

Radiolabeled in situ hybridization histochemistry. In addition to real-time quantitative PCR, mRNA levels of ENK and DYN were measured with radiolabeled in situ hybridization histochemistry in rats consuming a high-fat compared with low-fat diet for a 4-h interval. Antisense RNA probes and sense probes were labeled with [³⁵S]UTP (Amersham Biosciences, Piscataway, NJ) as described (35, 43). Alternate free-floating coronal sections were consecutively processed as follows: 10 min in 0.001% proteinase K, 5 min in 4% paraformaldehyde, and 10 min each in 0.2 N HCl and acetylation solution, with a 10-min wash in phosphate buffer between each step. After being washed, the sections were hybridized with ³⁵S-labeled probe (10³ cpm/ μ l) at 55°C for 18 h. Following hybridization, the sections were washed in 4 \times SSC, and nonspecifically bound probe was removed by RNase (Sigma) treatment for 30 min at 37°C. Next, sections were run through additional stringency washes with 0.1 M dithiothreitol (Sigma) in 2 \times SSC and 1 \times SSC and 0.1 \times SSC at 55°C. Finally, sections were mounted, air-dried, and exposed to Kodak BioMax MR film for 48–72 h at –80°C, when films were developed and macroscopically analyzed. The sense probe control was performed in the same tissue, and no signal was found.

Computer-assisted microdensitometry of autoradiographic images was determined as described (43, 57) on the MCID image analysis system (Imaging Research, St. Catharines, ON, Canada). Microscale ¹⁴C standards (Amersham Biosciences) were exposed on the same Kodak film with the sections and digitized. Gray level/optical density calibrations were performed by using a calibrated film strip ladder (Imaging Research) for optical density. Optical density was plotted as a function of microscale calibration values. It was determined that all subsequent optical density values of digitized autoradiographic images fell within the linear range of the function. The values obtained represent the average of measurements taken from 10–12 sections per animal. In each section, the optical density for the PVN, PFH, or ARC was recorded, from which the background optical density from a same-size area in the thalamus was subtracted. The mean value of the high-fat diet group in each hypothalamic area was reported as a percentage of the low-fat diet group.

RIA. The PVN tissue was homogenized in 1 ml of 0.1 M acetic acid and centrifuged at 14,000 g for 15 min at 4°C. All of the supernatant was removed, boiled for 10 min, and frozen at –80°C until use. For RIA, commercially available kits for met-ENK and DYN-A (Peninsula Laboratories, San Carlos, CA) were used.

Data analysis. The values in the figures and tables are expressed as means \pm SE. In each experiment, direct comparisons between the scores for the low-fat diet and high-fat diet groups were made by an unpaired *t*-test. The criterion for the use of the term “significant” in the text was that the probability value (*P*) for a given test be <0.05.

RESULTS

Experiment 1: effect of 1-wk high-fat diet on peptide expression. This experiment tested the effect of chronic high-fat diet consumption on ENK and DYN gene expression in the different hypothalamic nuclei. Two groups of rats (*n* = 10/group) were maintained for 1 wk on a solid high-fat (50% fat) or low-fat (10% fat) diet (see METHODS). The high-fat diet significantly increased 24-h caloric intake, body weight, fat pad weight, and levels of leptin, although it had no impact on insulin (Table 1). This diet, however, caused a significant, 50% rise in circulating levels of TG (Table 1). Measurements of ENK and DYN using real-time quantitative PCR revealed a significant change, with both peptides responding similarly to dietary fat (Fig. 1). Chronic high-fat diet intake over the 1-wk period produced a 20–40% increase in the expression of ENK and DYN, and this effect was observed similarly in each of the three hypothalamic areas: the PVN, PFH, and ARC.

Experiment 2: effect of 1-day high-fat diet on peptide expression. To minimize the changes in caloric intake, body weight, and fat pad weight that can be induced by a chronic high-fat diet, as shown in *experiment 1*, this experiment tested the effect of a shorter, 1-day period of high-fat diet consumption on ENK and DYN expression. A second set of rats (*n* =

Table 1. Measurements in rats fed a low-fat or high-fat diet for different time periods in experiments 1,2,4 and 6

