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Circulating Triglycerides Impact on Orexigenic Peptides and Neuronal Activity in Hypothalamus

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Little is known about the impact of circulating lipids on brain processes. Building on evidence that chronic fat consumption stimulates hypothalamic peptides in close association with elevated triglycerides (TG), this study examined whether an acute rise in TG levels induced by fat emulsion can affect these hypothalamic systems. In normal weight rats, ip injection of Intralipid (20%, 5 ml) during the first 4 h after injection produced a robust increase in TG levels and nonesterified fatty acids, but had no impact on glucose, insulin, or leptin levels. This was accompanied by a marked increase in the expression of particular orexigenic peptides, galanin, orexins, and the opioid, enkephalin, which are known to be positively related to fat ingestion. This effect, similarly induced by 4 h of high fat diet consumption, was detected in the paraventricular nucleus (PVN) for galanin, in the perifornical hypothalamus (PFH) for orexins, and in the PVN, PFH, as well as the arcuate nucleus (ARC) for enkephalin. It was not seen, however, for neuropeptide Y and agouti-related protein localized in the ARC, which are unaffected or reduced by dietary fat. This site specificity was confirmed by c-Fos immunostaining, a marker of neuronal activity, which was increased by Intralipid in the PVN and PFH, but not in the ARC, and was detected in 20% of orexin-expressing neurons in the PFH. These findings suggest that circulating lipids, through different mechanisms, may stimulate hypothalamic neurons, which synthesize specific feeding stimulatory peptides that possibly contribute to hyperphagia during consumption of a fat-rich diet. (*Endocrinology* 145: 3904–3912, 2004)

THERE IS STRONG evidence linking the ingestion of a fat-rich diet to the development of obesity (1, 2). The accrual of body fat rises in direct proportion to the amount of fat ingested, rather than the amount of carbohydrate, protein, or total caloric intake (1). Also, a strong preference for fat in outbred and inbred rodent strains is closely associated with a propensity toward obesity on a high fat diet (3). In addition to hyperphagia, the endocrine and metabolic changes produced by a fat-rich diet, which probably contribute to obesity, include hypertriglyceridemia, hyperglycemia, insulin resistance, and reduced energy expenditure as well as sympathetic nervous system activity (4–6).

Little is known about the central neurochemical mechanisms mediating these effects of dietary fat. Recent evidence demonstrates that chronic consumption of a high fat diet increases the expression of specific peptides in the hypothalamus. These fat-stimulated peptides include galanin, which is enhanced by a high fat diet, specifically in the paraventricular nucleus (PVN), where galanin peptide levels rise in direct proportion to the amount of fat in the diet (7, 8). They also include the orexins, which are stimulated by fat consumption in the perifornical hypothalamus (PFH) (9). Moreover, there is evidence that opioid peptides in the hypothalamus may also be stimulated by dietary fat (10, 11). It is of interest that these different peptides, when centrally injected, share an additional property, that of increasing food intake and producing a stronger feeding response during the consumption of diets rich in fat (12–16).

The mechanisms underlying this diet-induced increase in gene expression have yet to be defined. Because it can occur in normal weight and normophagic rats and is not a response to calories alone (9, 10, 17), it does not appear to be a direct consequence of the hyperphagia and obesity induced by a high fat diet. In addition, this diet-induced gene expression does not have a clear link to changes in the hormones, leptin and insulin. Whereas leptin injection inhibits the expression of these feeding stimulatory peptides (18, 19), levels of this hormone generally rise together with the hypothalamic peptides under natural feeding conditions (2, 9). Also, although insulin inhibits galanin expression (20), it has little or no impact on the orexins or opioid peptides in freely feeding animals (21, 22). Studies manipulating glucose or its utilization have similarly yielded mixed results, which include no change in galanin or a positive association with this peptide (7, 23), in contrast to an inverse relation between glucose and the activity of orexin neurons (22). Thus, although these different factors are likely to have some impact on peptide gene expression in rats consuming a high fat diet, these results lead one to consider other mechanisms, possibly related to circulating lipids, that underlie the response of these peptides to dietary fat.

Little is known about the effect of lipids, both triglycerides (TG) and fatty acids (FA), on hypothalamic peptide systems. In clinical and animals studies these metabolites are markedly elevated by both acute and chronic high fat diet conditions (4, 24), and they are also higher in obese compared with lean subjects consuming a fat-rich diet (9, 25). There is considerable research demonstrating the impact of lipids on gene expression in the liver (26, 27), and a few studies suggest that these metabolites may also affect central neural pro-

Abbreviations: AgRP, Agouti-related protein; ARC, arcuate nucleus; FA, fatty acid; ir, immunoreactivity; MA, mercaptoacetate; NEFA, nonesterified fatty acid; NPY, neuropeptide Y; n.s., nonsignificant; PFH, perifornical hypothalamus; PVN, paraventricular nucleus; TG, triglycerides.

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cesses, such as enzyme activity in the brain (28, 29). The hydrolysis of TG causes the release of FA, which are known to alter neuronal activity and gene expression in the brain (30, 31), and an inhibitor of FA synthase can modulate hypothalamic peptide expression (32). Also, in studies of galanin in the PVN and orexins in the PFH, mRNA levels of these peptides are found to be positively correlated with levels of circulating TG as well as the ingestion specifically of fat, and it is suppressed by an antagonist of fat metabolism (8, 9, 16, 23, 33). Although this peptide relationship to fat is a consistent phenomenon, this evidence provides only indirect support for an effect of circulating lipids on peptide-synthesizing neurons.

