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Increased enkephalin in brain of rats prone to overconsuming a fat-rich diet

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Abstract

Recent studies have shown that the opioid enkephalin (ENK), acting in part through the hypothalamic paraventricular nucleus (PVN), can stimulate consumption of a high-fat diet. The objective of the present study was to examine sub-populations of Sprague-Dawley rats naturally prone to overconsuming a high-fat diet and determine whether endogenous ENK, in different brain regions, is altered in these animals and possibly contributes to their behavioral phenotype. An animal model, involving a measure of initial high-fat diet intake during a few days of access that predicts long-term intake, was designed to classify rats at normal weight that are either high-fat consumers (HFC), which ingest 35% more calories of the high-fat than low-fat chow diet, or controls, which consume similar calories of these two diets. Immediately after their initial access to the diet, the HFC compared to control rats exhibited significantly greater expression of ENK mRNA, in the PVN, nucleus accumbens and central nucleus of the amygdala, but not the arcuate nucleus or basolateral amygdala. This site-specific increase in ENK persisted even when the HFC rats were maintained on a chow diet, suggesting that it reflects an inherent characteristic that can be expressed independently of the diet. It was also accompanied by a greater responsiveness of the HFC rats to the stimulatory effect of a PVN-injected, ENK analogue, D-ala2-met-enkephalinamide, compared to saline on consumption of the high-fat diet. Thus, normal-weight rats predicted to overconsume a fat-rich diet exhibit disturbances in endogenous ENK expression and functioning that may contribute to their long-term, behavioral phenotype.

Keywords

enkephalin; high-fat diet; hypothalamus; paraventricular nucleus; nucleus accumbens; central nucleus of the amygdala; qRT-PCR; radiolabeled in situ hybridization; digoxigenin-labeled in situ hybridization; DALA; consummatory behavior; rat

1. Introduction

The consumption of fat-rich food has greatly increased over the past several decades and has been implicated as a major contributor to the higher incidence of obesity, diabetes and early puberty onset in industrialized nations . This increase in fat consumption may be due, in part, to the increased availability of palatable fast foods or junk foods that are rich in fat .

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However, there are marked differences in the vulnerability of individuals to overconsuming fat, which may be due to genetic factors as well as early environmental exposure to a high-fat diet . Human and animal studies show that food preferences exhibited at a young age can predict adult eating patterns, with measures of these early preferences serving as useful tools for identifying individuals prone to overconsumption . Also, adult Sprague-Dawley rats with a long-term propensity to become obese on a high-fat diet are found to overconsume this diet during the first 1–2 weeks of access . Thus, measures of intake during initial exposure to a fat-rich diet may allow one, first, to identify individuals at normal weight that are prone versus resistant to overeating fat and, then, to investigate disturbances in brain mechanisms that may contribute to these different behavioral phenotypes over the long-term.

The question to be addressed in this study pertains to the brain mechanisms, perhaps involving orexigenic peptides, which promote the overconsumption of fat. One such peptide is believed to be galanin (GAL), expressed in the hypothalamic paraventricular nucleus (PVN), which acts in close relation to dietary fat. The stimulatory effect of GAL on feeding is stronger in individual subjects or rat strains that prefer fat or are maintained on a fat-rich diet, while attenuated by removal of fat from the diet, and the overexpression of the GAL gene in mutant mice causes an increase in consumption of a high-fat diet . In turn, GAL expression in the PVN is stimulated by acute or chronic consumption of a high-fat diet and is positively related to the amount of fat ingested and degree of fat preference exhibited by rats given a choice of macronutrient diets . These results suggest that GAL is bidirectionally related to a high-fat diet, functioning within a positive feedback loop to increase a rat's propensity to overconsume fat. This close relationship between GAL and fat is not seen with another orexigenic peptide, neuropeptide Y (NPY) in the arcuate nucleus (ARC), which preferentially stimulates the consumption of a carbohydrate-rich diet and, in turn, is found to be reduced in fat-preferring rats .

Evidence suggests that the opioid peptide enkephalin (ENK) may have similar characteristics to GAL in its positive relationship to dietary fat. Injection of an ENK analogue into the PVN preferentially increases intake of high-fat food . In addition, a few studies extending beyond the hypothalamus have shown that ENK administration in mesolimbic structures promotes the consumption of palatable, fat-rich food, possibly though their connections with the hypothalamus . These structures include the nucleus accumbens (NAc), which mediates reward-related behavior and hedonic aspects of palatable food intake, and the central nucleus of the amygdala (CeA), which mediates the emotional aspects of feeding . In the NAc, injection of the ENK analogue, [D-Ala(2),N-Me-Phe(4),Gly(5)-ol]-Enkephalin (DAMGO), preferentially increases the intake of fat relative to carbohydrate, and in the CeA where DAMGO also stimulates food intake, the opiate antagonist, naltrexone, selectively decreases the intake of a high-fat but not high-carbohydrate diet . In turn, ENK expression in Sprague-Dawley rats is stimulated in the PVN by acute or chronic intake of a high-fat diet and also by the circulating lipids, triglycerides (TG), which are invariably elevated by fat consumption . While there are no studies in outbred rats examining the role of endogenous ENK in animals naturally prone to overeating a fat-rich diet, there are two reports showing increased ENK concentration in the PVN of obese Zucker rats and mu-opioid receptor mRNA levels in the ARC of fat-preferring, Osborn**e**-Mendel rats . This evidence suggests that endogenous ENK may be similar to GAL in its close relationship to dietary fat and in contributing to the behavioral phenotype of rats that are naturally prone to overconsuming fat.

The present study was designed to investigate this possibility in sub-populations of outbred, Sprague-Dawley rats that can be differentiated at normal body weight as prone versus resistant to overeating a high-fat diet. Building on the above studies of early markers of dietary obesity, the first goal was to standardize a model for predicting a rat's long-term

propensity to overconsume fat, with a measure of intake during the first few days of high-fat diet access. In rats classified by this measure as high-fat consumers compared to controls, the next goal was to measure in these subgroups the expression of ENK, first, right after their initial exposure to the high-fat diet and, then, under basal conditions after the rats were returned to and maintained on a low-fat, chow diet. Endogenous ENK was measured in different areas of the hypothalamus, including the PVN, and also in the mesolimbic structures, the nucleus accumbens (NAc) and amygdala, to provide a broader perspective of this opioid's functions in mediating the phenotype of fat overconsumption. In the final experiment, these subpopulations were also examined with injections of an ENK analogue, to determine whether they exhibit differential responsiveness to its feeding-stimulatory effects. The results of these experiments provide evidence for a role of this opioid system, within and outside of the hypothalamus, in mediating the increased propensity of certain rats to overconsume a diet high in fat content.

2. Materials and methods

2.1 Subjects

Adult, male Sprague-Dawley rats (Charles River Breeding Labs, Kingston, NY) were individually housed (22°C, with lights off at 3:30 p.m. for 12 hr) in a fully accredited American Association for the Accreditation of Laboratory Animal Care facility. All animals were given one week to acclimate to lab conditions, during which time they were maintained *ad libitum* on laboratory chow (LabDiet Rodent Chow 5001, St. Louis, MO; 12% fat, 60% carbohydrate, and 28% protein; 3.0 kcal/g) and water. All procedures were approved by the Rockefeller University Animal Care and Use Committee and were in compliance with the National Institutes of Health Guide for Care and Use of Laboratory Animals.

