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Increased orexin and melanin-concentrating hormone expression in the perifornical lateral hypothalamus of rats prone to overconsuming a fat-rich diet

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Abstract

The goal of this study is to examine the expression pattern of orexigenic peptides, orexin (OX) and melanin-concentrating hormone (MCH), in the perifornical lateral hypothalamus (PFLH) in subpopulations of Sprague-Dawley rats differing in their propensity to overconsume a high-fat diet. Immediately after an initial 5-day screening test that predicts long-term consumption, rats identified as high-fat consumers (HFC), ingesting 35% more calories of a high-fat relative to low-fat chow diet, had significantly elevated mRNA expression of OX in the perifornical but not lateral hypothalamic area and of MCH mRNA in both areas, when compared to control rats that consume similar amounts of these diets. This same OX and MCH expression pattern was seen in HFC rats maintained for two weeks on a low-fat chow diet, indicating that increased expression of these orexigenic peptides, occurring independently of the high-fat diet, may be an inherent characteristic of these rats. These HFC rats were also more active and slightly more anxious than controls, as measured by line crossings and time spent in the periphery or middle segments of an open field. Together, these results demonstrate that animals prone to overeating a high-fat diet show a baseline increase in orexigenic peptide expression in the PFLH along with higher behavioral arousal, which together may contribute to their increased consummatory behavior.

Keywords

Orexin; Melanin-concentrating hormone; High-fat diet; Perifornical lateral hypothalamus; Overconsumption

1. Introduction

The consumption of palatable diets, such as those containing high amounts of fat, has increased significantly during the past several decades (WHO, 2003). This may be due, in part, to increased availability of more palatable, fatty products, such as fast foods or junk foods (Bowman and Vinyard, 2004; Jeffery et al., 2006; Paeratakul et al., 2003). However, the vulnerability to overconsume these foods varies markedly across individuals. Human and animal studies show that consummatory behavior and food preferences exhibited at a young

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age can predict adult eating patterns, with measures of these preferences providing useful tools for identifying individuals prone to overconsumption (Johnson et al., 1991; Serdula et al., 1993). Also, in adult animals first exposed to a high-fat diet, their initial intake of or natural preference for fat is found to be positively related to their long-term measures of caloric and fat intake, in addition to their ultimate weight gain and body fat accrual (Dourmashkin et al., 2006; Lauterio et al., 1994; Pagliassotti et al., 1994; Shor-Posner et al., 1994; Shor-Posner et al., 1991; Wang et al., 1998). Such measures allowing early identification of subpopulations prone versus resistant to overeating and weight gain on a high-fat diet permits one to examine disturbances in brain mechanisms that may be causally related to these differential phenotypes.

Studies of brain neurochemicals in genetically altered animal models with varying propensity to become obese have revealed marked differences between animals that are already obese compared to those that are lean (Alexander et al., 2006; Cai et al., 2000; Qu et al., 1996; Stricker-Krongrad et al., 2001; Yamamoto et al., 2002). Since these differences may simply be a consequence of the obesity, other studies have examined rats selectively bred for their propensity to become obese on a high-fat diet and observed early disturbances in brain systems that precede and thus may be causally related to the obesity (Barnes et al., 2006; Levin et al., 1997; White et al., 2005). While it is difficult to predict the propensity of outbred rats towards dietary obesity, studies in these animals have shown weight gain during the first few days on a high-fat diet to be a strong predictor (Chang et al., 2010; Dourmashkin et al., 2006; Lauterio et al., 1994; Leibowitz et al., 2007; Pagliassotti et al., 1994; Shor-Posner et al., 1991) and revealed an increased expression of hypothalamic orexigenic peptides in obesity-prone animals that are still at normal body weight (Dourmashkin et al., 2006; Leibowitz et al., 2007). These investigations provide new information on neurochemical systems that are disturbed in early stages and may contribute to the development of obesity on a high-fat diet. It remains unclear, however, whether these neurochemical changes in outbred rats are related to the obesity per se or to the rats' behavioral pattern of high-fat diet overconsumption that invariably accompanies the obesity.