The present investigation used the fat emulsion, Intralipid (Baxter Healthcare Corp., Deerfield, IL), to provide further tests of a possible relationship between circulating lipids and hypothalamic peptides. This study, building on a recent report from this laboratory showing Intralipid to stimulate orexin expression in the PFH (9), systematically examined the effects of Intralipid on hormones and metabolites as a function of time after injection. It additionally tested the impact of Intralipid on three hypothalamic peptides, galanin, enkephalin, as well as orexin, and performed double-labeling immunofluorescence to examine changes in c-Fos activity in orexin-containing neurons. Under well-defined conditions of acute administration, Intralipid in normal weight and normophagic rats was found to increase circulating lipids without altering glucose levels or hormones known to influence the peptides. This fat emulsion, in a peptide- and site-specific manner, increased the expression of certain hypothalamic peptides and activated neurons that synthesize these peptides, suggesting a possible role for circulating lipids in modulating central mechanisms controlling food intake and body weight.

Materials and Methods

Animals

Adult male Sprague Dawley rats (Charles River Breeding Laboratories, Kingston, NY) were individually housed (22 C, with lights off at 1500 h 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 NIH Guide to the Use and Care of Animals and also with the approval of the Rockefeller University animal care committee. All protocols fully conformed to the Guiding Principles for Research Involving Animals and Human Beings (34). The rats (275–300 g) were given 1 wk to acclimate to laboratory conditions and were maintained *ad libitum* on water and laboratory chow, consuming 23–28 g/d.

Test procedures

One week before the Intralipid experiments, the rats were handled daily and were adapted to the procedure of ip injections of saline. The actual test involved a single, i.p. injection of Intralipid (5 ml, 20% solution) or saline 4–5 h before the onset of nocturnal feeding, with food removed at the time of the injection. In the first two experiments, the rats received three such tests in which blood was collected via tail vein puncture at one of three different time periods (0.5–4 h) after injection. These three tests were given in random order, 2 d apart. In the other experiments the rats were given a single test and were killed by decapitation 4 h after injection. Trunk blood was collected, and brains were rapidly removed and prepared for real-time quantitative PCR, Northern blot, or immunocytochemistry, as described below.

For the diet experiment the rats were given a 4-h test period at dark onset with a low or high fat diet to determine whether a brief period of exposure to dietary fat, similar to Intralipid, can stimulate peptide gene expression. To adapt them to the diet, the rats were first given two pretests of 2-h diet exposure at dark onset. The constituents of these diets were: fat with 75% lard (Armour Inc., Omaha, NE) and 25% vegetable oil (Pure Wesson Oil; Canagra, Fullerton, CA); carbohydrate, with 30% dextrin, 30% cornstarch (ICN Pharmaceuticals, Costa Mesa, CA), and 40% sucrose (Domino Food Inc., Yonkers, NY); and protein with casein (Bioserv, Frenchtown, NJ) and 0.03% L-cysteine hydrochloride (ICN Pharmaceuticals). These diets were supplemented with minerals (USP XIV Salt Mixture Briggs, ICN Pharmaceuticals) and vitamins (Vitamin Diet Fortification Mixture, ICN Pharmaceuticals). Diet composition was calculated as the percentage of total kilocalories, with the low fat diet containing 10% fat, 65% carbohydrate, and 25% protein (3.75 kcal/g) and the high fat diet containing 50% fat, 25% carbohydrate, and 25% protein (4.75 kcal/g).

Hormone and metabolite assays

Serum from trunk blood was assayed for insulin and leptin using assay kits from Linco Research, Inc. (St. Charles, MO). Serum glucose and TG levels were measured using a glucose Trinder Kit or TG Assay Kit (Sigma-Aldrich Corp., St. Louis, MO), and nonesterified FA (NEFA) levels were analyzed using a NEFA C Kit (Wako, Richmond, VA). The metabolite assays were performed using an E-Max Microplate Reader.

Brain dissection

Immediately after decapitation, 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. The sections were placed on a glass slide, and three hypothalamic areas, the PVN (Bregma −1.3 to −2.1 mm), PFH and arcuate nucleus (ARC) (Bregma −2.8 to −3.3 mm), were rapidly microdissected under a microscope, using the fornix and third ventricle as landmarks (Fig. 1). 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 and Northern blot analysis

As previously described (9), total RNA from pooled microdissected hypothalamic samples was extracted with TRIzol reagent. RNA was treated with ribonuclease-free deoxyribonuclease I before RT. For quantitative PCR, cDNA and minus RT were synthesized using an oligodeoxythymidine primer with or without SuperScript II reverse transcriptase. The SYBR Green PCR core reagents kit (Applied Biosystems, Foster City, 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 sec 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 levels of target gene expression were quantified relative to the level of β-actin, using the standard curve method. The primers, designed with ABI Primer Express version 1.5a software based on published sequences, were: 1) β-actin, 5′-GGCCAACCGTGAAAAGATGA-3′ (forward) and 5′-CACAGCCTGGATGGCTACGT-3′ (reverse); 2) galanin, 5′-TTCCCACCACTGCTCAAGATG-3′ (forward) and 5′-TGGCTGACAGGGTTGCAA-3′ (reverse); 3) orexin, 5′-AGATACCATCTCTCCGGATTGC-3′ (forward) and 5′-CCAGGGAACCTTTGTAGAAGGA-3′ (reverse); 4) enkephalin, 5′-GGACTGCGCTAAATGCAGCTA-3′ (forward) and 5′-GTGTGCATGCCAGGAAGTTG-3′ (reverse); 5) neuropeptide Y (NPY), 5′-CACAGAAAATGCCCCCAGAA-3′ (forward) and 5′-GTCAGGAGAGCAAGTTTCATTTCC-3′ (reverse); and 6) agouti-related protein (AgRP), 5′-GCAGAGGTGCTAGATCCACAGAA-3′ (forward) and 5′-AGGA-