2.2 Diets

The high-fat diet used in this report has been described in detail in previous publications . This diet (5.2 kcal/g) had 50% fat composed of 75% lard (Armour Star, Peoria, IL) and 25% vegetable oil (Crisco, Orrville, OH), 25% carbohydrate from 30% dextrin (ICN Pharmaceuticals, Costa Mesa, CA), 30% cornstarch (ICN Pharmaceuticals, Costa Mesa, CA), and 40% sucrose (Domino Foods Inc., Yonkers, NY), and 25% protein from casein (Bio-Serv, Frenchtown, NJ) and 0.03% L-cysteine hydrochloride (ICN Pharmaceuticals, Costa Mesa, CA). This solid diet was supplemented with minerals (USP XIV Salt Mixture Briggs; ICN Pharmaceuticals, Costa Mesa, CA) and vitamins (Vitamin Diet Fortification Mixture; ICN Pharmaceuticals, Costa Mesa, CA).

2.3 Test procedures

Each of the 6 experiments had a similar feeding paradigm. Following 1 week of adaptation to the laboratory conditions, rats were maintained for 3 additional days on lab chow, with daily intake measures of chow calories recorded for the purpose of establishing baseline intake. All rats were then adapted to the 50% high-fat diet by receiving a small meal (15 kcal) of this diet for 3 consecutive days with chow present. After this adaptation period, the lab chow was removed, and all rats were allowed *ad libitum* access to this high-fat diet for 5 days, with measures of body weight and food intake recorded daily. At the end of this 5-day period, the rats were rank ordered and subgrouped according to their average daily intake of the high-fat diet, with the 33% highest classified as high-fat overconsumers (HFC), the 33% lowest as controls (n=6–10/group), and the middle group omitted from further analysis. In each experiment, the HFC rats consumed approximately 126 kcal (116–137 kcal) of the high-fat diet compared to only 91 kcal (81–99 kcal) of lab chow. This is in contrast to the control rats, which consumed similar amount of the high-fat diet, an average of 96 kcal (86–102 kcal), and the chow diet, averaging 88 kcal (73–96 kcal). When comparing the high-fat

diet intake to chow intake, as well as to the high-fat diet intake of the controls, the HFC rats consumed on average 30–35% more calories per day. With Experiment 1 focusing on the behavioral and physiological measures of these subgroups, Experiments 2 and 4 analyzed gene expression of ENK peptide, using quantitative real-time polymerase chain reaction (qRT-PCR), in the PVN, NAc, CeA and ARC. In Experiments 3 and 5, ENK was examined using *in situ* hybridization (ISH) with radiolabeled probes in the PVN, NAc and CeA (but not the ARC where ENK mRNA showed no group difference using qRT-PCR), and also using *in situ* hybridization with digoxigenin (DIG)-labeled probes, to examine cell density in different parts of the PVN, the shell and core of the NAc, and both the CeA and the basolateral nucleus of the amygdala (BLA), which is located immediately lateral to the CeA. In Experiments 2–5, rats were sacrificed by rapid decapitation 2 hr before dark onset, with food removed 2 hr prior to sacrifice to avoid the influence of food intake on peptide expression.

The main goal of Experiment 1 was to determine if consumption of the high-fat diet during the first few days of access can predict a rat's long-term intake. Two groups of rats (N=30) were characterized as HFC (n=10) or controls (n=10) at the end of the 5-day period on the high-fat diet. Group 1 was allowed to continue on the high-fat diet for an additional 2 weeks, to determine if their initial, 5-day high-fat diet intake predicts their subsequent chronic intake, while Group 2 was switched to the chow diet for 2 weeks, to stabilize their eating patterns and body weight, and was then returned to the high-fat diet for 2 weeks, to determine if their overconsuming phenotype still persists. After their 22 days of behavioral experiments on the high-fat diet, the rats of Group 1 were sacrificed, and their 4 fat pads (retroperitoneal, gonadal, inguinal and mesenteric) were dissected and weighed, as described previously .

In Experiment 2, ENK gene expression in the hypothalamic and mesolimbic areas was measured in the HFC and control rats, to determine if they exhibit any differences while having access to the high-fat diet. All rats (N=18) were allowed to consume this diet for 5 days, were characterized as HFC or controls (n=6/group), and were then sacrificed by rapid decapitation at the end of this 5-day period. Their brains were rapidly removed and dissected for analysis of ENK gene expression in the PVN, NAc, CeA and ARC using qRT-PCR. Trunk blood was collected for analysis of serum triglycerides (TG), leptin and insulin.

In Experiment 3, an additional set of rats (N=18) was characterized as HFC or controls (n=6/group) and, once again, sacrificed at the end of the 5-day period on the high-fat diet. To confirm the results of Experiment 1, brains were removed for the analysis using ISH with radiolabeled (n=6/group) and DIG-labeled probes (n=6/group), which together provide a more anatomically precise and sensitive quantitative procedure for measuring gene expression.

In Experiment 4, to determine whether differential expression of ENK reflects inherent differences between the two subgroups in the absence of the high-fat diet, rats (N=18) first characterized as HFC or controls (n=6/group) were switched to lab chow for 2 weeks. At the end of this 2-week period, when these groups were similar in their daily caloric intake, the HFC and control rats were sacrificed via rapid decapitation and their brains rapidly removed and dissected for analysis of ENK gene expression in the PVN, NAc, CeA, and ARC using qRT-PCR. Trunk blood was collected for analysis of serum TG, leptin and insulin.

In Experiment 5, to provide a more anatomically precise analysis of peptide changes, a set of rats (N=18) was characterized as HFC or control (n=6/group) and was then switched to chow diet for 2 weeks as described in Experiment 4. Rats were sacrificed at the end of the 2-

week period, and their brains were removed for the analysis using ISH with radiolabeled (n=6/group) and DIG-labeled probes (n=6/group).

In Experiment 6, the HFC and control rats were characterized in a manner described above (n=7/group) and were implanted with stainless steel cannula aimed at the PVN (see Methods below). Following one week of recovery from surgery, the rats were over a 3-day period again adapted to the high-fat diet. Following this adaptation period, the animals were given 2 sets of injections at dark onset, of D-ala2-met-enkephalinamide (DALA, 10 nmol; American Peptide Company, Inc., Sunnyvale, CA) or saline vehicle in counterbalanced order (see below), with chow removed at the time of injection. The dose of DALA was chosen based on literature showing PVN injection at this dose to increase food intake . For each set of injections, the HFC and control rats, injected on 2 consecutive days with either vehicle or peptide, were given a high-fat diet 30 min later and allowed to consume this diet *ad libitum* for the next 60 min. Calories consumed during this period, as well as body weights, were recorded and averaged.

2.4 Hormone and metabolite determinations

Insulin and leptin were assayed from serum using RIA kits from Linco Research Inc, MO. Triglycerides were measured with an E-Max Microplate Reader using a Triglyceride Assay Kit from Sigma, St Louis, MO.

2.5 Surgery

Subjects were anesthetized with ketamine (80 mg/kg, i.p.) and xylazine (10 mg/kg, i.p.), supplemented with ketamine as needed. Hand-manufactured guide shafts, made of 21-gauge stainless steel, 12 mm in length, were implanted perpendicularly and unilaterally dorsal to the PVN. The coordinates were A–P −1.8 (relative to bregma), L 0.4 (relative to midsaggital sinus), and D–V 5.3 (relative to level skull surface), with half on the left side and half on the right side. Injectors protruded 3 mm beyond the guide shafts to reach the PVN. One week of recovery was provided after surgery before testing. Between procedures, stainless steel obturators were left in the guide shafts to prevent occlusion.

2.6 Injection procedure

Injections were delivered through concentric microinjectors made of 26-gauge stainless steel outside and fused-silica tubing inside (74 μm ID, 154 μm OD, Polymicro Technologies, Phoenix AZ) that protruded 3 mm beyond the guide shaft to reach the PVN (V 8.3). A volume of 0.5 μl was delivered during 1 min by a syringe pump (Razel Scientific Instruments, St. Albans, VT), and the microinjector remained in place for another 1 min to allow diffusion into the injection site. Following the conclusion of the behavioral tests, rats were sacrificed for verification of the injection sites. With brains sliced into 40 μm sections and slide-mounted for microscopic verification as described, the injection sites were found to be located within the PVN, in its ventral, medial parvocellular, and lateral magnocellular regions.