To address this question, we have recently established a method in Sprague-Dawley rats for reliably predicting, with a measure of initial acute consumption of a fat-rich diet, animals that are prone versus resistant to overconsuming this diet in a chronic situation (Chang et al., 2010). Using this animal model, we have chosen to investigate a possible role of the hypothalamic orexigenic peptides, orexin (OX) and melanin-concentration hormone (MCH), which are both expressed predominantly in the perifornical lateral hypothalamus (PFLH) and have widespread projections throughout the brain (Bittencourt et al., 1992; de Lecea et al., 1998; Nahon et al., 1989; Peyron et al., 1998; Sakurai et al., 1998; Skofitsch et al., 1985). There is evidence showing OX to preferentially stimulate consumption of a high-fat diet (Clegg et al., 2002), mediate the reinforcing properties of fat-rich foods (Nair et al., 2008), and to be elevated with anticipation of or in response to consumption of a fat-rich meal (Choi et al., 2010; Wortley et al., 2003). Further, like OX, MCH stimulates food intake (Abbott et al., 2003; Rossi et al., 1999; Sakurai et al., 1998) and plays a distinct role in the consumption of different reinforcing substances (Benoit et al., 2005; Duncan et al., 2005; Duncan et al., 2006; Richards et al., 2008; Schneider et al., 2007). Evidence implicating both OX and MCH in locomotor activity (Chung et al., 2009; Novak et al., 2006; Smith et al., 2006; Thorpe and Kotz, 2005) suggests that these peptides may have a broader function in mediating behavioral activation that accompanies the consumption of preferred substances. While studies have revealed disturbances in OX and MCH expression in already obese rats (Cai et al., 2000; Elliott et al., 2004; Qu et al., 1996; Stricker-Krongrad et al., 2001; Yamamoto et al., 2002), it is unknown whether these peptides are disturbed and possibly contribute to the differential propensity of individual rats to overconsume a high-fat diet.

The present study was aimed at measuring the expression of OX and MCH mRNA in the PFLH of Sprague-Dawley rats characterized as prone or resistant to overeating a high-fat diet based on their initial 5-day intake of this diet. In these subgroups, OX and MCH peptides were first analyzed immediately after their few days of access to a high-fat diet. Then, to determine whether any observed differences can be seen independently of the high-fat diet, the peptides were measured in the subgroups after being returned to a lower-fat chow diet for two weeks. To gain a broader understanding of these peptides' functions, additional measurements were taken of locomotor activity in these subgroups and also of peptide expression in the sub-regions of the PFLH, which have been differentially related to the arousing and rewarding aspects of often overconsumed substances (Harris and Aston-Jones, 2006). The goal of this study was to determine whether the PFLH OX and MCH peptide systems are disturbed and possibly contribute to an increased propensity to overconsume a diet high in fat.

2. Materials and Methods

2.1. Subjects

Adult, male Sprague-Dawley rats weighing on average 250 g at the start of all experiments (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, 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 and Use Committee. All animals were given 1 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) and water. All protocols fully conformed to the Guiding Principles for Research Involving Animals and Human Beings.

2.2. Diets

For the experimental period, rats were maintained ad libitum on a high-fat (50%) diet, which was the same as that described in prior publications (Dourmashkin et al., 2006; Leibowitz et al., 2004). It consisted of fat from 75% lard (Armour) and 25% vegetable oil (Crisco), of carbohydrate from 30% dextrin, 30% cornstarch (ICN Pharmaceuticals), and 40% sucrose (Domino), and of protein from casein (Bioserv) 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). The macronutrient composition was calculated as percentage of total kcal, with the high-fat diet containing 50% fat, 25% carbohydrate, and 25% protein (5.2 kcal/g). This semi-solid diet was stored at 4°C until use. On a daily basis, fresh diet was weighed out in metal bowls and placed into the appropriate animals cages.

2.3. Test procedures

Following the one week of adaptation to the laboratory conditions, rats were maintained for 3 additional days on standard lab chow, during which time measurements of daily caloric intake were taken to establish a baseline of consumption. The rats were then given 3 days of adaptation to the 50% high-fat diet by providing a small meal (15 kcal) of this diet with chow present. Following this period of adaptation, chow was removed, and all rats were given ab libitum access to this high-fat diet for 5 days. Daily measures of food intake and body weight were recorded. Building on our previously published model for predicting rats that overconsume a high-fat diet (Chang et al., 2010; Dourmashkin et al., 2006; Leibowitz et al., 2007; Leibowitz et al., 2004), rats at the end of this 5-day period were rank-ordered and based on their average daily intake on the fat-rich compared to chow diet, were classified as high-fat overconsumers (HFC) (highest tertile) or control rats (lowest tertile) (n=6/group), with the middle group (n=8)

omitted from all further experiments. The HFC rats generally consumed 112–130 kcal/day of high-fat diet and 81–98 kcal/day of the lower-fat chow diet, whereas the control rats consumed 85–99 kcal/day of high-fat diet and 78–95 kcal/day of the chow diet. These two rat subpopulations were tested in Experiments 1–4, as described below. They were sacrificed in a fed state by rapid decapitation, just prior to dark onset, and their brains were prepared for peptide analyses.