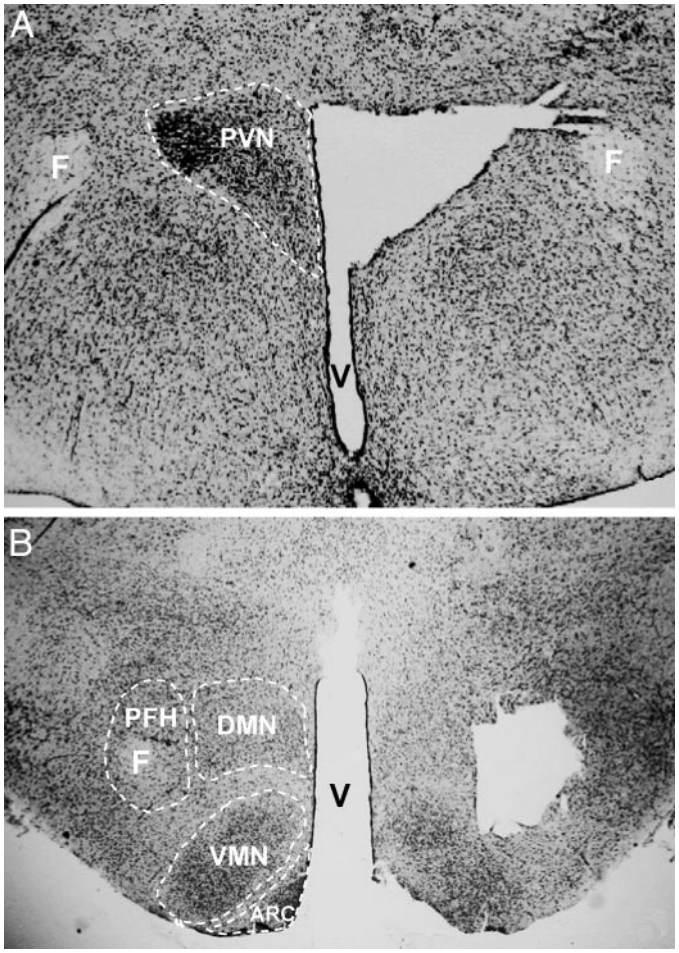

FIG. 1. Coronal sections of the rat hypothalamus showing microdissections of the PVN, PFH, and ARC. A, PVN dissection. *Left*, Intact PVN; *right*, dissected PVN. B, PFH and ARC dissections. *Left*, Intact structures; *right*, dissected PFH and ARC. F, Fornix; DMN, dorsomedial hypothalamic nucleus; VMN, ventromedial hypothalamic nucleus; V, third ventricle.

CTCGTGCAGCCTTACAC-3′ (reverse). The concentrations of primers were 100–200 nM. All reagents, unless indicated, were purchased from Invitrogen (Carlsbad, CA).

The expressions of galanin and orexins in the PVN and PFH were also examined with Northern hybridization using total RNA and a Northern Max Kit (Ambion, Austin, TX), as previously described (35). RNA (10 μg) from each sample was used for Northern blotting. Galanin and orexin cDNA probes were gifts from Dr. M. E. Vrontakis (36) and Dr. L. de Lecea (37), respectively. β-Actin cDNA probe was purchased commercially (Ambion, Austin, TX). Probes were radiolabeled by nick translation DECA Prime II (Ambion, TX) and [^{32}P]deoxy-ATP (PerkinElmer, Wellesley, MA). The blots were exposed to Biomax (MR) film (Eastman Kodak Co., Rochester, NY) for up to 4 d.

Immunocytochemistry

Four hours after i.p. injection of Intralipid, animals were deeply anesthetized with sodium pentobarbital (100 mg/kg, ip) and transcardially perfused with 150 ml 0.9% normal saline, followed by 200 ml 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4). Brains were removed immediately and postfixed in the same fixative at 4 C for 4 h, then transferred in 25% sucrose phosphate buffer (0.1 M; pH 7.4) at 4 C for 48 h. Thirty-micrometer free-floating cryostat sections were used for immunocytochemistry. Sections were sequentially incubated as follows: 30 min in 80% methanol 0.03% H_2O_2/PBS, 30 min in 5% normal goat serum 0.5% Triton X-100/PBS, 18 h at 4 C in rabbit anti-c-Fos serum (1:500; Santa Cruz Biotechnology, Inc., Santa Cruz, CA), 1 h in biotinylated goat antirabbit IgG (1:200; Vector Laboratories, Inc., Burlingame, CA), 2 h in standard Vectastain ABC (1:300; Vector laboratories, Inc.), and 10 min in 0.035% diaminobenzidene/0.005% H_2O_2 in 0.05 M cocadylate buffer (SPI-CHEM, West Chester, PA). Between each step, the sections were rinsed four times, for 5 min each time, in PBS (0.01 M; pH 7.4). They were mounted, air-dried, dehydrated, cleared, and coverslipped.

Double-labeling immunofluorescence

Adjacent sections from the animals described above were used to examine, by double-labeling immunofluorescence, the colocalization of immunoreactivity (ir) for c-Fos and the orexins in the PFH, which do not require colchicine for visualization. Free-floating sections were incubated sequentially as follows: 1 h in 10% normal donkey serum and 0.5% Triton X-100/PBS, 18 h at 4 C in rabbit anti-c-Fos (1:50) and goat anti-orexin (1:200; Santa Cruz Biotechnology, Inc.), 2 h in fluorescein isothiocyanate-conjugated donkey antigoat IgG (1:100) and Cy3-conjugated donkey antirabbit IgG (1:200; Jackson ImmunoResearch Laboratories, Inc., West Grove, PA). Between each step, the sections were rinsed three times, for 10 min each time, in PBS. Lastly, sections were mounted and coverslipped with Vectashield (Vector Laboratories, Inc.) and examined using an LSM 510 laser confocal microscope (Zeiss, Oberkocken, Germany); immunofluorescence double-labeled neurons containing c-Fos-ir and orexin-ir in the PFH were counted and imaged. All procedures were conducted at room temperature unless indicated.