2.7 Brain dissection

For mRNA analysis, immediately after sacrifice, the brain was placed in a matrix slicing guide with the ventral surface facing up. The first coronal cut was made in the anterior middle optic chiasm (Bregma −0.8 mm), according to the atlas of Paxinos and Watson . The second cut was 1.5 mm rostral to this (Bregma −0.8 to 0.7 mm), then another rostral 1.5 mm cut was made (Bregma 0.7 to 2.2 mm). The first slice was discarded and the second was used for microdissection of the NAc (Bregma 0.7 to 2.2 mm). Two additional 1.0 mm and one 0.5 mm slices (Bregma −0.8 to −2.8 mm, −2.8 to −3.3 mm) were made caudal to the

original slice, with the first used for microdissection of the PVN (Bregma −0.8 to −1.8 mm), the second for the CeA (Bregma −1.8 to −2.8 mm), and the third for the ARC. These sections were placed on a glass slide and rapidly dissected under a microscope. The NAc was dissected bilaterally in the shape of an oval, with the dorsal tip beginning at the lateral ventricle, the medial aspect at the semilunar nucleus, the ventral edge along the ventral pallidum, and the lateral aspect located medial to the lateral stripe of the striatum. The PVN was dissected as a reversed isosceles triangle, 1.0 mm bilateral to the third ventricle and between the fornix structures . The CeA was dissected bilaterally as an oval, immediately medial to the BLA and 0.2 mm dorsolateral to the optic tract. For the ARC, the area adjacent to the ventral aspect 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 0.2 mm at the bottom .

2.8 Real-time quantitative PCR analysis

As previously described, total RNA from pooled microdissected samples was extracted with TRIzol reagent. After treatment with RNAse-free DNAse I, 1 μg of total RNA was reverse transcribed (RT) into cDNA in a 25-μl reaction with 200 units of Superscript II reverse transcriptase (Invitrogen, Carlsbad, CA) using 150 ng oligo$(dT)_{15}$ primers as per the manufacturer's instructions. Minus RT was synthesized by replacing SuperScript II reverse transcriptase with water. The SYBR Green PCR core reagents kit (Applied Biosystems, Foster City, CA) was used, with cyclophilin as an endogenous control. Several housekeeping genes, including cyclophilin, β-actin and GAPDH, were assessed as endogenous controls, with cyclophilin producing the most consistent and reproducible results for our primers and cDNAs. qRT-PCR was performed in MicroAmp Optic 96-well Reaction Plates (Applied Biosystems, Foster City, CA). This was done on an ABI PRISM 7900 Sequence Detection system (Applied Biosystems, Foster City, CA), under the condition of 2 min at 50°C, 10 min at 95°C, and 40 cycles of 15 sec at 95°C and 1 min at 60°C. Each study consisted of 4 independent runs of qRT-PCR in triplicate, and each run included a standard curve, non-template control, and negative RT control. The levels of target gene expression were quantified relative to the level of cyclophilin, using the standard curve method. The primers, designed with ABI Primer Express V.1.5a software from published sequences, were: (1) cyclophilin: 5'-GTGTTCTTCGACATCACGGCT - 3' (forward) and 5'- CTGTCTTTGGAACTTTGTCTGCA −3' (reverse) and (2) ENK: 5'-GGACTGCGCTAAATGCAGCTA −3'(forward) and 5'-GTGTGCATGCCAGGAAGTTG −3' (reverse). The concentrations of primers were 100 nM. All reagents, unless indicated, were from Invitrogen (Carlsbad, CA).

2.9 Radiolabeled in situ hybridization histochemistry

Besides qRT-PCR, mRNA levels of ENK were measured using radiolabeled ISH, which allows for more anatomically precise quantification of changes in gene expression than qRT-PCR. Antisense and sense RNA probes were labeled with ^{35}S-UTP (Amersham Biosciences, Piscataway, NJ), as previously described . 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 PB between each step. After the wash, the sections were hybridized with a ^{35}S-labeled probe (10^3 cpm/mL) at 55°C for 18 h. Following hybridization, the sections were washed in 5X sodium chloride and sodium citrate (SSC), and the nonspecifically bound probe was removed by RNase (Sigma-Aldrich, St. Louis, MO) treatment for 30 min at 37°C. Sections were then run through further stringency washes with 0.1 M dithiothreitol (Sigma-Aldrich, St. Louis, MO) in 2X SSC and 1X SSC and 0.1X SSC at 55°C. Sections were finally mounted, air-dried, and exposed to a Kodak BioMax MR film for 18 to 24 h at −80°C, when films were developed and microscopically 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, on the MCID image analysis system (Image Research Inc., St. Catherines, ON, Canada). Gray-level/optical density calibrations were performed with a calibrated film-strip ladder (Image Research Inc., St. Catherines, ON, Canada) for optical density. This was plotted as a function of microscale calibration values. 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 sections per animal. Within each section, the optical density for the nucleus was recorded, from which the background optical density from a same-size area in the corpus callosum was subtracted.

2.10 Digoxigenin-labeled in situ hybridization histochemistry

In situ hybridization histochemistry with DIG-labeled probes was also used to quantify ENK. This technique specifically measures the density of neurons expressing the peptide gene above threshold levels. Brains were cut into 30 μm thick sections with a cryostat. DIG-labeled cRNA probes of ENK were synthesized by *in vitro* transcription as previously described . Free-floating coronal sections were processed for DIG-ISH as for radiolabeled ISH until the high stringency wash, with the exception of replacement with the DIG-labeled probe. After the high stringency wash, the sections were blocked and incubated in AP-conjugated sheep anti-digoxigenin antibody (Sheep Anti-DIG-AP, Fab fragments, 1:1000; Boehringer Mannheim) overnight. After washing in Tris buffer (0.1 M, pH 9.5), the signal was revealed with NBT/BCIP and the sections mounted, dehydrated and coverslipped as described . Gene expression level was measured by semiquantification with Image-Pro Plus software (Version 4.5, Media Cybernetics Inc., Silver Spring, MD) as described and expressed as the density of mRNA-containing cells, “cells/mm^2”.

2.11 Data analysis

The data in the figures and table are expressed as mean ± SEM. Statistical analyses of these data were performed using an unpaired two-tailed *t*-test. For Experiment 1, correlations were made using a Pearson’s product moment coefficient. For Experiment 6, a 2-way repeated measures ANOVA was performed, with group as the between-subject factor and injected substance as the within-subject factor.

3. Results

3.1 Experiment 1: 5-day intake of high-fat diet as related to long-term consumption and weight gain

Building on a rat model showing initial weight gain associated with intake on a high-fat diet to predict long-term propensity for obesity on this diet, this experiment was designed to determine whether the behavioral measure of high-fat diet intake during the first few days of access can predict a rat’s propensity to overconsume this diet when chronically available. Two groups of Sprague Dawley rats (N=30/group), first maintained on lab chow for 3 days, were given *ad libitum* access for 5 days to the high-fat diet (50% fat), with Group 1 allowed to consume the high-fat diet for an additional 2 weeks and Group 2 switched to lab chow for 2 weeks and then back to the high-fat diet for another 2 weeks. In both Groups 1 and 2, the control rats, consuming approximately 90 kcal/day on the chow diet, ingested a similar amount of the high-fat diet, averaging 96 kcal/day, during their first 5 days on the high-fat diet, while the HFC rats, consuming approximately 91 kcal/day on the chow diet, consumed 35% more calories of the high-fat diet (123 kcal/day) during the 5-day period (Table 1). Compared to control rats, the HFC rats in both groups consumed approximately 28% more high-fat diet and became slightly but not significantly heavier (+22 g) by the end of the 5-day period (Table 1). In Group 1 maintained for 2 more weeks on the high-fat diet, the HFC rats compared to controls continued to consume more calories (+30%) and became