Time	Exp. No.	Diet	Caloric Intake, kcal	Body Weight, g	Fat Pad Weight, g	Leptin, ng/ml	Insulin, ng/ml	TG, mg/dl
1 week	1	Low fat	100 \pm 7	463 \pm 9	1.7 \pm 0.2	4.7 \pm 0.8	2.7 \pm 0.4	82 \pm 10
		High fat	115 \pm 6*	490 \pm 10*	3.0 \pm 0.5*	11.9 \pm 1.6*	2.6 \pm 0.3	124 \pm 9*
1 day	2	Low fat	89 \pm 9	448 \pm 7	2.8 \pm 0.4	8.8 \pm 0.8	2.5 \pm 0.4	96 \pm 8
		High fat	96 \pm 7	447 \pm 5	2.9 \pm 0.2	11.4 \pm 0.7*	2.3 \pm 0.4	156 \pm 13*
60 min	4	Low fat	15 \pm 3	403 \pm 6	2.3 \pm 0.3	5.3 \pm 0.6	1.7 \pm 0.4	80 \pm 15
		High fat	29 \pm 3*	403 \pm 8	2.1 \pm 0.2	4.5 \pm 0.9	1.8 \pm 0.3	159 \pm 21*
15 min	6	Low fat	15 \pm 0.2	370 \pm 11	1.6 \pm 0.2	5.3 \pm 0.9	2.3 \pm 0.3	102 \pm 10
		High fat	15 \pm 0.1	379 \pm 6	1.7 \pm 0.2	5.0 \pm 1.3	1.9 \pm 0.2	177 \pm 35*

Data are means \pm SE; *n* = 10/group. **P* < 0.5 for comparisons between high-fat and low-fat diet groups.

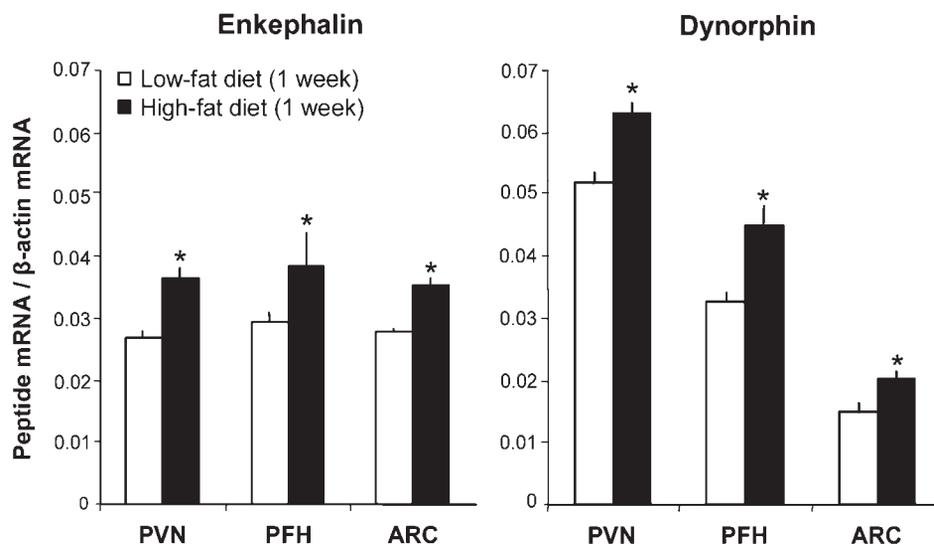


Fig. 1. Effects of high-fat diet for 1 wk (115 kcal/day) on the expression of enkephalin and dynorphin in the paraventricular nucleus (PVN), perifornical hypothalamus (PFH), and arcuate nucleus (ARC) as measured by real-time quantitative PCR. The data (means \pm SE) showed a significant increase in both peptides, in all areas, in the high-diet compared with the low-fat diet group. * $P < 0.05$ compared with low-fat diet group.

10/group) was given a solid high-fat or low-fat diet for 24 h. After just 1 day, the high-fat diet had little effect on the measures of caloric intake, body weight, and fat pad weight, and it produced a small increase in leptin, whereas it had no effect on insulin (Table 1). The greatest change was observed in circulating levels of TG, which were increased by 60% in the high-fat diet group (Table 1).

This rise in lipids, similar to that seen in *experiment 1* after 1 wk on the diet, was associated with a similar change in peptide expression measured by real-time quantitative PCR. The high-fat diet for 1 day significantly stimulated, by 35–100%, both ENK and DYN expression in the PVN and PFH while producing a somewhat smaller, 15–20% increase in the ARC (Fig. 2). Thus, in addition to confirming the change in peptide gene expression observed after 1 wk of diet consumption (*experiment 1*), these results with a briefer period on the diet give some indication that the opioid peptides in the PVN and PFH may be more responsive to dietary fat than those in the ARC.

Experiment 3: effect of 1-wk and 1-day high-fat diet on peptide levels. To determine whether this increase in peptide expression translates into a change in peptide production, this

experiment in two additional sets of rats ($n = 20$ /set) used RIA to measure peptide levels in the PVN of rats consuming a high-fat vs. low-fat diet for 1 wk or 1 day, using similar procedures as described in *experiments 1* and *2*. These two sets of rats yielded very similar results to those presented in Table 1, with 1 day in contrast to 1 wk on the high-fat diet having no effect on caloric intake, body weight, and fat pad weight and with both feeding paradigms producing a 50% increase in levels of TG.