Quantitation of c-Fos-ir

For quantitation of c-Fos-ir, sections were viewed using a Leitz microscope (Rockleigh, IL) with ×10 illumination objective, and images were captured with a DXM 1200 digital camera (Nikon, Melville, NY) and analyzed using Image-Pro Plus software (version 4.5, Media Cybernetics, Inc., Silver Spring, MD) on a Gray value scale from 1–255. The coordinates of the hypothalamic areas examined according to Paxinos and Watson (38) were: PVN (Bregma −1.4 to −1.8 mm), ARC (Bregma −2.8 to −3.12 mm), and PFH (Bregma −2.8 to −3.6 mm). Ten sections at the same level in each area of each animal were examined. The area of interest was outlined, and the number of c-Fos-ir nuclei in the area was counted and expressed as the density of c-Fos-ir cells per square millimeter. Analyses were performed by an observer blind to the identity of the animals.

Data analysis

Hypotheses regarding group differences were tested using either a one- or two-way ANOVA, followed by a Bonferroni *post hoc* test for multiple comparisons between groups or an unpaired *t* test, where appropriate. The criterion for statistical significance was a value of $P < 0.05$.

Results

Intralipid increased circulating lipids

To establish the acute effects of Intralipid on circulating metabolites, rats (n = 7/group) with food absent were injected with saline or Intralipid (5 ml, 20% solution, ip), and blood was collected via tail vein puncture at 0.5, 1, or 4 h after injection. Intralipid caused a marked increase in TG levels across the 4-h test period [$F(1,36) = 23.6$; $P < 0.001$], with the largest effect (+170%) apparent at the fourth hour (Fig. 2). There was a somewhat smaller increase (25–50%) in levels of NEFA across the 4 h [$F(1,36) = 17.8$; $P < 0.001$], but no change in glucose levels [$F(1,36) = 0.47$; nonsignificant (n.s.)] when comparing saline and Intralipid scores at 0.5 h (153 ± 6 *vs.* 160 ± 7 mg/dl), 1 h (148 ± 7 *vs.* 153 ± 8 mg/dl), and 4 h (156 ± 10 *vs.* 157 ± 11 mg/dl).

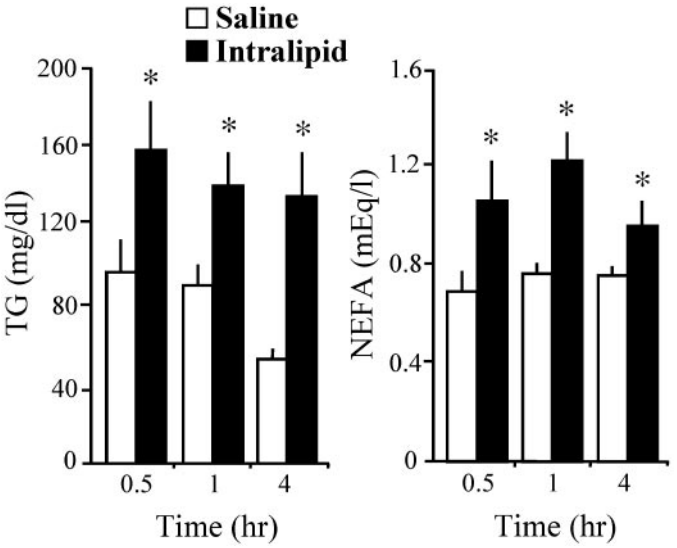

FIG. 2. Measurements of TG and NEFA 0.5, 1, and 4 h after ip administration of saline or Intralipid (5 ml, 20%) in rats. Given are the mean ± SEM. *, $P < 0.05$ for comparisons between saline and Intralipid scores.

Intralipid elevated lipids without altering glucose, insulin, and leptin

This experiment was designed to repeat the Intralipid test using a different method of blood collection. Rats were killed by decapitation, which yielded sufficient trunk blood for assays of circulating hormones as well as metabolites. Subjects (n = 8/group) were injected with saline or Intralipid (5 ml, 20%) and were killed 1, 2, and 4 h after injection by decapitation. As in the first experiment, Intralipid caused a marked rise in TG [$F(1,42) = 24.4$; $P < 0.001$] and NEFA [$F(1,42) = 11.8$; $P < 0.001$] levels across the 4-h period while producing no change in glucose [$F(1,42) = 0.22$; n.s.; Table 1). In contrast, Intralipid had no effect on levels of insulin [$F(1,42) = 0.21$; n.s.] and leptin [$F(1,42) = 1.13$; n.s.] over the 4-h period. This demonstrates some degree of specificity in the effects of Intralipid under the acute injection conditions of these tests.

Hypothalamic peptide gene expression was stimulated by Intralipid

Real-time quantitative PCR was used to examine changes in hypothalamic peptide expression in response to Intralipid, with peptide mRNA measured in relation to β-actin. Rats (n = 5/group) with food absent were injected with saline or Intralipid (5 ml, 20%) at dark onset and were killed 4 h later. Their brains were removed; specific hypothalamic areas (PVN, PFH, and ARC) were dissected; and mRNA was prepared. As indicated above, Intralipid compared with saline increased levels of TG (82 ± 17 *vs.* 135 ± 12 mg/dl; $P < 0.01$) and NEFA (0.62 ± 0.08 *vs.* 0.83 ± 0.09 mEq/liter; $P < 0.05$); it produced no change, however, in glucose (151 ± 8 *vs.* 153 ± 7 mg/dl; n.s.), insulin (1.6 ± 0.13 *vs.* 1.4 ± 0.15 ng/ml; n.s.), or leptin (2.3 ± 0.73 *vs.* 2.2 ± 0.59 ng/ml; n.s.).