significantly heavier (+48 g) by the end of this extended period ($p<0.05$) (Table 1), while accumulating larger fat pads (28±1.6 vs. 16±1.0 g, $p<0.05$). The initial 5-day measure of high-fat diet intake across all rats was strongly, positively correlated with their intake during the 3rd week of access ($r=+0.78$, $p<0.001$), demonstrating that initial consumption of fat is a strong predictor of long-term consummatory patterns. It was also correlated with their final body weight ($r=+0.61$, $p<0.05$) and body fat accrual ($r=0.64$, $p<0.05$). In Group 2, the HFC and control rats, switched to chow for 2 weeks and then back to the high-fat diet for 2 weeks, consumed the same number of chow calories and remained similar in body weight by the end of the second week; however, when re-exposed to the high-fat diet during the next 2 weeks, the HFC rats again increased their intake (+35%), which was positively correlated with their initial 5-day intake ($r=+0.82$, $p<0.01$), while the control rats still maintained similar caloric intake throughout this extended period (Table 1). Together, these data demonstrate that the rats' caloric intake during the first few days on a high-fat diet strongly predicts long-term patterns of fat consumption, whether the high-fat diet is chronically or intermittently available, and it provides a reliable, early measure for identifying subgroups of HFC and control rats that differ markedly in their chronic intake of this diet.

3.2 Experiment 2: ENK expression in HFC rats on a high-fat diet as measured by qRT-PCR

This experiment investigated whether the opioid ENK is disturbed in the rats prone to overconsuming a high-fat diet. A new set of rats (N=18) was first differentiated into HFC and control subgroups based on their intake measure during the first 5 days of access to a high-fat diet (n=6/group). As in Experiment 1, the HFC rats during these initial days consumed significantly more high-fat diet, as compared to the chow diet (+34%) or the control rats on the high-fat diet (+30%), while the control rats exhibited no significant difference between their intake of these two diets. The first question to address was whether the HFC and control subgroups, examined at the end of 5 days on the high-fat diet, exhibited differences in their expression of ENK in the hypothalamus and mesolimbic areas, as measured using qRT-PCR. In the HFC rats compared to controls, these measurements revealed increased expression of ENK in specific brain areas, both within and outside of the hypothalamus. This effect was seen in the PVN (+27%, $p<0.05$), but not the ARC (−5%, ns), and also in the NAc (+30%, $p<0.01$) and CeA (+40%, $p<0.01$) (Fig. 1). These results show that, after only a few days of consumption of the high-fat diet, ENK mRNA in rats that naturally overconsume this diet is disturbed in several brain areas involved in controlling different behavioral processes that may contribute to the overconsumption. In response to their higher intake, this effect in the HFC rats as compared to controls was accompanied by significantly elevated levels of circulating TG at the end of the 5-day period (142±9 vs. 93±6 mg/dl, $p<0.01$). Their measures of body weight (393±14 vs. 371±16 g, ns), insulin (2.3±0.3 vs. 1.6±0.4, ns) and leptin (7.3±1.9 vs. 4.6±0.7, ns), however, were slightly but not significantly higher, indicating that these parameters are unlikely to be related to the increased ENK expression in the HFC rats. A possible involvement of the elevated fat intake or TG levels at the end of the 5-day period, which are known to stimulate ENK expression in the PVN, remains to be determined.

3.3 Experiment 3: ENK in HFC rats on a high-fat diet as measured by ISH with radiolabeled and DIG-labeled probes

To confirm the results of Experiment 2 with more anatomically precise and sensitive quantitative procedures for measuring gene expression, a set of rats (N=18) was classified in a similar manner, and at the end of their 5 days on the high-fat diet, ENK expression was measured using ISH with radiolabeled and DIG-labeled probes. Consistent with the results of Experiment 2, the radiolabeled ISH analysis revealed significantly increased ENK mRNA levels in 3 different brain areas of the HFC rats, namely, the PVN (+24%, $p<0.01$), NAc (+23%, $p<0.01$), and CeA (+33%, $P<0.01$) (Table 2), as illustrated in the photomicrographs

(Fig. 2). Further analyses using the DIG-labeled probe revealed anatomical specificity in the change in the density of ENK-expressing neurons. Within the PVN, this effect was localized to the anterior-medial, parvocellular area of the nucleus, where the ENK-expressing cells are most concentrated and their density increased by 40% ($p<0.01$) (Table 3 and Fig. 3). In the NAc, the increase in cell density was of similar magnitude in both the shell and core (36%, $p<0.01$), while the significant increase in cell density in the CeA (+20%) was not observed in the BLA (6%, ns) immediately lateral to the CeA (Table 3 and Fig. 3). These results substantiate the finding that HFC rats have enhanced expression of ENK in both the hypothalamic and mesolimbic areas, although it remains unclear whether this phenomenon seen in rats while consuming the high-fat diet is a cause or consequence of the greater fat consumption or elevated TG levels characteristic of the HFC rats, as shown in Experiment 2.

3.4 Experiment 4: ENK expression in HFC rats on a low-fat chow diet as measured by qRT-PCR

The purpose of this experiment was to determine whether the gene expression changes reported in Experiments 2 and 3, rather than being produced by the overeating of a high-fat diet, reflect an inherent difference between the HFC and control rats that can be revealed in the absence of this diet. After 5 days on this diet, a separate set of rats (N=18) was characterized as the HFC or controls, in a manner similar to Experiments 1–3, but was then switched to the chow diet. After 2 weeks on this low-fat diet, the HFC and control rats were similar in their measures of caloric intake (92±8 vs. 89±10 kcal/day) and circulating TG levels (96±6 vs. 99±8 mg/dl), as well as their body weight (411±7 vs. 419±6 g), insulin (2.2±0.5 vs. 1.5±0.3 ng/ml) and leptin levels (6.0±0.9 vs. 4.9±0.5 ng/ml). Similar to the rats consuming the high-fat diet in Experiments 2 and 3, the HFC rats on the chow diet examined by qRT-PCR still showed significantly greater expression of ENK in the PVN (+30%, $p<0.01$), NAc (+35%, $p<0.01$) and CeA (+37%, $p<0.01$), but not the ARC (+4%, ns) (Fig. 4). This effect, occurring despite the similarities in these rats' behavioral, physiological and endocrine parameters, suggests that the increase in ENK expression is not a consequence of the overconsumption of the high-fat diet but, instead, reflects an innate difference in opioid peptide gene expression in rats that are prone to overconsuming a fat-rich diet.

3.5 Experiment 5: ENK in HFC rats on a chow diet measured by ISH with radiolabeled or DIG-labeled probes

In order to confirm the qRT-PCR results from Experiment 4, an additional set of rats (N=18) was characterized as HFC or controls after 5 days on the high-fat diet and then switched to the chow diet. At the end of the 2-week period on chow, the rats were sacrificed and their brains analyzed using ISH with radiolabeled or DIG-labeled probes. The increase in ENK expression in the HFC rats on the chow diet, as shown in Experiment 4 using qRT-PCR, was confirmed here with ISH using radiolabeled (Table 2 and Fig. 5) and DIG-labeled (Table 3) probes. This effect obtained with radiolabeled ISH was detected in the PVN (+43%, $p<0.01$), NAc (+34%, $p<0.01$), and CeA (+43%, $p<0.01$). The analysis using DIG revealed an increase in the density of ENK-expressing cells, in the anterior-medial parvocellular subdivision of the PVN (+40%, $p<0.01$), both the shell (+29%, $p<0.01$) and core (+29%, $p<0.01$) of the NAc, and the CeA (+27%, $p<0.01$). Together, these results provide strong evidence for an inherent difference in the gene expression of this opioid peptide in animals, at normal weight and on a low-fat diet, that are prone versus resistant to over-consuming a high-fat diet.