As with measurements of peptide mRNA in *experiments 1* and *2*, the high-fat diet produced a significant increase in peptide levels of both ENK and DYN in the PVN (Fig. 3). The magnitude of this diet effect, ranging from 40 to 60%, was similar for the two peptides and the two feeding paradigms. Thus the change in peptide expression in the PVN demonstrated in *experiments 1* and *2* resulted in an increase in the production of both ENK and DYN peptides, with both effects occurring in association with a marked rise in TG levels.

Experiment 4: effect of 60-min high-fat diet on peptide gene expression. This experiment tested whether a brief, 60-min period of high-fat diet consumption can affect peptide gene expression and TG levels in a similar manner as the 1-day and

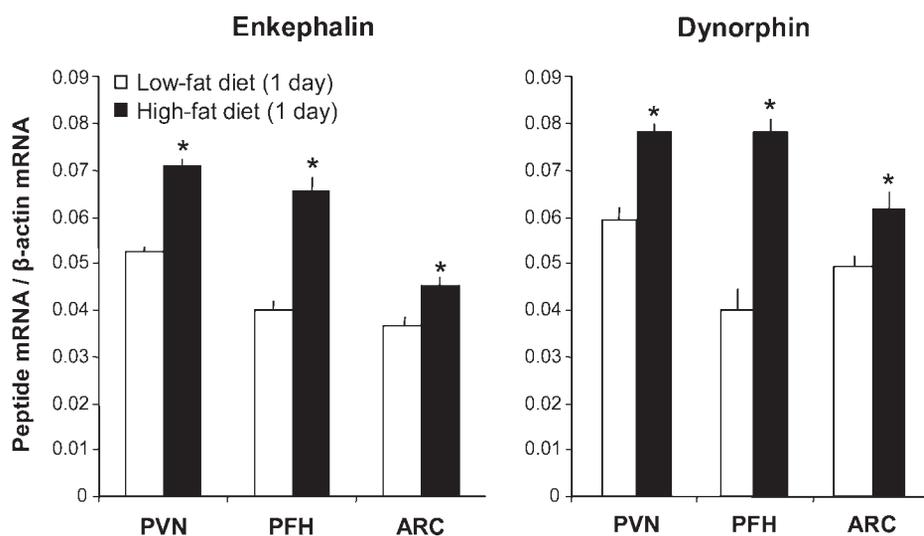


Fig. 2. Effects of high-fat diet for 1 day (95 kcal) on the expression of enkephalin and dynorphin in the PVN, PFH, and ARC as measured by real-time quantitative PCR. The data (means \pm SE) revealed a significant increase in both peptides, in all areas, in the high-fat compared with low-fat diet group. * $P < 0.05$ compared with low-fat diet group.

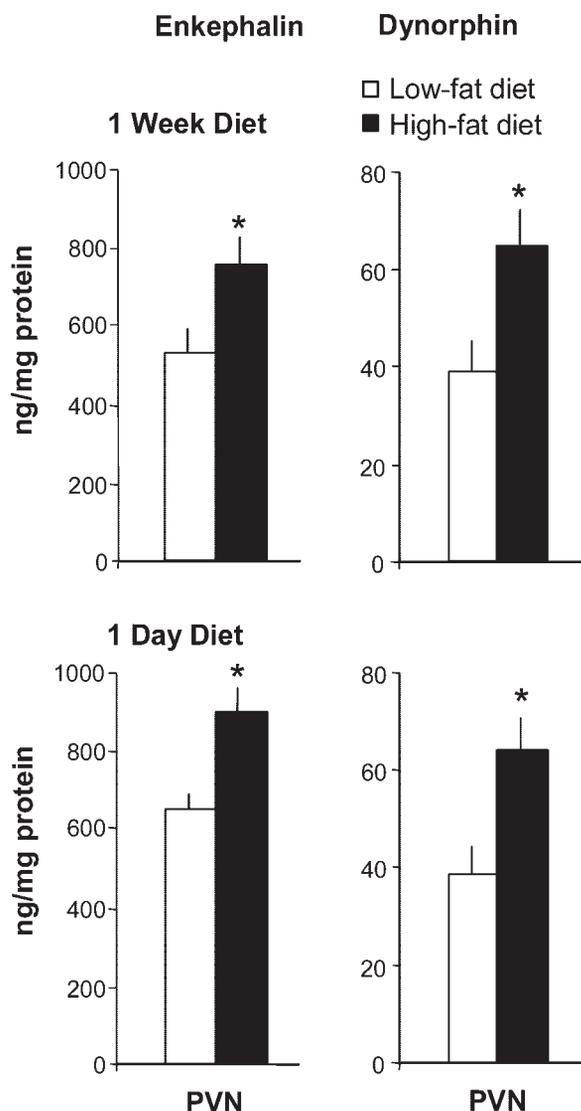


Fig. 3. Effects of high-fat diet for 1 wk or 1 day on levels of enkephalin and dynorphin peptide in the PVN as measured by RIA. The data (means \pm SE) obtained from these 2 feeding paradigms showed a significant increase in both peptides in the PVN of the high-fat compared with low-fat diet group. * $P < 0.05$ compared with low-fat diet group.