In Experiment 1, rats (N=20) were characterized as HFC or control (n=6/group) in the manner described above. After the 5-day exposure to a high-fat diet, they were sacrificed, and their brains were rapidly removed and dissected on ice for measurements of OX and MCH gene expression in the PFLH using quantitative real-time polymerase chain reaction (qRT-PCR). Trunk blood was collected for analysis of serum triglyceride (TG), leptin and insulin levels. Experiment 2 used an additional set of rats (N=20), characterized as HFC or control (n=6/group), that were sacrificed right after the 5 days of access to the high-fat diet and examined using in situ hybridization (ISH) with radiolabeled and digoxigenin (DIG)-labeled probes, which together provide a more anatomically precise and sensitive quantitative procedure for measuring gene expression. With evidence suggesting that OX and MCH function to increase behavioral arousal, Experiment 3 was conducted in a new set of animals (N=20), whose activity and anxiety levels were measured in an open field for 5 min, 2 h into the dark cycle when baseline activity is high in rats. After open field testing, the rats were given 5 days of access to the high-fat diet and, based on their daily intake, subgrouped into HFC or controls (n=6/group) as before. In contrast to Experiments 1 and 2, however, the rats were taken off the high-fat diet and switched to the low-fat chow diet for 2 weeks, to remove any effect of the dietary fat. Their caloric intake and body weight were recorded daily, and then all rats were sacrificed, their brains analyzed for OX and MCH gene expression using qRT-PCR, and trunk blood collected for analysis of serum TG, leptin and insulin levels. Experiment 4 examined another set of rats (N=20) in the same manner as Experiment 3, except that peptide expression in the HFC and control rats (n=6/group) was analyzed using ISH with radiolabeled probes.

2.4. Hormone and metabolite determinations

Serum from trunk blood was assayed for insulin and leptin, using assay kits from Linco Research Inc, MO. Serum TG levels were measured with an E-Max Microplate Reader using a TG assay kit (Sigma-Aldrich Co., St. Louis, MO).

2.5. Open field measurements

In Experiment 3, all animals were tested in an open field for locomotor activity and anxiety, as measured by total line crossings, time spent in the peripheral squares (outer 12), and time spent in the middle squares (inner 4). The apparatus for the open field test was a 72” by 72” box constructed from plywood painted black and the bottom composed of 16 equal size squares. Each rat was placed in the center of the field, and the number of lines crossed and time spent in periphery versus middle was recorded for 5 minutes. Between tests, the apparatus was thoroughly cleaned with 70% EtOH and allowed to dry. All testing was carried out in a temperature, noise and light controlled room.

2.6. Brain dissections

In Experiments 1 and 3, immediately after sacrifice, brains were removed for peptide measurements using qRT-PCR. Brains were placed in a matrix with the ventral surface facing up, and three 1.0 mm coronal sections were made, with the middle optic chiasm as the anterior boundary (Paxinos and Watson, 1986). For microdissection, the sections were placed on a glass slide, and the PFLH (Bregma − 2.8 to − 3.6 mm) was removed under a microscope, using the fornix and third ventricle as landmarks. The PFLH was taken from the area surrounding the fornix, within a range of 0.2 mm medial and ventral to the fornix and 0.3 mm dorsal and 1.0

mm lateral. These dissections were stored in RNA*later* (Sigma-Aldrich Co., St. Louis, MO) until processed.

2.7. Quantitative real-time PCR analysis

In Experiments 1 and 3, qRT-PCR was used to measure OX and MCH mRNA levels in the PFLH. As previously described (Chang et al., 2004), total RNA from pooled microdissected hypothalamic samples was extracted with Trizol reagent and treated with RNase-free DNase 1. The brain regions were pooled in order to maximize the amount and purity of extracted RNA from brain regions of interest. The cDNA and minus RT were synthesized using an oligo-dT primer with or without SuperScript II reverse transcriptase. The qRT-PCR experiments were conducted with an Applied Biosystems (ABI) system. With Applied Biosystems Primer Express V1.5a software, primers were designed to have a melting temperature of 58–60°C and to produce an amplicon of 50–160 base pairs. The last five bases on the 3' end contained no more than 2 G and/or C bases, to reduce the possibility of nonspecific product formation.

The SYBR Green PCR core reagents kit (ABI, CA) was used with cyclophilin (cyc) as an endogenous control. PCR was performed in MicroAmp Optic 96-well Reaction Plates (ABI) on an ABI PRISM 7900 Sequence Detection system, 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 4 independent runs of 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 cyc by standard curve method, based on threshold with Ct value of 18–25 for the different genes. Initial expression experiments were performed using cyc, β- actin and GAPDH as controls. We found that cyc gave the most reliable data, with no region or treatment specific changes in quantity. Therefore, we continued to use cyc to normalize our data for OX and MCH expression. The primers, designed with ABI Primer Express V.1.5a software based on published sequences, were: 1) cyc: 5'- GTGTTCTTCGACATCACGGCT -3' (forward) and 5'- CTGTCTTTGGAACTTTGTCTGCA -3' (reverse); and 2) OX: 5'- AGATACCATCTCTCCGGATTGC -3' (forward) and 5'-CCAGGGAACCTT TGT AGAAGGA-3' (reverse); and 3) MCH: 5'- ATCGGTTGTTGCTCCTTCTCTG -3' (forward) and 5'- TCT GCT TGG AGC CTG TGT TCT T - 3' (reverse). The concentrations of primers were 100 to 200 nM, and all reagents, unless indicated, were from Invitrogen (Carlsbad, CA). The specificities of qRT-PCR products were confirmed by both a single dissociation curve of the product and a single band with a corresponding molecular weight revealed by an agarose gel electrophoresis. In addition to the non-template control and a negative RT control, the specificity of the qRT-PCR was verified with an anatomical negative control by using the corpus callossum in the same brains.