Measurements of peptide mRNA as a ratio to β-actin revealed marked alterations in the expression of the orexigenic peptides, galanin, orexins, and enkephalin (Fig. 3). Intralipid produced a 60% increase in galanin mRNA [$F(1,12) = 9.14$; $P < 0.01$]. This effect was site-specific [$F(2,12) = 6.01$; $P < 0.01$] and was evident in the PVN ($P < 0.01$), but not in the PFH or ARC (Fig. 3). An even larger increase in orexin mRNA (+100%) was demonstrated in the PFH ($P < 0.01$), whereas somewhat smaller rises in enkephalin mRNA (+20–40%) were evident in the PVN and PFH as well as the ARC [$F(1,16) = 106.5$; $P < 0.001$]. This increased expression of the three peptides in the PVN and PFH is in distinct contrast to NPY or AgRP localized to the ARC, which showed no difference in mRNA level between the saline and Intralipid scores (0.492 ± 0.022 *vs.* 0.512 ± 0.023 for NPY/β-actin; 0.135 ± 0.018 *vs.* 0.149 ± 0.021 for AgRP/β-actin; n.s.). These results with galanin and the orexins were confirmed using Northern blot. In a separate set of rats (n = 5/group) killed 4 h after injection of saline or Intralipid (5 ml, 20%), measurements of galanin mRNA in the PVN and of orexin mRNA in the PFH revealed a considerably higher level in the Intralipid rats, as illustrated in Fig. 4.

TABLE 1. Effects of Intralipid on circulating metabolites and hormones

	TG (mg/dl)	NEFA (mEq/liter)	Glucose (mg/dl)	Insulin (ng/ml)	Leptin (ng/ml)
1 h					
Sal	98 ± 19	0.73 ± 0.1	158 ± 7	1.9 ± 0.2	2.2 ± 0.3
IL	129 ± 10	0.92 ± 0.1[a]	165 ± 8	2.0 ± 0.2	2.7 ± 0.5
2 h					
Sal	56 ± 6	0.65 ± 0.1	151 ± 7	1.2 ± 0.1	2.9 ± 1.1
IL	120 ± 26[a]	1.15 ± 0.1[a]	140 ± 9	1.1 ± 0.2	2.6 ± 1.5
4 h					
Sal	73 ± 9	0.75 ± 0.1	158 ± 10	1.0 ± 0.2	2.4 ± 0.9
IL	126 ± 15[a]	0.98 ± 0.1[a]	162 ± 12	1.3 ± 0.1	3.0 ± 0.7

Sal, Saline; IL, Intralipid.
[a] $P < 0.05$ for comparisons between Sal and IL scores.

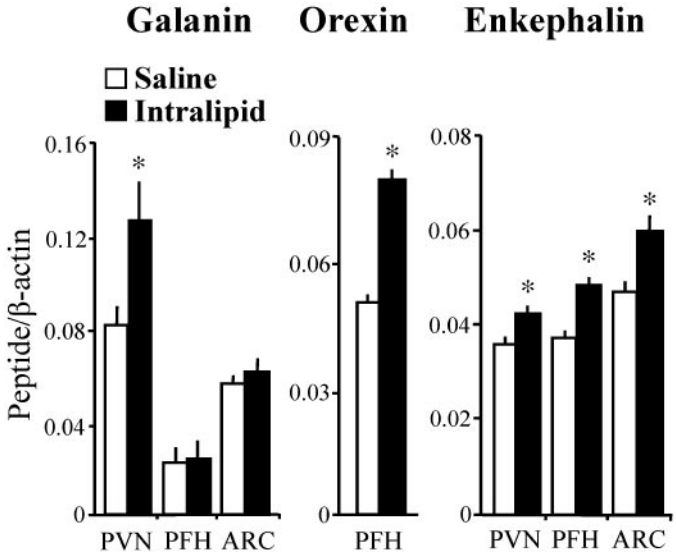

FIG. 3. Galanin, orexin, and enkephalin mRNA (ratio to β-actin mRNA) in the PVN, PFH, and ARC 4 h after ip administration of saline or Intralipid (5 ml, 20%). Given are the mean ± SEM. *, $P < 0.05$ for comparisons between saline and Intralipid scores.

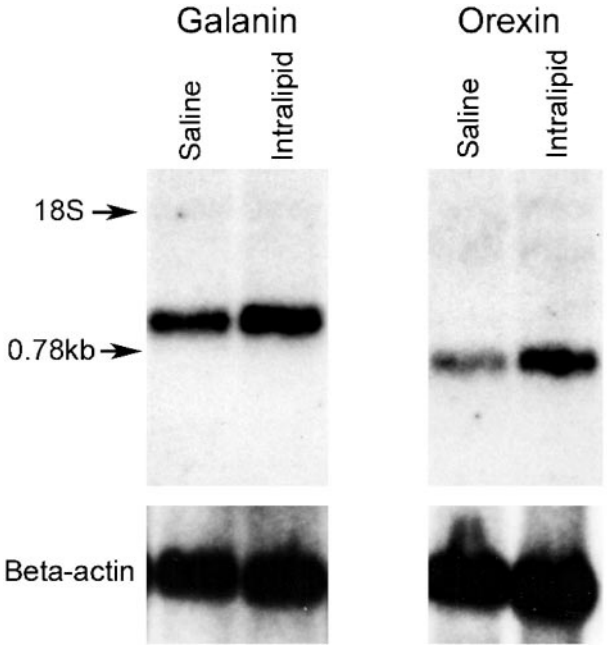

FIG. 4. Northern blot analysis showing galanin mRNA in the paraventricular nucleus and orexin mRNA in the perifornical hypothalamus 4 h after ip administration of saline or Intralipid (5 ml, 20%). A β-actin probe was used on the same blot to normalize the amount of mRNA.