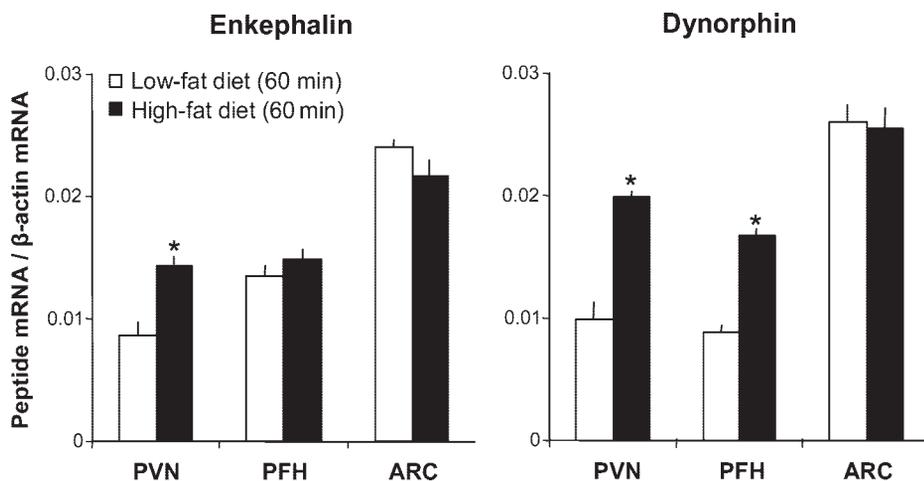


Fig. 4. Effects induced by 60 min of high-fat diet intake (30 kcal) on the expression of enkephalin and dynorphin in the PVN, PFH, and ARC as measured by real-time quantitative PCR. The data (means \pm SE) revealed a significant increase in the expression of both peptides in the PVN, but not in the ARC, of the high-fat diet group. * $P < 0.05$ compared with low-fat diet group.

1-wk feeding paradigms and whether this effect can occur in the absence of a change in leptin. In this experiment, the rats ($n = 10/\text{group}$) consumed a liquid high-fat or low-fat diet (~ 30 ml) given for 60 min at 2 h prior to dark onset (see METHODS). This brief period of diet exposure, while increasing caloric intake during the 60-min test period, had little effect on the measures of body weight and fat pad weight in addition to leptin and insulin (Table 1). However, it produced a marked, twofold rise in circulating TG levels, even greater than that seen with the longer periods of diet exposure (Table 1).

With this rise in TG, the expression level of ENK and DYN measured via real-time quantitative PCR was significantly increased, by 50%, specifically in the PVN (Fig. 4). In the PFH this brief period of diet consumption produced no change in ENK mRNA, whereas DYN mRNA was significantly elevated, and in the ARC neither peptide was affected. These results demonstrate that ENK and DYN in the PVN after 60 min of high-fat diet intake respond in a similar manner as they did after 1 day or 1 wk on the diet (*experiments 1* and *2*). With this brief period of diet consumption, the data also show that ENK in the PFH is less responsive than it is in the PVN and that both opioid peptides are unresponsive in the ARC.

To confirm whether high-fat diet consumption for 60 min also produces a change in peptide levels 1 h after completion of the meal, an additional set of rats ($n = 9/\text{group}$) was examined with the same diets and test paradigm, and their peptide levels in the PVN were measured via RIA as in *experiment 3*. After this 60-min period of food intake, levels of TG were elevated by the high-fat compared with low-fat diet from 86 ± 3.4 to 182 ± 11 mg/dl ($P < 0.001$), similar to the results for the chronic diet paradigm presented in Table 1. The levels of opioid peptides in the PVN were also increased, with a significant effect obtained for ENK (624 ± 71 vs. 446 ± 28 ng/mg protein, $P < 0.03$) and a trend for DYN (60 ± 9.7 vs. 48 ± 6.6 , $P > 0.10$) that failed to reach statistical significance.