2.8. Radiolabeled in situ hybridization histochemistry

In Experiments 2 and 4, mRNA levels of OX and MCH were also measured by radiolabeled ISH histochemistry in animals characterized as HFC or controls (n=6/group). The animals were sacrificed by rapid decapitation, and the brains were immediately removed and fixed in 4% paraformaldehyde PB (0.1M, pH 7.2) for 48–72 h, cryoprotected in 25% sucrose for 48–72 h, and then frozen and stored at −80°C. The antisense and sense OX RNA probes were donated by Dr. Luis de Lecea (Harvard University) and MCH RNA probes by Dr. Nicholas A. Tritos (Stanford University) and labeled with ^{35}S-UTP (Perkin Elmer, Waltham, MA) as described (Wortley et al., 2003). Free-floating 30 μm coronal sections were 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 10 min wash in PB between each step. After washing, the sections were hybridized with ^{35}S-labeled probe (10^3 cpm/ ml) at 55°C for 18 h. Following hybridization, the sections were washed in 4 × SSC, and nonspecifically-bound probe was removed by RNase (Sigma-Aldrich Co., St. Louis, MO) treatment for 30 min at 37°C. Then,

sections were run through a series of stringency washes with 0.1 M dithiothreitol (Sigma-Aldrich Co., St. Louis, MO) in 2 × SSC and 1 × SSC and 0.1 × SSC at 55°C. Finally, sections were mounted, air-dried and exposed to Kodak BioMax MR film for 8–18 h at −80°C, developed and microscopically analyzed. The sense probe control was performed in the same tissue, and no signal was found.

Gene expression level was determined with a computer-assisted microdensitometry of autoradiographic images on the MCID image analysis system (Image Research, Inc., St. Catherines, Canada) as described (Lucas et al., 1998; Reagan et al., 2004) Microscale ^{14}C standards (Amersham Biosciences, Piscataway, NJ) were exposed on the same Kodak film with the sections and digitized. Gray level/optical density calibrations were performed by using a calibrated film strip ladder (Imaging Research, St. Catherines, ON, Canada) for optical density. Optical density was plotted as a function of microscale calibration values. It was determined that all subsequent optical density values of digitized autoradiographic images fell within the linear range of the function. The values obtained represent the average of measurements taken from 10–12 sections per animal. In each section, the optical density for the PFLH was recorded, from which the background optical density from a same size area in the thalamus was subtracted. The mean value of OX and MCH expression of the HFC group in each experiment is reported as percentage of the control group.

2.9. Digoxigenin-labeled in situ hybridization histochemistry

As previously described (Chang et al., 2008), digoxigenin-labeled antisense RNA probes and 30-μm free-floating cryostat sections were used for ISH histochemistry in Experiment 2. AP-conjugated sheep anti-digoxigenin Fab fragments (1:1000, Roche, Nutley, NJ) and NBT / BCIP (Roche, Nutley, NJ) were used to visualize the signal. Gene expression level was measured by semi-quantification with Image-Pro Plus software, version 4.5 (Media Cybernetics, Inc., Silver Spring, MD) and was expressed as cells/mm^2, reflecting density of mRNA containing cells. Densitometry was performed adjacent, anatomically matched sections. In all analyses, the cell number was counted only on one plane in each section, and only those cells containing a nucleus in the plane (> 10 μm^2) were counted, thereby excluding fractions of cells. All OX and MCH cells lateral to the fornix were considered to be in the lateral hypothalamus (LH), and all neurons located and 0.4 mm medial and dorsal to the fornix were considered to be in the perifornical region (PF). The average cell density in each region for the different groups was compared and statistically analyzed, with the analyses being performed by an observer who was blind to the identity of the rats.

2.10. Data analysis

The values in the figures are expressed as mean ± SEM. Statistical analyses of these data were performed using an unpaired two-tailed *t*-test. The probability values given in the text or legends to the figures and tables reflect the results of these tests.

3. Results

3.1. Experiment 1: Increased OX and MCH mRNA in HFC rats as measured by qRT-PCR

With the rats rank-ordered based on intake values on the high-fat compared to chow diet and separated into two subgroups (n=6/group), the HFC rats were found to consume 35% more calories of the high-fat diet compared to the chow diet ($p<0.05$), in contrast to the control rats that consumed similar amounts of the two diets (Table 1). After the 5-day access to the high-fat diet, the HFC compared to control rats, consuming 29% more kcal/day, became slightly but not significantly higher in their measures of weight gain or levels of insulin and leptin but had significantly elevated levels of circulating TG (Table 1). The measurements of OX and MCH mRNA expression using qRT-PCR analysis revealed a clear difference between these groups.