Intralipid injection activated c-Fos and increased colocalization with orexins

To assess the impact of Intralipid on neuronal activity in the hypothalamus, an additional set of rats (n = 4/group) was injected with saline or Intralipid (5 ml, 20%) and killed 4 h after injection. Their brains were prepared for immunostaining of c-Fos in the hypothalamus, which reflects stimulus-induced neuronal activation on a cellular level (39). Also, double-labeling immunofluorescence was used to determine whether c-Fos activation could actually be detected in peptide-synthesizing neurons, specifically in the orexin neurons of the PFH.

Injection of Intralipid compared with saline significantly increased c-Fos expression in neuronal nuclei of the hypothalamus [$F(1,18) = 61.1$; $P < 0.001$]. This effect was site specific [$F(2,18) = 22.4$; $P < 0.001$] and was observed specifically in neurons of the PVN ($P < 0.001$) and PFH ($P < 0.001$), but not in the ARC (Figs. 5 and 6). Whereas saline-injected rats exhibited almost no c-Fos-ir neurons, Intralipid rats had a dense concentration of Fos-expressing neurons in specific areas. These were evident predominantly in the anterior parvocellular region of the PVN (Fig. 6, *top*), with only a few Fos-positive cells seen in the posterior magnocellular neurons and no immunostaining apparent in the supraoptic nucleus. Also, neurons exhibiting c-Fos staining were evenly distributed throughout the PFH and lateral hypothalamic area (Fig. 6, *bottom*). Although some c-Fos-ir neurons were observed in the ARC, this nucleus exhibited no difference between saline- and Intralipid-injected rats (Fig. 5).

Neurons expressing orexin-ir were concentrated in the PFH, as previously reported (37, 40). In saline-injected control rats, no double-labeled neurons containing c-Fos-ir and orexin-ir were detected in this area. Intralipid administration, however, markedly increased Fos protein in orexin-synthesizing neurons. Approximately 20% of the orexin-ir neurons (18 ± 5 of 92 ± 10) contained c-Fos-ir, indicated by yellow staining (see *white arrows*) in PFH neurons in Fig. 7.

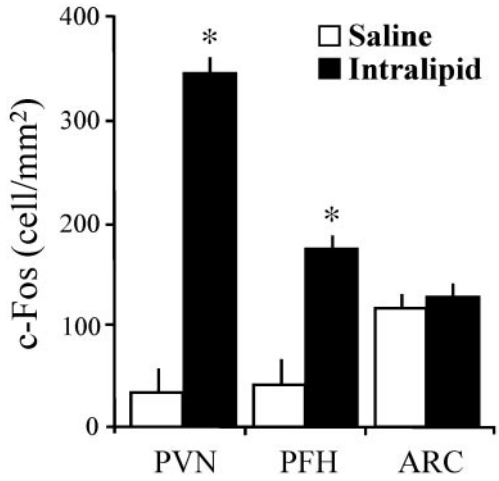

FIG. 5. Effects of Intralipid (5 ml, 20%) compared with saline on c-Fos immunoreactivity in the PVN, PFH, and ARC. Given are the mean ± SEM. *, $P < 0.05$ for comparisons between saline and Intralipid scores.

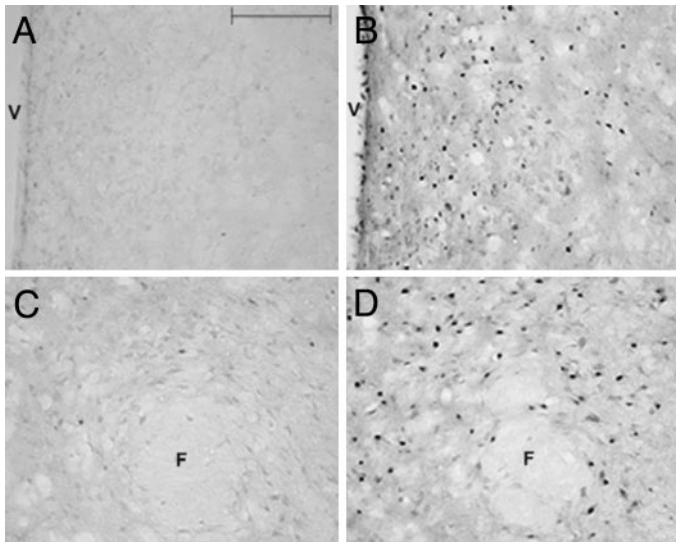

FIG. 6. Photomicrographs illustrating the effect of Intralipid (B and D) compared with that of saline (A and C) on c-Fos immunoreactivity in the PVN (*top*) and PFH (*bottom*). V, Third ventricle; F, fornix. *Bar*, 50 μm.

Hypothalamic peptide gene expression was similarly stimulated by 4-h high fat diet exposure

This experiment tested whether a 4-h period of high fat diet consumption at dark onset can affect peptide gene expression in a manner similar to the 4-h period after Intralipid injection. Rats (n = 5/group) previously adapted to the test diet were given 30 kcal of a low fat (10% fat) or high fat (50% fat) diet at dark onset. After consuming the 30 kcal of the diet over the next 4 h, the rats were killed as described for the Intralipid experiment. As shown in Table 2, consumption of