3.6 Experiment 6: Responsiveness of HFC rats on a chow diet to the feeding-stimulatory effect of an ENK analogue

Building on the results showing HFC rats to exhibit higher expression of endogenous ENK, this experiment tested whether the these rats also show greater responsiveness to the

feeding-stimulatory effect of an ENK analogue, DALA. The rats (n=7/group) were characterized as HFC or controls, switched to a chow diet for 2 weeks, and then implanted with a cannula aimed at the PVN. One week after recovery from surgery, the rats were injected in counterbalanced order with either saline vehicle or DALA (10 nmol), and their intake of a high-fat diet was measured over a 60-min period. The results revealed a stimulatory effect of DALA on intake that was considerably stronger in the HFC animals. Specifically, a two-way repeated measures ANOVA yielded a significant, main effect of DALA ($F(1,12) = 72.6$, $p<0.001$), which compared to saline significantly increased high-fat diet intake in both the HFC and control rats. Further, the ANOVA revealed an interaction effect between the treatment and animal subgroups ($F(1,12) = 11.5$, $p<0.05$). After saline injection, the HFC rats during the 60-min period of high-fat diet access consumed 34% more calories compared to the control rats ($p<0.01$) (Fig. 6), indicating that the overeating phenotype of HFC rats can be detected even during a brief re-exposure to this diet. Also, after injection of DALA, the HFC rats despite their already high baseline were more responsive to the feeding-stimulatory effect of the opioid agonist, showing a 60% increase in their intake of the high-fat diet as compared to only 36% for the control rats. These results suggest that, in addition to having higher expression of endogenous ENK, the HFC rats are more sensitive than controls to the feeding stimulatory effect of extracellular ENK.

4. Discussion

4.1 Model for identifying rats prone to overconsuming a high-fat diet

The first purpose of the present study was to design a protocol for characterizing outbred Sprague-Dawley rats, according to their initial consumption of a high-fat diet, and determine whether this measure is a reliable predictor of long-term intake of this diet when chronically available. Previous studies have demonstrated that Sprague-Dawley rats, identified as obesity prone based on their weight gain during initial exposure to a high-fat diet, exhibit increased intake of a high-fat diet throughout the period of time when this diet is available . They also show that Wistar rats on pure macronutrient diets, which exhibit a preference for fat, continue to show this preference throughout the full 4-week period of diet access, ultimately leading to greater weight gain . The results obtained here take additional steps toward standardizing procedures that allow one, with an early measure, to identify rats that have an increased propensity to consume a fat-rich diet over the long term. They show that a specific measure of initial intake of a high-fat diet can reliably predict overconsumption and that this prediction occurs not only under conditions when the diet is chronically available but also when it is removed and then restored after the rats were maintained for 2 weeks on a low-fat, chow diet. The consistent increase in high-fat diet intake (+30–35%) in HFC relative to control rats, with no difference in their caloric intake on the chow diet, demonstrates that the HFC rats are particularly vulnerable to the feeding-stimulatory effect of exposure to a high-fat diet.

4.2 Differences in ENK expression exhibited by HFC and controls rats on a high-fat diet

The results of the present study show that HFC rats, at the end of 5 days on the high-fat diet, exhibit higher gene expression of ENK in the PVN as well as mesolimbic brain areas when compared to control rats. This effect was observed in two separate experiments, with measurements of ENK mRNA levels and density of ENK-expressing cells performed using qRT-PCR and also ISH with both radiolabeled and DIG-labeled probes. With the HFC rats consuming more of the high-fat diet compared to the control rats, the question remains as to whether this difference between the groups is a consequence of their excess consumption of a fat-rich diet or their significantly elevated levels of TG that accompany this overconsumption. This could be the case in the PVN, where ENK is found to be stimulated by acute or chronic consumption of a high-fat diet compared to low-fat diet and to be

elevated in association with a rise in TG levels . However, it is less likely for ENK in the NAc or CeA, where there is little evidence showing fat or TG to affect endogenous ENK and one published report showing ENK mRNA in the NAc to be unaffected or reduced by acute or chronic consumption of a fat-rich diet with added sucrose . Although slightly elevated after 5 days of high-fat diet consumption, levels of insulin and leptin were not significantly different between the HFC and control groups, suggesting that these hormones are not involved in the observed changes in ENK. This agrees with a study showing the stimulatory effect a high-fat diet on PVN ENK to occur in the absence of any changes in these two adiposity hormones .

4.3 Differences in ENK expression exhibited by HFC and control rats on a chow diet

The results obtained in the experiments on rats switched for 2 weeks to a chow diet provide more definitive evidence that the disturbances in ENK in HFC rats are evident even in the absence of the high-fat diet. With normal caloric intake and circulating TG levels, the HFC rats compared to controls still exhibited a marked increase in ENK expression in the different hypothalamic and extra-hypothalamic areas. Thus, the phenotypic difference between these two subgroups, extending beyond the PVN to include the NAc and CeA, is more likely an inherent characteristic of the HFC subpopulation, rather than a consequence of the overconsumption of the high-fat diet. Although there are no prior studies of ENK in models of fat overconsumption, the expression of this opioid in various extra-hypothalamic regions, including the NAc, is similarly found to be elevated in naïve, alcohol-preferring animals, indicating that ENK may have a more general function in driving excessive ingestive behaviors. While these persistent changes in ENK could reflect long-lasting effects of the initial, high-fat diet access, this is not supported by other studies, showing a stimulatory effect of dietary fat on dynorphin expression to be transient or even reversed after a few weeks on a chow diet . Together, these results indicate that HFC rats are inherently different from control rats with respect to their endogenous expression of ENK and that the differences in opioid function may contribute to their overconsumption phenotype.

4.4 Behavioral consequence of elevated ENK in the PVN of HFC rats

Injection studies provide additional support for the idea that the elevated expression of ENK in HFC rats has a functional role in promoting their excess consumption of a fat-rich diet. Previous investigations in Sprague-Dawley rats have shown that PVN injection of DALA stimulates intake of chow and that PVN administration of another ENK analogue, DAMGO, preferentially stimulates intake of fat . In the present study, PVN injection of DALA compared to saline was similarly found to increase ingestion of a high-fat diet, in both the HFC and control rats. Notably, the effect observed in the HFC rats was significantly larger (+60%) than that observed in the control rats (+36%), a difference that occurred despite the naturally higher baseline intake of the HFC rats after injection of saline. This indicates that, in addition to having higher levels of endogenous ENK in the PVN and mesolimbic areas, the HFC rats are also more sensitive to the stimulatory effect of this opioid on the consumption of fat, providing further support for the role of the ENK system in driving overconsumption of a high-fat diet.

4.5 Significance of the elevation on ENK in the NAc and CeA

In addition to its feeding-stimulatory actions in the PVN, the elevated expression of ENK in the NAc and CeA in HFC rats may reflect a different role for ENK, possibly mediating the rewarding and reinforcing aspects of food intake . Within both the shell and core subregions of the NAc, ENK cells are found to express the inhibitory, D2 dopamine receptor, suggesting that ENK expression may increase in response to a low level of extracellular dopamine and consequent receptor activity. Consistent with this proposal are recent findings

in inbred rats prone to overeating a high-fat diet, which have lower basal levels of NAc dopamine that, particularly in the shell, may compel HFC rats to consume more high-fat diet in order to obtain greater reward. The injection of an ENK analogue into this region is found to increase the consumption of fat and also palatable food in general . The present findings demonstrate that ENK expression in HFC rats is elevated in both subregions of the NAc, suggesting that this opioid, in addition to acting through the shell to affect motivational valence, also acts through the core to affect response-reinforcement learning . In the CeA, endogenous ENK expression is also elevated in HFC compared to control rats, with no change observed in the BLA. While both regions of the amygdala are involved in appetitive conditioning, the CeA encodes the general affective significance of an emotional event such as access to palatable food, while the BLA encodes the sensory features involved in this event . Thus, enhanced ENK expression in the CeA may stimulate high-fat diet intake due to the emotional salience of its availability. This nucleus has an important role in the opioid-mediated enhancement of food consumption, and local injection of DAMGO stimulates food intake, while the opiate antagonist naltrexone decreases intake of a high-fat but not high-carbohydrate diet . Together, these findings showing increased expression of ENK in the NAc and CeA of HFC rats support this opioid's role in driving fat intake due to positive reinforcement.