Experiment 5: effect of 60-min high-fat diet on peptide mRNA measured using in situ hybridization. To provide a more quantitative analysis of this change in peptide expression, the next set of rats ($n = 10/\text{group}$) was tested on the same 60-min feeding paradigm as in *experiment 4*, with the peptide gene expression measured using radiolabeled in situ hybridization. The results of this experiment confirmed those obtained in *experiment 4*, demonstrating a marked increase in peptide

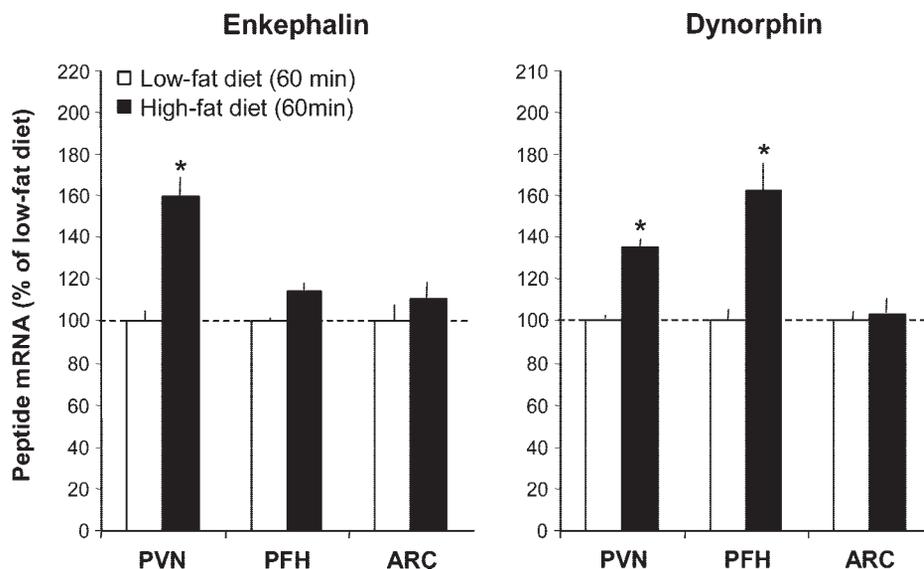


Fig. 5. Changes induced by 60 min of high-fat diet intake (30 kcal) on the expression of enkephalin and dynorphin in the PVN, PFH, and ARC as measured by radiolabeled in situ hybridization histochemistry. The data (means \pm SE) revealed a significant increase in expression of both peptides in the PVN, but not the ARC, of the high-fat diet group. * $P < 0.05$ compared with the low-fat diet group.

mRNA specifically in the PVN of the high-fat diet rats, in association with a rise in TG levels. After 60 min of liquid high-fat diet intake, these rats showed no change in caloric intake, body weight, fat pad weight, and hormone levels, but they showed a twofold increase in TG levels (143 vs. 73 mg/dl, $P < 0.01$) similar to that shown in Table 1 for the rats in *experiment 4*.

The radiolabeled in situ hybridization technique revealed a distribution pattern of GAL-, ENK- and DYN-expressing neurons similar to that previously described (15, 27, 35), with a dense signal seen in the PVN and PFH and a relatively weak signal in the ARC. Group comparisons showed significantly increased mRNA levels for both peptides in the PVN of the high-fat diet rats (expressed as %low-fat diet), as shown in Fig. 5 and illustrated in the photomicrographs of Fig. 6. Although ENK mRNA was increased by 65% in the PVN, this peptide showed little change in the PFH and ARC. The expression of DYN, however, was significantly affected in both the PVN and

PFH, but not in the ARC. These results are very similar to those obtained in *experiment 4* using real-time quantitative PCR, showing a close association between increased peptide expression in the PVN and a rise in circulating TG.

Experiment 6: effect of a 15-min high-fat diet with equal caloric density and palatability. Since the high-fat diets used in *experiments 1–5* were more calorically dense and thus perhaps more palatable than the low-fat diets, the increase in peptide expression and levels may be attributed to these specific properties of the fat-rich diet. To test this possibility, a final set of rats ($n = 10$ /group) was examined with the liquid high-fat or low-fat diets described by Warwick (69), which are similar in caloric density and equally palatable. To reduce the amount of diet exposure even further, the rats were given these diets for only 15 min at 2 h prior to the start of the nocturnal feeding cycle and were then killed 45 min later. This brief period of diet exposure had no effect on the measures of caloric intake, body weight, fat pad weight, and levels of insulin or leptin, although there was a marked increase (100%) in circulating levels of TG (Table 1).

With this rise in TG, the expression level of ENK and DYN measured via real-time quantitative PCR was significantly increased, by 100%, in the PVN of the high-fat diet group (Fig. 7). Whereas the PFH showed a smaller change in DYN on the high-fat diet, ENK in this area was unresponsive, and both peptides were unaffected in the ARC. Once again, these results show that the opioid peptides are more responsive in the PVN than in the PFH and least responsive in the ARC to dietary fat and elevated TG levels. In addition, they demonstrate that differential palatability, caloric density, and total calories consumed are not critical factors in the diet-induced stimulation of peptide expression.