As depicted in Fig. 1, OX mRNA expression in the PFLH region was 80% greater in the HFC compared to control rats ($p<0.001$), with an even larger difference (+150%, $p<0.001$) seen with MCH in the PFLH (Fig. 1). These results provide initial evidence for increased orexigenic peptide expression in the PFLH of HFC rats that overconsume a high-fat diet relative to a chow diet.

3.2. Experiment 2: Increased OX and MCH mRNA in HFC rats as measured by ISH

To confirm and provide an anatomical analysis of the expression changes observed in Experiment 1, a second set of animals (N=20) was characterized in a similar manner and examined using ISH with radiolabeled and DIG-labeled probes. Similar to the results reported with qRT-PCR, the HFC compared to control rats exhibited enhanced expression of OX (+30%, $p<0.05$) and MCH (+37%, $p<0.05$) mRNA in the PFLH, as shown with the radiolabeled probe (Fig. 2a,b). Further, the DIG analysis, which permits a more precise examination of the separate PF and LH subdivisions, revealed a significant increase in the density of peptide-expressing cells in this region. The number of OX-expressing cells was enhanced in the PF region ($p<0.05$) but not the LH, while the density of MCH-expressing cells was increased in both the PF and LH ($p<0.05$) (Fig. 3a,b). These results focus attention on OX in the PF as being specifically related to the overconsumption of fat, in contrast to MCH which is affected more broadly throughout both the PF and LH subregions.

3.3. Experiment 3: Increased OX and MCH mRNA in HFC rats on a chow diet as measured by qRT-PCR

This experiment had two goals, first, to determine whether HFC and control rats differ in their activity and anxiety levels prior to any high-fat diet exposure and, second, to assess whether HFC compared to control rats show differences in peptide expression in the absence of the high-fat diet. In the rats subsequently characterized as HFC compared to control rats, the measurements in an open field revealed a significantly higher locomotor activity ($p<0.05$) and also a trend towards higher anxiety levels as indicated by slightly reduced time spent in the middle ($p=0.12$) and increased time spent in the periphery ($p=0.15$) of the open field (Table 1). Similar to Experiments 1 and 2, HFC animals over the initial 5-day period consumed about 33% greater calories of a high-fat compared to chow diet ($p<0.05$) and 36% more calories of the high-fat diet compared to control rats ($p<0.05$), while control animals showed no significant differences in their consumption of these two-diets (Table 1). After returning these rats to a chow diet for 2 weeks, allowing them to normalize their caloric intake and circulating TG levels (Table 1), the results of peptide analyses using qRT-PCR revealed very similar results to those obtained in Experiments 1 and 2 in rats still consuming the high-fat diet. These HFC rats switched to a chow diet showed greater expression of OX (+40%, $p<0.001$) and MCH (+60%, $p<0.001$) in the PFLH as compared to control animals (Fig. 4). These group differences in activity levels and peptide expression in animals on a chow diet suggest that OX and MCH mRNA may be endogenously elevated in the HFC compared to control rats at baseline, independent of high-fat diet consumption.

3.4. Experiment 4: Increased OX and MCH expression in rats on a chow diet as measured by ISH

In order to substantiate the group differences in OX and MCH peptide expression measured by qRT-PCR, we tested a separate set of animals (N=20) characterized as HFC or control on a high-fat diet and then switched to chow for an additional 2 weeks. Whole brains were processed for ISH using radiolabeled probes. The analysis revealed significantly greater mRNA expression of OX (+35%, $p<0.05$) and MCH (+39%, $p<0.05$) in the PFLH region of the HFC compared to control rats (Fig. 5a,b). This evidence supports the idea that the greater

expression of orexigenic peptides in the PFLH of HFC rats may be an inherent characteristic that exists independently of high-fat diet consumption.

4. Discussion

The present study measured differences in orexigenic peptide expression of animals classified based on their intake of a high-fat relative to lower-fat chow diet. Compared to control rats, animals characterized as HFC, at normal body weight, showed increased mRNA expression of OX and MCH in the PFLH. These peptide changes, observed while the animals were consuming the high-fat diet, persisted even after the animals were switched for 2 weeks to a lower-fat chow diet and allowed to resume normal eating patterns and circulating levels of TG. These results indicate that the HFC rats are inherently different from control animals even in the absence of the high-fat diet and, with additional behavioral tests, are found to exhibit greater activity and slightly greater anxiety levels in association with these peptide changes. Together, these studies demonstrate that animals prone to overconsumption of a high-fat diet have elevated expression of orexigenic peptides that may contribute to their behavioral phenotype.