4.6 Conclusion

The HFC animal model described here is the first to standardize a protocol for characterizing normal-weight animals based specifically on acute consummatory behavior, a few days of high-fat diet intake compared to chow intake, which predicts long-term dietary fat consumption. The results of this study demonstrate that animals prone to overconsumption of a diet rich in fat, prior to significant weight gain or changes in adiposity hormones, have disturbances in expression of the opioid ENK that mediates the rewarding and reinforcing aspects of fat intake. Consistent with the positive relationship that exists between dietary fat and ENK, they show that HFC rats are inherently different from controls with respect to their endogenous levels of ENK mRNA, as well as their feeding responses to an ENK analogue. Further examination of the neurochemical differences that exist in this distinct sub-population of animals may shed light on the mechanisms contributing to their natural propensity to overconsume a fat-rich diet.

Research Highlights

- Initial high-fat diet intake during a few days of access predicts long-term intake
- Rats prone to overeating fat have increased enkephalin in several brain regions
- Rats predicted to overeat fat eat more in response to enkephalin injection
- Disturbances in endogenous enkephalin may contribute to the overeating of fat

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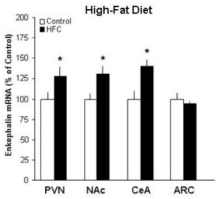

Fig. 1.

Expression of enkephalin mRNA is increased in hypothalamic and mesolimbic areas of high-fat consumers (HFC) vs. controls (n=6/group) following 5-days of high-fat diet consumption, as measured by qRT-PCR. Data are mean ± S.E.M. *$p<0.05$ vs. controls. Abbreviations: ARC: arcuate nucleus, CeA: central nucleus of the amygdala, NAc: nucleus accumbens, PVN: hypothalamic paraventricular nucleus.

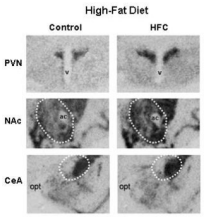

Fig. 2.

Photomicrographs illustrating increased enkephalin expression in high-fat consumers (HFC) vs. controls following 5-days of high-fat diet consumption, as assessed by radiolabeled *in situ* hybridization. Abbreviations: ac: anterior commisure, CeA: central nucleus of the amygdala, NAc: nucleus accumbens, opt: optic tract, PVN: hypothalamic paraventricular nucleus, v: third ventricle.

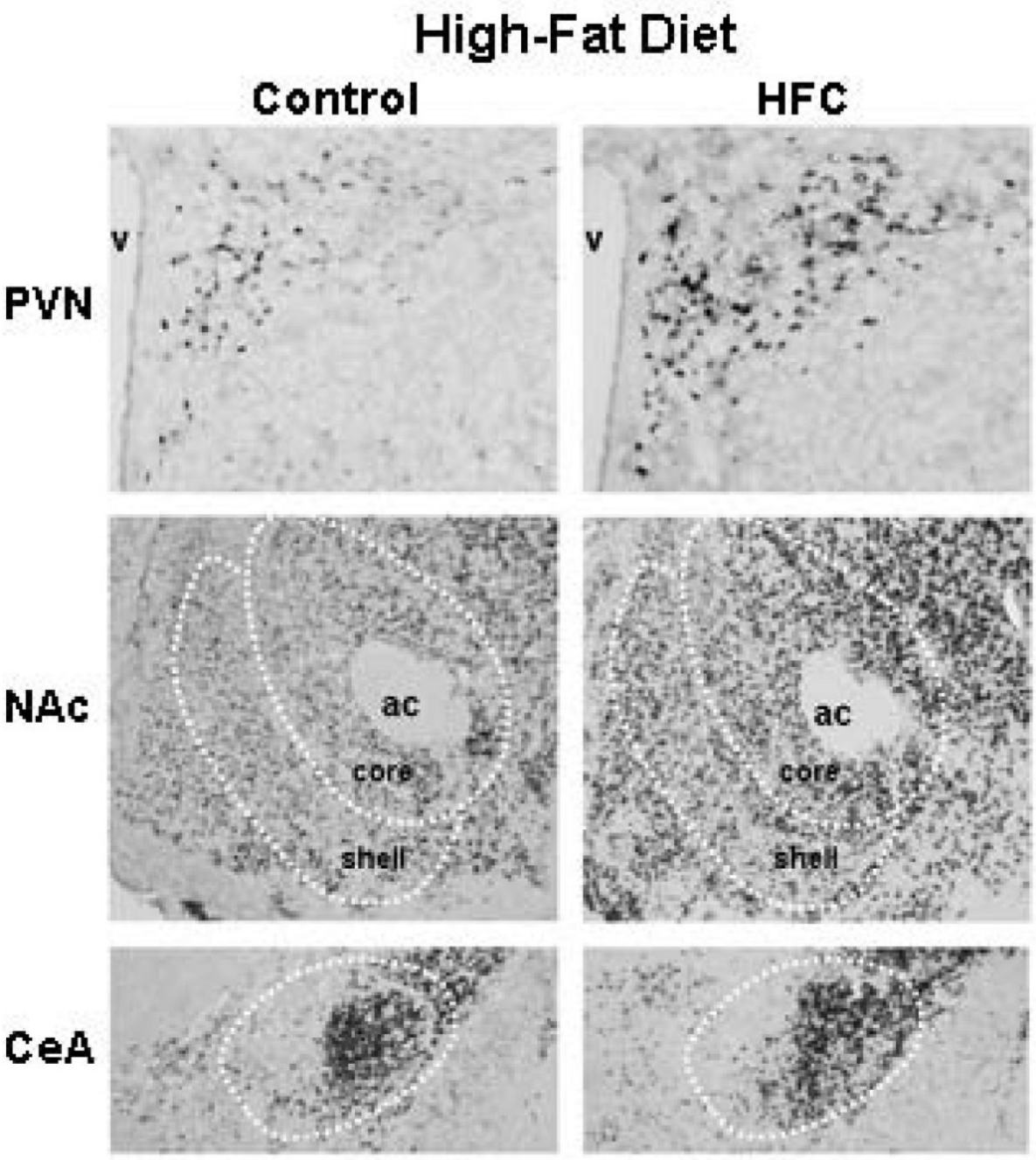

Fig. 3.

Photomicrographs illustrating increased density of ENK-expressing cells in high-fat consumers (HFC) vs. controls following 5-days of high-fat diet consumption, as assessed by digoxigenin-labeled *in situ* hybridization histochemistry. See legend to Fig. 2 for abbreviations.