DISCUSSION

Stimulatory effect of dietary fat on opioid peptides in the PVN. The results of these six experiments consistently demonstrate a robust, stimulatory effect of high-fat diet consumption on the opioid peptides in the hypothalamus. This effect, previously described for ENK after a 4-h period of diet expo-

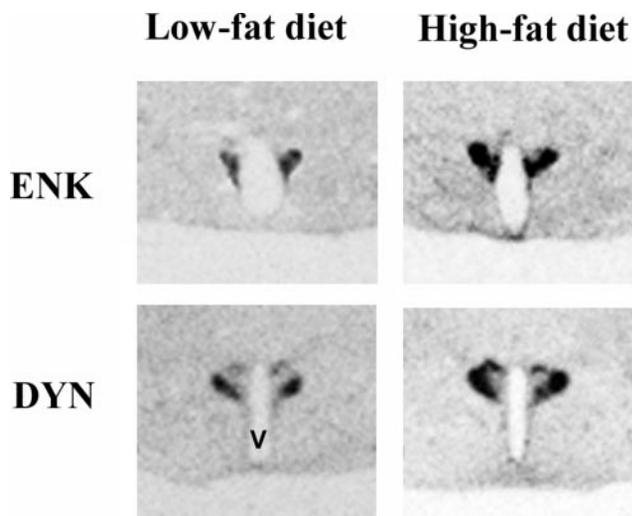


Fig. 6. Photomicrographs illustrating the stimulatory effect of a brief, 60-min period of high-fat diet consumption on mRNA levels of enkephalin and dynorphin in the PVN, as graphed in Fig. 5. V, third ventricle.

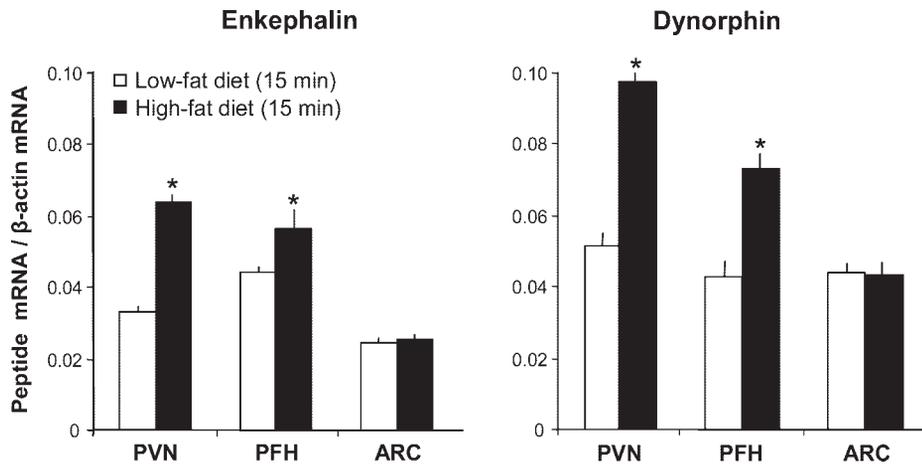


Fig. 7. Changes induced by 15 min of high-fat diet consumption (15 kcal) on the expression of enkephalin and dynorphin in the PVN, PFH, and ARC as measured by real-time quantitative PCR. With diets of equal palatability and caloric density, this brief period of high-fat diet consumption significantly increased the expression of both peptides in the PVN. * $P < 0.05$ compared with the low-fat diet group.

sure (13), was similarly seen here for both ENK and DYN after variable periods of high-fat diet intake. The strongest and most consistent change in peptides was evident in the PVN. In this nucleus, ENK and DYN were increased by 50–100% after high-fat diet consumption. This dietary effect was revealed with measurements of mRNA levels in the PVN using both real-time quantitative PCR and radiolabeled in situ hybridization histochemistry. Consistent with evidence obtained with measurements of DYN mRNA in the PVN after chronic high-fat diet consumption over 1–7 wk (39, 71), the present findings revealed a stimulatory effect of 1-wk consumption of a solid fat-rich diet on ENK mRNA in the PVN, with similar results obtained for DYN mRNA. They additionally showed this change in gene expression of both peptides to occur after just 1 day on this diet. Furthermore, with measurements using RIA, they demonstrated an increase in ENK and DYN peptide levels in the PVN after 1 wk or 1 day of diet exposure. This latter result, consistent with our previous findings showing mRNA and peptide to respond similarly (36, 68), suggests that peptide synthesis is increasing in response to the elevated gene expression and thus may have functional consequences. With the animals killed 1 h after a 60-min period of consuming a liquid high-fat diet, a similar increase in PVN mRNA was observed, consistent with rapid changes in gene expression observed after injection of alcohol or glucose (14, 17, 60). This was accompanied by a rise in peptide levels, which was statistically significant for ENK in the PVN, but only a trend for DYN, due to high variability in the scores. With the same robust response detected even after a 15-min meal of this liquid diet, these results underscore the sensitivity of the PVN opioid systems to changes in dietary fat content.