4.1. Increased expression of OX and MCH in the PFLH of rats overconsuming a high-fat diet

The specific question addressed in the present study is whether the orexigenic peptides, OX and MCH, are involved in the greater high-fat diet intake observed in the subgroup of HFC rats. Compared to control rats which consume similar amounts of a high-fat compared to chow diet, these HFC rats when examined over the initial 5-day period consume 35% more calories from the high-fat diet than the chow diet and 29% more calories from the high-fat diet than the control rats. With this early eating pattern found to predict long-term intake of this same diet (Chang et al., 2010; Dourmashkin et al., 2006; Lauterio et al., 1994; Leibowitz et al., 2007; Pagliassotti et al., 1994; Shor-Posner et al., 1991), disturbances in the brain detected during this acute period may be causally related to the chronic behavioral patterns. Along with greater caloric intake, the HFC compared to control animals showed elevated circulating TG levels after 5 days on the high-fat diet. These lipids are found to be closely related to and predictive of increased consumption of fat (Gaysinskaya et al., 2007)and therefore may have a direct role in enhancing consummatory behavior of the HFC animals. Our results demonstrate that orexigenic peptides in the PFLH are significantly higher in the HFC rats examined on the fifth day of access to the high-fat diet, a period when hyperphagia is most evident (Melhorn et al., 2010). Although OX and MCH may be differentially altered by a high-fat diet when it is chronically overconsumed and circulating adiposity hormones and glucose levels are altered (Burdakov et al., 2005), these two peptides are similarly enhanced during shorter periods of high-fat exposure (Chang et al., 2004; Karatayev et al., 2009b; Wortley et al., 2003), suggesting their involvement in short-term regulation of food intake. Consistent with previous reports (Chang et al., 2004; Karatayev et al., 2009b; Wortley et al., 2003), this increase in OX and MCH peptide expression occurs in close relation to elevated TG levels in the HFC rats, suggesting that these lipids may play a role in stimulating the expression of these peptides that, in turn, promote further feeding.

4.2. Differential regulation of PF and LH OX neurons in rats exhibiting excess consumption of fat-rich diet

The DIG-labeled probes in our ISH experiment allowed us to detect specific regional differences in peptide expression in the PF and LH regions. Although MCH expression was increased in both the PF and LH regions of the HFC compared to control rats, the OX expression was elevated exclusively in the PF subregion, suggesting that OX neurons in the PF are more closely related to manipulations involving a high-fat diet. This conclusion is consistent with our previous studies, which showed a similar effect specifically in the PF region in rats consuming a high-fat diet compared to a low- or moderate-fat diet (Karatayev et al., 2009b;

Wortley et al., 2003). It also agrees with two additional reports, showing increased activation of OX neurons specifically in the PF region of rats anticipating a palatable fat-rich diet (Choi et al., 2010) or after central administration of an opioid analogue, which is known to promote high-fat diet consumption (Zheng et al., 2007). This region specificity may stem from the proposed dichotomy in OX function, with OX neurons in the PF involved in arousal (Estabrooke et al., 2001; Harris and Aston-Jones, 2006) and those in the LH having a more prominent role in reward-driven behaviors (Harris and Aston-Jones, 2006; Harris et al., 2005). These differential functions may be attributed to the anatomical distinctions between the PF and LH OX neurons, which predominantly project to arousal centers (Espana et al., 2005; Peyron et al., 1998) and major reward centers (Fadel and Deutch, 2002), respectively. Since the majority of MCH neurons reside in the LH region and no studies to date have revealed heterogeneity among the PF and LH neuronal populations, the increased expression of MCH in both regions of HFC rats suggests that this peptide functions through a common PFLH pathway to stimulate consummatory behavior. However, our findings of increased OX gene expression specifically in the PF region of HFC animals, together with studies suggesting the involvement of this region in arousal mechanisms, suggest that HFC animals may be more behaviorally active in association with their increase in consummatory behavior on a high-fat diet.

4.3. Increased activity predicts increased high-fat diet consumption

In order to test behavioral arousal in HFC compared to control animals, we measured locomotor activity in these two subpopulations of rats. Our results suggest that HFC animals, in addition to showing greater consumption of a high-fat diet, are more behaviorally active and show a trend towards greater anxiety than control animals even prior to high-fat exposure. This is consistent with a recent report showing animals with increased nocturnal activity in an open field to consume more calories when given a diet rich in fat (Hesse et al., 2010). It also agrees with additional evidence relating OX and MCH to behavioral arousal and anxiety. Hypothalamic and extra-hypothalamic injection of OX peptide is found to stimulate behavioral activity in Sprague-Dawley rats (Kiwaki et al., 2004; Novak et al., 2006; Thorpe and Kotz, 2005) and also increased anxiety in an elevated-plus-maze (Suzuki et al., 2005). Also, ventricular injection of MCH increases anxiety-like behavior in an elevated-plus-maze (Smith et al., 2006) and MCH receptor knockout mice show reduced anxiety (Lalonde and Qian, 2007) while nucleus accumbens injections of MCH increase cocaine-induced locomotor activity (Chung et al., 2009). These studies suggest that the HFC rats have an additional phenotype of increased activity and anxiety levels, which along with increased consummatory behavior on a high-fat diet may be related to their disturbances in OX and MCH peptide systems.