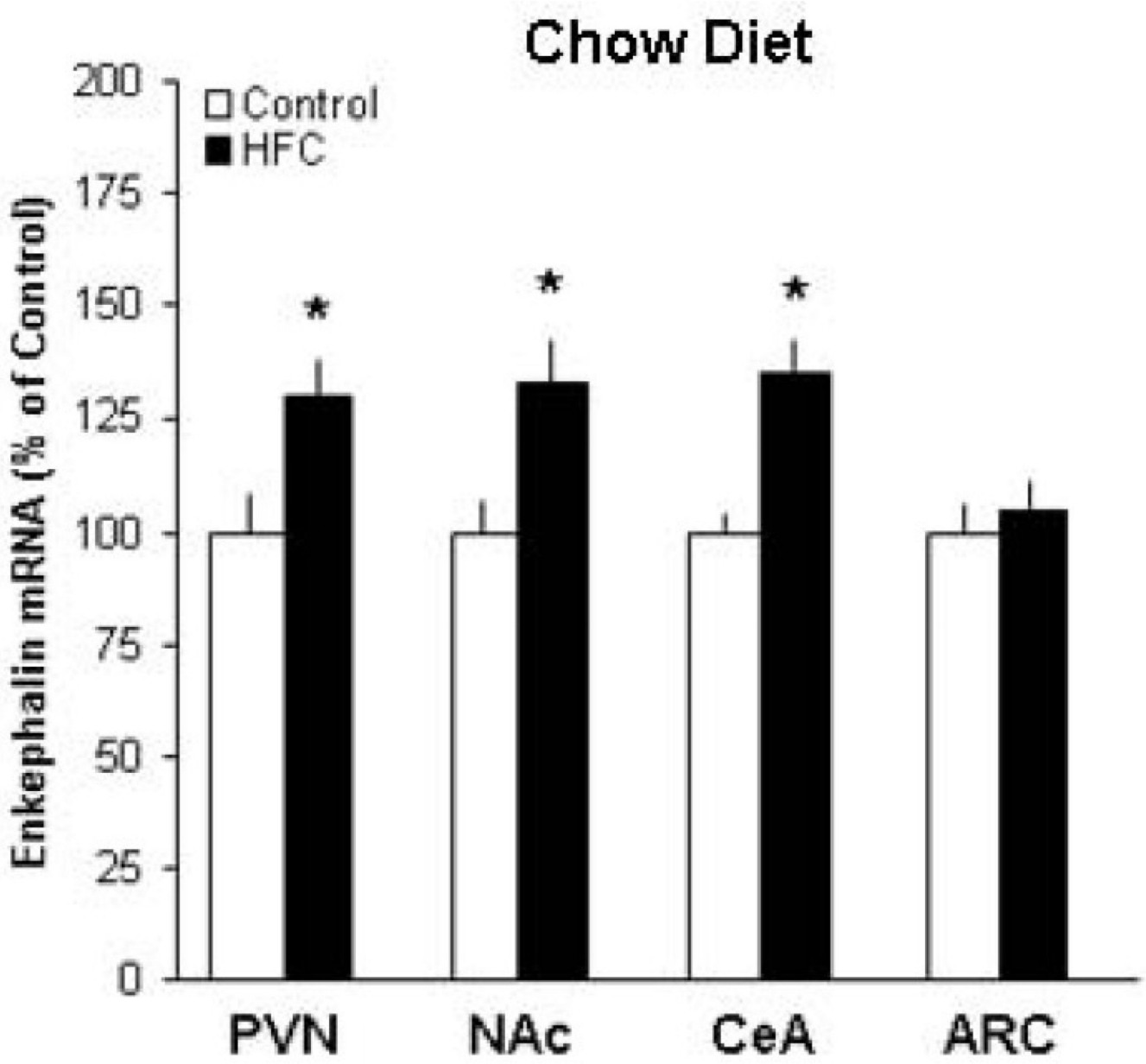

Fig. 4.

Expression of enkephalin mRNA is increased in hypothalamic and mesolimbic areas of high-fat consumers (HFC) vs. controls (n=6/group) on a chow diet, as measured by qRT-PCR. Data are mean ± S.E.M. *$p<0.05$ vs. controls. See legend to Fig. 1 for abbreviations.

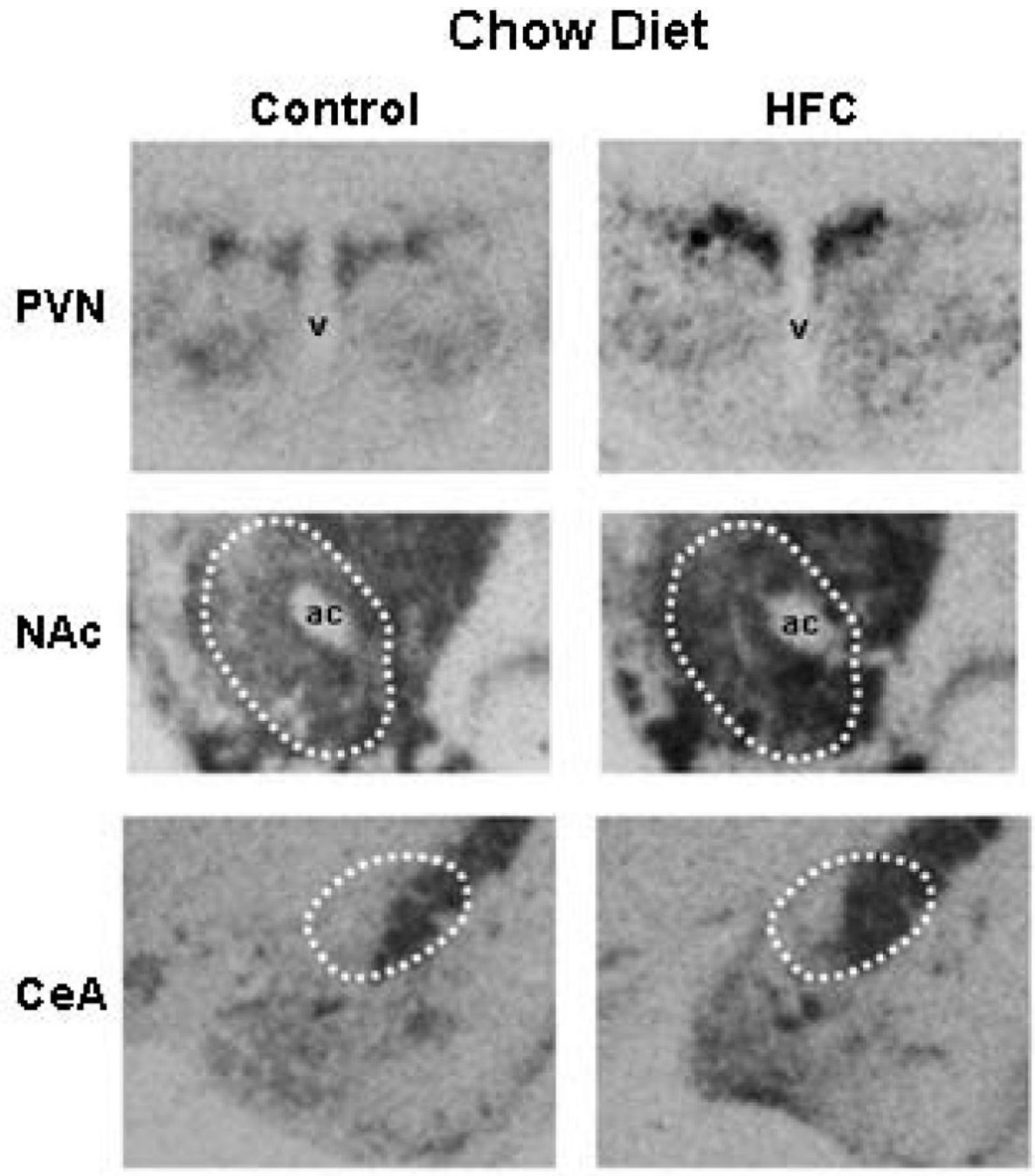

Fig. 5.
Photomicrographs illustrating increased enkephalin expression in high-fat consumers (HFC) vs. controls on a chow diet, as assessed by radiolabeled *in situ* hybridization. See legend to Fig. 2 for abbreviations.