Stimulatory effect of dietary fat on opioid peptides in the PFH and ARC. These opioid peptides in the PFH were similar to the PVN in the direction and magnitude of their response to 1 wk as well as 1 day of high-fat diet consumption. Whereas 4 h of high-fat diet intake also significantly stimulated ENK in the PFH (13), the present experiments with briefer periods of diet exposure, 15 and 60 min, failed to reveal any change in this peptide, although they showed an increase in DYN expression in the PFH. Thus ENK in this hypothalamic area is less responsive than it is in the PVN, whereas DYN is similarly responsive in these two sites to dietary fat. Compared with the PVN and PFH, however, the opioid peptides in the ARC are consistently less responsive to fat consumption. Although they

are stimulated in this nucleus by the 1-wk feeding paradigm similarly to that seen with measurements of DYN after 1 or 3 wk of high-fat diet consumption (4, 39, 71), ENK and DYN in the ARC exhibited a smaller change than in the PVN and PFH after 1 day of diet consumption and little or no change after presentation of the diet for 15 or 60 min. Thus the opioid peptides in this basal hypothalamic nucleus clearly differ from those in the dorsal hypothalamic regions in their relation to dietary fat, responding only to chronic diet manipulations that produce an increase in body weight. The opioids in the PVN also differ from those in the NAc, where ENK mRNA is significantly reduced in rats maintained for 3 wk (3 h/day) on a high-fat diet and unaffected by acute ingestion of the diet (30).

Peptide stimulation independent of increased caloric intake and palatability. It has long been postulated that opioids in the brain play a major role specifically in mediating the increased palatability and hedonistic impact of energy-dense foods (23, 24, 40). They may also respond specifically to an increase in caloric intake, as suggested by evidence that 1 wk of high-fat diet consumption stimulates DYN mRNA in the PVN and ARC only when the rats consume a greater number of calories than on the low-fat diet (71). The results of the present experiments, however, suggest that an increase in caloric intake, caloric density, or palatability is not required for the phenomenon of fat-induced stimulation of the opioid peptides in the PVN. In the different feeding paradigms (Table 1), high-fat diet consumption significantly increased ENK and DYN expression, as well as peptide levels, even when the calories ingested were similar between the high-fat and low-fat groups. It was also apparent when the caloric density of the diets was controlled, as in the 15-min liquid diet paradigm of *experiment 6*. Although a high-fat diet is generally more palatable compared with a low-fat diet (59), and although this effect may contribute to the stimulation of opioid peptides (71), the present results do not support this possibility with respect to opioid changes in the hypothalamus. They demonstrate that opioid expression in the PVN can be stimulated with just 15 min of consuming a liquid high-fat diet that is equally palatable to the low-fat diet, suggesting that this variable is not an essential feature of the diet. This is supported by further evidence showing that, similar to consumption of a high-fat diet, injection of a lipid emulsion, Intralipid, that bypasses the ingestion process and stimuli related to palatability can in-

crease the expression of ENK in the PVN (13, 36). Thus something other than palatability must be involved in the effect of dietary fat on hypothalamic opioid peptides.

Increased opioid peptides independent of changes in body fat, leptin, and insulin. The results of the present experiments with variable periods of diet exposure demonstrate that high-fat diet consumption can stimulate the opioids ENK and DYN independently of any change in body fat. Both ENK and DYN were significantly elevated in the PVN after 1 wk on the high-fat diet, when there was only a small increase in fat pad weight, and they were similarly affected after 1 day, 60 min, or 15 min of high-fat diet consumption, when body fat was unaffected. Furthermore, in a published report (31) that measured these peptides specifically as a function of body weight, the expression of DYN in the PVN was actually reduced in obese compared with lean rats, whereas it was unchanged in the ARC.

Other evidence suggests that the diet-induced increase in opioid peptides can also occur independently of changes in the hormones leptin or insulin, which rise in association with an increase in adiposity (6, 61). Levels of insulin remained stable in all of the high-fat diet feeding paradigms, and leptin levels were unaltered in the 60-min and 15-min feeding paradigms that significantly stimulated both ENK and DYN in the PVN. Moreover, PVN expression of ENK was stimulated by injection of a fat emulsion, which had no effect on insulin or leptin (13). Although there appear to be no studies that have directly tested the effect of these hormones on ENK and DYN in the hypothalamus, acute leptin injection is found to increase Fos-IR in DYN neurons of the ARC (21). Thus, rather than affecting opioids in the PVN, leptin may have some role in stimulating DYN and possibly ENK in the ARC, where leptin receptors are particularly dense and peptide-expressing neurons are particularly sensitive to leptin (16). This is supported by the findings here showing that ENK and DYN in the ARC are stimulated after 1 wk or 1 day on a fat-rich diet that elevates leptin levels but are unaffected when the diet is presented for a briefer period that produces no change in leptin. Any involvement of leptin in the stimulatory effect of a chronic high-fat diet on opioids in the ARC is unlikely to be related to the greater palatability of the diet, since this hormone, like insulin, actually reduces the ingestion and rewarding properties of palatable diets (32, 64).