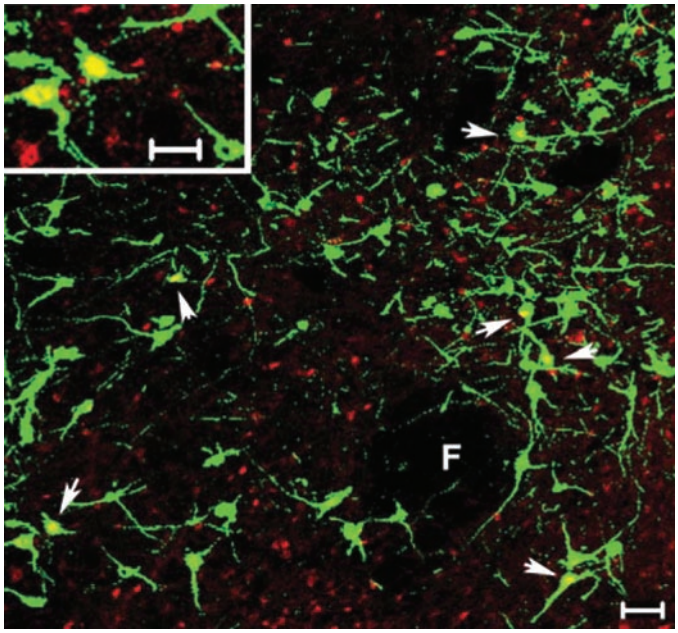

FIG. 7. Double-labeled neurons (*yellow*), indicated by *white arrows*, in the PFH of Intralipid-injected rats, reflecting colocalization of c-Fos-ir (*red*) and orexin-ir (*green*). Saline-injected control rats showed no double-labeled neurons (not shown). The *inset* in the *upper left corner* amplifies neurons in the *lower left corner* that coexpress c-Fos and orexins. F, Fornix. *Bar*, 50 μm.

the high fat diet for 4 h compared with a low fat diet had very similar effects as Intralipid in terms of its impact on circulating lipids and hypothalamic peptides. The high fat diet significantly increased levels of TG and NEFA and stimulated the expression of galanin, orexin, and enkephalin, as indicated by the ratio of peptide/β-actin. The main difference between these paradigms was seen in the measures of insulin and leptin on the high fat diet, which, in contrast to Intralipid, caused a significant reduction in the levels of both hormones (Table 2).

TABLE 2. Effects of a 4-h high fat diet compared with those of a low fat diet

	Low fat	High fat
Metabolites		
TG (mg/dl)	137 ± 9	190 ± 29[a]
NEFA (mEq/liter)	0.59 ± 0.1	1.53 ± 0.1[a]
Hormones		
Insulin (ng/ml)	4.9 ± 0.3	1.7 ± 0.4[a]
Leptin (ng/ml)	9.3 ± 0.6	6.8 ± 0.8[a]
Peptides		
Galanin[b]		
PVN	0.080 ± 0.001	0.091 ± 0.003[a]
PFH	0.021 ± 0.000	0.022 ± 0.000
ARC	0.058 ± 0.004	0.061 ± 0.005
Orexin[b]		
PFH	0.050 ± 0.002	0.067 ± 0.003[a]
Enkephalin[b]		
PVN	0.036 ± 0.002	0.041 ± 0.001[a]
PFH	0.041 ± 0.001	0.046 ± 0.001[a]
ARC	0.034 ± 0.002	0.035 ± 0.002

[a] $P < 0.05$ for comparisons between low and high fat diet scores.
[b] Ratio of peptide mRNA/β-actin mRNA.

Discussion

These experiments establish an acute Intralipid injection paradigm as a tool for consistently and markedly raising circulating lipids in normal weight rats consuming standard laboratory chow, without altering glucose or hormones known to modulate hypothalamic peptides. This fat emulsion elevated TG levels by over 150% for at least 4 h and produced a somewhat smaller increase in NEFA levels. These effects are consistent with other reports using iv as well as ip administration (41–43) and are similar in magnitude to those observed in natural feeding conditions with acute or chronic consumption of a high fat diet (4, 24). However, they occur in the absence of changes in glucose as well as insulin and leptin, which are generally elevated in association with hyperphagia and a rise in body fat accrual during a fat-rich diet (2, 4). These results agree with other Intralipid studies showing no change in glucose and leptin in rats (44, 45) and humans (46, 47). Whereas insulin can be increased by Intralipid under some conditions (45, 48), other reports show no change, as indicated here (44, 46), perhaps due to differences in the mode and duration of Intralipid administration and in the magnitude of changes in FA levels.

With this Intralipid-induced rise in lipids, there was a marked increase in the expression of specific hypothalamic peptides. This stimulatory effect, a 20–100% rise in peptide mRNA relative to β-actin, was evident for galanin, the orexins, and enkephalin in particular hypothalamic areas. For galanin, the elevated expression after Intralipid was detected in the PVN, but not the PFH or ARC, where galanin-expressing neurons are concentrated (49). Also, the increase in orexin expression, consistent with a study using *in situ* hybridization (9), was evident in the PFH, where the orexin-synthesizing neurons exist (37, 40). For enkephalin mRNA, a significant rise occurred in the PVN and PFH as well as the ARC, where this peptide is concentrated (50).

These effects are similar to those seen in natural feeding paradigms involving dietary fat, indicating that the peptides are responding to specific signals, presumably related to circulating lipids, that are shared by Intralipid and a high fat diet. The consumption of a high fat diet, which raises TG and NEFA levels, stimulates the expression of galanin in the PVN, orexins in the PFH (7, 9, 51), and also enkephalin in these hypothalamic nuclei, as shown in the present study. The results obtained here with 4 h of high fat diet consumption are very similar to those observed 4 h after Intralipid injection. In the different feeding paradigms (9, 51), the expression levels of galanin in the PVN and orexins in the PFH were positively correlated with TG levels, with generally weaker relationships detected for NEFA as well as for glucose, insulin, and leptin. This specific relationship between hypothalamic peptides and circulating TG is supported by the present results with Intralipid. This fat emulsion increased peptide expression and TG levels in the absence of changes in hormones, as well as other factors related to body weight, adiposity, eating behavior, taste, and palatability. Also, these peptides can shift independently of changes in leptin and insulin. This is demonstrated by the findings that they rise significantly in response to both a chronic high fat

diet, which elevates leptin levels (51), and 4 h of high fat diet consumption, which reduces leptin and insulin.