4.4. Increased OX and MCH expression in the PFLH of HFC rats while on a chow diet

An important remaining question of the present study is whether the increase in OX and MCH expression is an inherent characteristic of the HFC rats, which can occur independently of exposure to the high-fat diet. This question was addressed in Experiments 3 and 4, which tested rats that after the initial 5-day access to the high-fat diet were switched off this diet and on to a lower-fat chow diet for 2 weeks, allowing their food intake and TG levels to be normalized. The greater expression of OX and MCH in the PFLH was found to persist in the HFC rats even when they were maintained on the chow diet. Although these differences in peptide expression may reflect long-term changes induced by the brief, initial exposure to the high-fat diet, several recent studies suggest that the effects of acute consumption of a high-fat diet on peptide expression are short-lived. For example, changes in expression of proopiomelanocortin, brain-derived neurotrophic factor and tyrosine hydroxylase induced by high-fat diet consumption are no longer evident when animals are switched to a lower-fat diet (Archer et al., 2005; Levin and Dunn-Meynell, 2002; Li et al., 2009). Moreover, increased expression of hypothalamic

dynorphin induced by long-term consumption of a sweet-fat diet is no longer seen or even reversed after a few weeks on a chow diet (Archer et al., 2005; Chang et al., 2007; Levin and Dunn-Meynell, 2002). Therefore, our findings of a persistent increase in OX and MCH expression in HFC animals, even after two weeks on a low-fat diet, suggest that these animals have endogenous differences in the PFLH peptides that contribute to their increase in consummatory behavior when given access to a meal high in fat content. In light of recent evidence suggesting that OX may protect against weight gain (Funato et al., 2009), possibly by increasing activity levels in animals (Novak and Levine, 2009), our results of increased OX and also MCH peptide expression, which is accompanied by higher activity levels in HFC animals that go on to consume more high-fat diet, may reflect a protective mechanism that helps to maintain homeostatic energy levels.

4.5. Function of OX and MCH in initiating consummatory behavior

The increased expression of OX and MCH in HFC animals suggests that these peptides may be involved in initiating and promoting consummatory behavior, specifically of a palatable fat-rich diet. This idea agrees with other findings, showing OX neurons to be activated during the anticipation of a fat-rich meal (Choi et al., 2010)and both OX and MCH neurons to be activated by an acute, high-fat feeding paradigm (Karatayev et al., 2009b; Wortley et al., 2003). Further, injection of OX preferentially increases consumption of a high-fat diet (Clegg et al., 2002). There are several reports showing acute injection of OX or MCH to produce a transient enhancement of food consumption, while chronic injections have no effect or even suppress food intake (Abbott et al., 2003; Clegg et al., 2002; Haynes et al., 1999; Rodgers et al., 2000; Sakurai et al., 1998; Sweet et al., 1999; Yamanaka et al., 1999). This evidence indicates that these peptides regulate consummatory behavior and energy balance over the short term, perhaps to mediate early stages of excess intake. The present findings, showing OX and MCH to be increased after a short 5-day period of high-fat consumption or even at baseline prior to high-fat diet exposure, agree with this idea and further suggest that these peptide systems are involved in the initiation of high-fat diet consumption in HFC animals but may recruit other peptide systems, to generate a persistent, long-term pattern of overconsumption.

4.6. Neural circuits of increased consummatory behavior in HFC rats

The increased consumption of a high-fat diet in HFC animals is most likely influenced by OX and MCH acting through different projection sites in hypothalamic or limbic regions. Both OX and MCH neurons originating in the PFLH are known to send projections to the nearby hypothalamic paraventricular nucleus (PVN) (Peyron et al., 1998; Qi et al., 2009), a region intimately involved in consummatory behavior, and also to the nucleus accumbens (NAc) (Fadel and Deutch, 2002; Georgescu et al., 2005), a region with a prominent function in reinforced behavior. Injections of the OX or MCH peptide into the PVN (Abbott et al., 2003; Dube et al., 1999) or NAc (Georgescu et al., 2005; Schneider et al., 2007) are found to enhance food intake, suggesting that these two brain regions are involved in mediating the actions of these PFLH peptides on consummatory behavior. Although the exact neurochemical mechanisms that contribute to the increased consummatory behavior are not well understood, there is evidence focusing on the opioid enkephalin in the PVN and the inhibitory amino acid γ-aminobutyric acid (GABA) in the NAc. This evidence shows that the injection of OX stimulates the expression of enkephalin in the PVN, where local injections of this peptide increase food intake and preferentially stimulate fat consumption (Karatayev et al., 2009a; Naleid et al., 2007). Also, OX and MCH injection stimulate GABA in the NAc, where local injections of this amino acid also potentiate food intake (Martin et al., 2002; Meister, 2007; Stratford and Kelley, 1997). These findings suggest that these neurochemicals in the PVN and NAc may provide the next link in the neural circuit mediating the greater amount of high-fat diet consumption exhibited by the HFC rats.