Role for circulating TG in stimulating opioid peptides in the PVN. The most robust change in blood induced by the different periods of high-fat diet exposure that stimulated the opioids was an increase in circulating levels of TG. Consistent with published reports in animals and humans (11, 61), these lipids were elevated by 50–100% on a high-fat compared with a low-fat diet. This change in lipids occurred whether the diet was consumed for 1 wk, 1 day, or as little as 15 min. Also, it was invariably accompanied by a significant increase in ENK and DYN, most consistently in the PVN. This association between TG and the endogenous opioids is supported by a study with injection of a lipid emulsion (13). Peripheral administration of 20% Intralipid significantly elevated circulating levels of TG to the same degree as a high-fat diet, and this was accompanied by a significant increase in ENK mRNA in the PVN, an effect that may last up to 4 h after injection as long as TG are elevated. Little is known about the mechanisms underlying the effect of lipids, either TG or fatty acids, on hypothalamic peptide systems.

Either directly or indirectly, these lipid metabolites can alter central neural processes by affecting neuronal activity, enzyme activity, and gene expression (3, 13, 18, 49, 53, 73). Recent studies (13, 56) show that injection of Intralipid or linoleic acid produces an increase in neuronal activity and peptide mRNA, specifically in the PVN and PFH. This effect can be detected in hypothalamic neurons that express the orexin peptides that, like the opioids, stimulate feeding and, in turn, are stimulated by the consumption of a high-fat diet and elevated TG (13). However, it does not occur in the ARC, where, as discussed in sections above, the peptides are less responsive to fat but more sensitive to changes in leptin. Although the molecular mechanisms elevating ENK and DYN transcripts within 15 and 60 min remain to be characterized, evidence (44) demonstrates a direct stimulatory effect of short-chain fatty acids on ENK gene expression in PC12 rat cells via an intact PKA signaling pathway, with a possible involvement of lipid-activated transcription factors. It is of interest that, in addition to their effects on peptide expression, fatty acids may also modulate the binding of opioid peptides to their receptor (58).

Functional consequences of elevated opioid peptides in the PVN. The stimulation of opioid gene expression in the PVN by a high-fat diet, in association with a rise in circulating TG, was accompanied by an increase in opioid peptide levels as measured by RIA. This suggests that the increased mRNA levels enhanced the production and possibly the release of these peptides, allowing them to have functional consequences in the PVN. As revealed by pharmacological studies, a primary effect of these peptides after hypothalamic injection is the stimulation of feeding behavior, particularly of a fat-rich diet (7), and the opposite effect is obtained with injection of μ - and κ -antagonists, but not δ -antagonists (5, 28). Chronic consumption of a high-fat diet has long been known to enhance total caloric intake as well as raise circulating levels of TG (36, 69). Also, a small high-fat meal (15 kcal) or injection of a fat emulsion (10 kcal), both of which elevate TG levels, stimulates food intake in subsequent meals (12, 42, 50, 69, 70). The present findings help to elucidate possible mechanisms underlying this phenomenon of fat-induced hyperphagia. They suggest that dietary fat stimulates the expression and production of PVN opioid peptides, which in turn promote additional eating behavior, and that circulating TG raised by the fat-rich diet are active participants in this process.

Opioid peptides in relation to GAL in the PVN. These results observed with the opioid peptides are very similar to those obtained with measurements of GAL in the hypothalamus. As with ENK and DYN, the expression of GAL in the PVN, but not the ARC, is consistently stimulated by acute or chronic consumption of a high-fat diet and closely related to circulating TG levels (2, 13, 35, 36, 47, 52, 55). Also, the feeding response induced by GAL injection is stronger in rats that are given a high-fat diet or naturally overeat fat (8, 41, 67), and feeding on a fat-rich diet is reduced by injection of a GAL antagonist (38, 51). These similarities between the opioids and GAL in relation to dietary fat suggest that these peptides in the hypothalamus may function in a similar manner and possibly interact in the process of controlling feeding behavior (34). This is further supported by evidence that GAL is colocalized with DYN in neurons of the PVN (48) and that GAL-induced feeding is blocked by an opioid antagonist (8, 20). Thus, GAL may act in

part through the endogenous opioid system to stimulate food intake on a palatable, high-fat diet. Another active participant in this process may be dopamine in the forebrain, which has a primary function in the motivation to eat palatable foods. The release of dopamine in the NAc is stimulated by both GAL and the opioids (9, 37) and also by the ingestion of a palatable, fat-rich meal (45, 72).

Thus, it is hypothesized that a high-fat diet promotes hyperphagia, in part, by increasing the expression and production of opioid peptides as well as GAL in the PVN, which in turn promote overeating on a fat-rich diet. This effect of dietary fat, which occurs independently of factors related to palatability, caloric density, body fat, and body weight, involves circulating TG and possibly stimuli related to the metabolism of these lipids.

GRANTS

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