The evidence that the peptides are more closely related to TG than to NEFA levels suggests that it is the process of TG hydrolysis to FA, perhaps via lipoprotein lipase in hypothalamic capillary endothelium (52, 53), rather than circulating FA from peripheral tissues, that may be involved in determining the level of peptide expression and synthesis. These hypothalamic systems are well positioned to provide the central mechanisms for a proposed role of TG-FA uptake in the control of feeding behavior (28). The present results with acute, 4-h Intralipid or high fat diet involving 10–30 kcal suggest that in the short term, this control by nutrient signaling involves fat-stimulated peptides that potentiate feeding. However, under conditions where lipids are chronically and increasingly elevated, it may additionally recruit systems that suppress feeding. This is supported by evidence with Intralipid infusions, showing no change in feeding with 30 kcal Intralipid, but a significant suppression with 360 kcal (54), and also by a recent study showing chronic high fat diet consumption to stimulate the expression of the satiety peptide, cocaine- and amphetamine-regulated transcript (55).

The results with double-labeling immunofluorescence further strengthen this idea of an effect of TG-FA on neurons expressing the orexigenic peptides. Administration of Intralipid increased c-Fos immunostaining in the hypothalamus, suggesting increased neuronal activity. This effect was detected in the PVN, consistent with previous results (56). It was evident specifically in the anterior, parvocellular region, but not in the magnocellular region or the supraoptic nucleus, areas that should exhibit a change if ip Intralipid is affecting blood osmolality. It was additionally seen in the PFH, where a high fat diet has been shown to increase c-Fos expression (57), and FA administration stimulates neuronal activity (30). In addition, Intralipid increased c-Fos colocalization with the orexins in 20% of the peptide-synthesizing neurons in the PFH. This provides support for an effect of lipids specifically on hypothalamic neurons containing the orexigenic peptides.

Of particular note is the anatomical and peptide specificity of this hypothalamic relationship to circulating lipids. In contrast to the peptides in the PVN and PFH, those expressed primarily in the ARC are generally unresponsive or inversely related to circulating lipids. Specifically, Intralipid failed to stimulate the expression of NPY and AgRP in the ARC. This agrees with evidence that these peptides are unaffected or actually reduced by fat consumption or injections of FA (58–61). Intralipid also had no effect on c-Fos activity in the ARC. Whereas this is unexpected in light of the stimulatory effect of Intralipid on enkephalin in this nucleus, it agrees with the results obtained with the 4-h high fat diet, which produced no change in enkephalin mRNA in the ARC, and also with the pattern observed for galanin shown here and in other publications (7, 8, 51). In addition to being unaffected by Intralipid, galanin in the ARC is unresponsive to consumption of a high fat diet and is unrelated to fat preference in rats given a free choice feeding paradigm. Thus, in marked contrast to the fat-stimulated peptides in the PVN and PFH, those in the ARC appear to be differently regulated and generally unresponsive to fat. In the case of NPY and AgRP, for example, these peptides are closely related to dietary carbohydrate (58, 62, 63), circulating levels of glucose and corticosterone (63–65), shifts in leptin and insulin (66), and changes in malonyl-coenzyme A, perhaps related to alterations in glucose metabolism (67–69). A causal relationship between fat metabolism and galanin is suggested by evidence that pharmacological blockade of fat oxidation with mercaptoacetate (MA) produces the opposite effect of dietary fat, namely, a suppression of PVN galanin mRNA and peptide (16, 23).

In addition to the hypothalamic fat-stimulated peptide systems, there is evidence that brainstem structures, and perhaps brainstem galanin, are also responsive to changes in fat metabolism. MA stimulates Fos-like immunoreactivity in different sites of the brainstem, including the area postrema-nucleus of the solitary tract, lateral parabrachial nucleus, and central nucleus of the amygdala (70). Furthermore, a feeding response induced by MA is blocked by lesions of these structures as well as by hindbrain injections of the galanin antagonist, M40 (71–74). These extrahypothalamic systems responsive to MA, however, appear to function independently of and distinctly from the PVN peptide systems. The feeding induced by MA is unaffected by PVN lesions (75) and is actually suppressed by Intralipid injection (76). Also, MA preferentially stimulates the consumption of protein (23, 77), whereas PVN galanin injection increases predominantly fat and carbohydrate intake (12). The distinct functions of these PVN and extrahypothalamic systems and also of other hypothalamic systems besides the fat-stimulated peptides are underscored by two additional findings. MA has no effect on NPY in the ARC, but reduces PVN galanin expression (16, 23, 64), possibly contributing to a suppression of feeding, and central FA injection decreases NPY gene expression while similarly reducing food intake (61). This evidence supports the existence of multiple systems in the brain that are responsive to FA and their metabolism. These include the hypothalamic fat-stimulated peptides acting to enhance feeding when increased by fat-rich diets and other extrahypothalamic systems that stimulate ingestion, perhaps of protein, when FA metabolism is inhibited.

Central injection studies demonstrate that the fat-stimulated peptides, galanin, orexins, and the opioids, have in common a specific behavioral effect, that of stimulating eating behavior and producing a significantly stronger feeding response on a diet rich in fat (12–16). Together with these injection studies, the present results with Intralipid and 4-h high fat diet suggest the possibility of a positive feedback loop, by which dietary fat and TG-FA under acute conditions can stimulate hypothalamic peptides that, in turn, potentiate feeding on a fat-rich diet. Although the physiological significance of this nonhomeostatic, positive feedback requires further study, one possibility is that it functions under conditions when food is scarce, and periods of gorging on energy-dense foods, perhaps involving mechanisms of reward, are essential to survival (78, 79). When food, particularly dietary fat, is plentiful, however, this system may become overactive, contributing to the initial hyperphagia

on a high fat diet (1, 2), but ultimately recruiting systems that inhibit feeding (55). The present findings distinguish these fat-stimulated peptides in the PVN and PFH from the NPY and AgRP systems in the ARC, which are unresponsive to Intralipid and, instead, are stimulated under conditions of negative energy balance, when leptin levels are low (66).

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