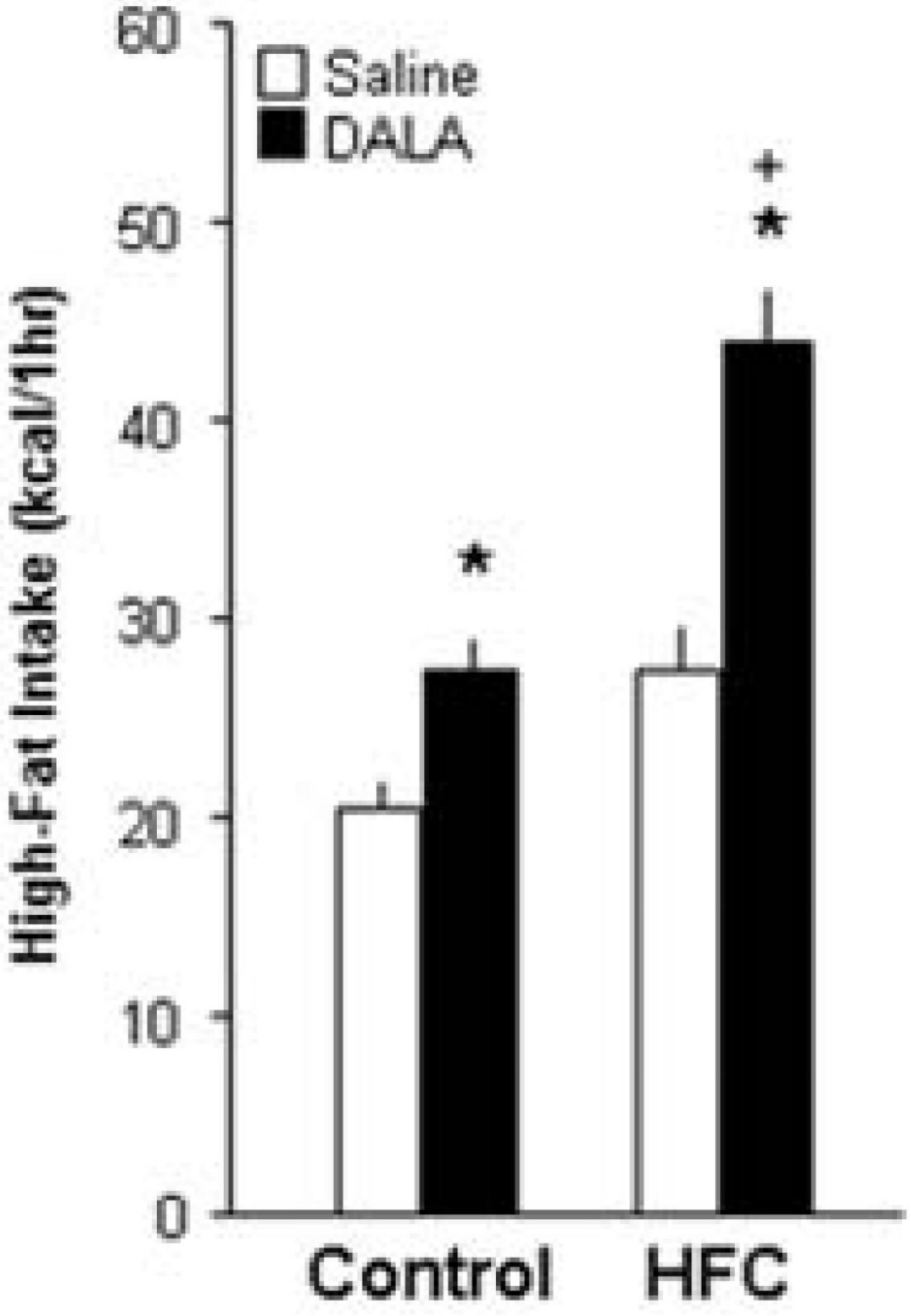

Fig. 6.

Injection of DALA into the hypothalamic paraventricular nucleus stimulates 1-h consumption of a high-fat diet in both high-fat consumers (HFC) and controls but does so to a greater extent in HFC rats. Data are mean ± S.E.M. *$p<0.05$ vs. saline injection; $^{+}$ $p<0.05$ vs. control.

Table 1

Caloric intake and body weights of high-fat consumers (HFC) compared to controls in Groups 1 and 2 during different periods of chow and high-fat diet access (Experiment 1).

	Group 1		Group 2	
	Control	**HFC**	**Control**	**HFC**
Intake (kcal/day)				
Chow (before high-fat diet)	91 ± 6	89 ± 4	90 ± 5	92 ± 4
High-fat diet (5 d)	95 ± 4	121 ± 10^{ab}	97 ± 4	124 ± 9^{ab}
High-fat diet (2 wk)	94 ± 5	118 ± 10^{a}	---	---
Chow (2 wk)	---	---	89 ± 6	92 ± 8
High-fat diet (re-exposure, 2 wk)	---	---	96 ± 7	126 ± 9^{ab}
Body weight (g)				
Chow (before high-fat diet)	302 ± 10	300 ± 8	294 ± 10	298 ± 11
High-fat diet (5 d)	371 ± 9	392 ± 12^{b}	369 ± 11	393 ± 14^{b}
High-fat diet (2 wk)	427 ± 15	475 ± 19^{a}	---	---
Chow (2 wk)	---	---	405 ± 9	409 ± 8
High-fat diet (re-exposure, 2 wk)	---	---	472 ± 6	498 ± 8^{ab}

Data are mean ± S.E.M.

^{a} p<0.05 vs. control;

^{b} p<0.05 vs. chow.

Table 2

Increased expression of ENK in high-fat consumers (HFC) compared to controls as assessed by radiolabeled *in situ* hybridization after 5-days of high-fat diet access (Experiment 3) or after being switched back to a chow diet (Experiment 5).

	ENK mRNA			
	High-fat diet		Chow diet	
	Control	HFC	Control	HFC
PVN	1.72 ± 0.02	2.14 ± 0.07*	1.05 ± 0.04	1.41 ± 0.09*
NAc	3.43 ± 0.07	4.25 ± 0.15*	1.38 ± 0.07	1.86 ± 0.08*
CeA	1.75 ± 0.02	2.28 ± 0.09*	1.22 ± 0.07	1.60 ± 0.06*

Data are mean ± S.E.M. Measure of ENK mRNA level is indicated by optical density.

*$p<0.05$ vs. controls.

CeA: central nucleus of the amygdala; NAc: nucleus accumbens; PVN: hypothalamic paraventricular nucleus.

Table 3

Increased density of ENK-expressing cells in high-fat consumers (HFC) compared to controls as assessed by dioxigenin-labeled *in situ* hybridization histochemistry after 5-days of high-fat diet access (Experiment 3) and after being switched back to chow (Experiment 5).

	ENK mRNA			
	High-fat diet		Chow diet	
	Control	HFC	Control	HFC
PVN	1.60 ± 0.10	2.24 ± 0.04*	1.41 ± 0.06	1.98 ± 0.09*
NAc Shell	13.00 ± 0.32	17.80 ± 0.29*	11.90 ± 0.52	13.80 ± 0.29*
NAc Core	13.30 ± 0.16	17.90 ± 0.24*	12.20 ± 0.97	14.40 ± 0.51*
CeA	2.88 ± 0.04	3.45 ± 0.10*	3.69 ± 0.18	4.69 ± 0.22*
BLA	2.52 ± 0.22	2.67 ± 0.15	2.58 ± 0.26	2.47 ± 0.33

Data are mean ± S.E.M. Measure of ENK mRNA level is indicated by cell density (cells/$\mu m^2 \times 10^{-4}$).

* $p<0.05$ vs. controls.

BLA: basolateral nucleus of the amygdala, CeA: central nucleus of the amygdala, NAc: nucleus accumbens, PVN: hypothalamic paraventricular nucleus.