4.7. Conclusion

The HFC animal model described here is one of the first to characterize animals based specifically on varying degrees of acute consummatory behavior, a few days of high-fat diet intake compared to baseline chow intake that predicts long-term eating patterns. Our results suggest that animals prone to increased consumption of a diet rich in fat may have specific disturbances in the OX and MCH peptide systems in the PFLH. Evidence suggests that these peptides, in addition to responding to fat consumption, may function to further promote fat intake by increasing behavioral activation, stimulating consummatory behavior, and possibly even protecting against excess weight gain in the short-term. In light of evidence supporting a role for OX and MCH in short-term regulation of consummatory behavior, most likely via projections to the PVN or NAc, our results in HFC animals demonstrate that increased expression of these peptides may inherently regulate the early stages of high-fat diet overconsumption. It should be noted that, rather than being disturbed in the HFC, it may be the control animals that are abnormal by failing to show enhanced peptide expression that may protect against fat accumulation. Further examination of the neurochemical differences that exist in this distinct sub-population of animals may shed light on the mechanisms contributing to increased consummatory behavior particularly as it occurs with palatable foods.

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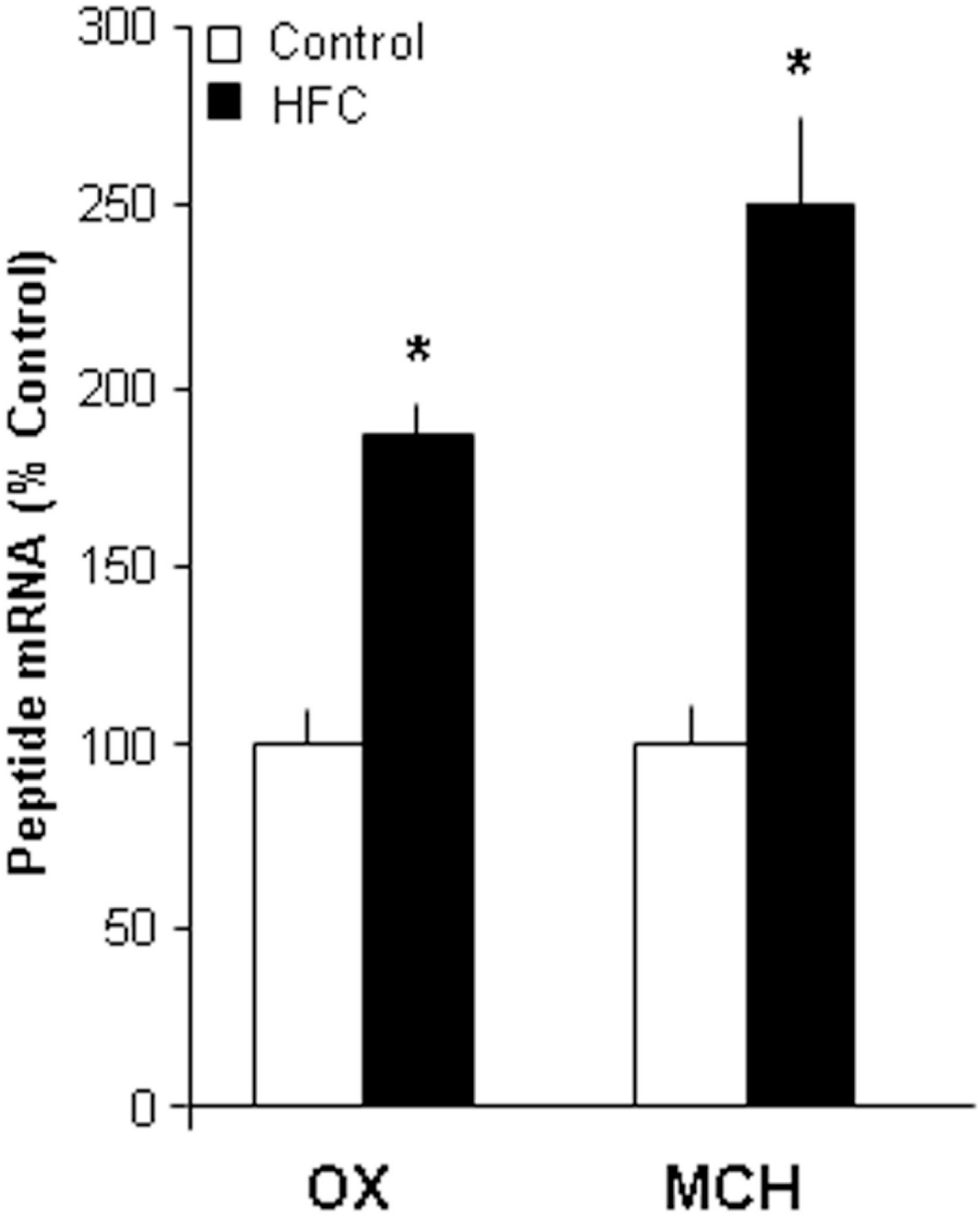

Fig. 1.

Expression of OX and MCH mRNA in the PFLH of HFC or control rats immediately after 5 days of high-fat diet consumption, as measured by qRT-PCR (Experiment 1). The data (mean ± SEM) revealed a significant increase in expression of OX and MCH in the PFLH of HFC compared to control rats (* $p<0.001$).

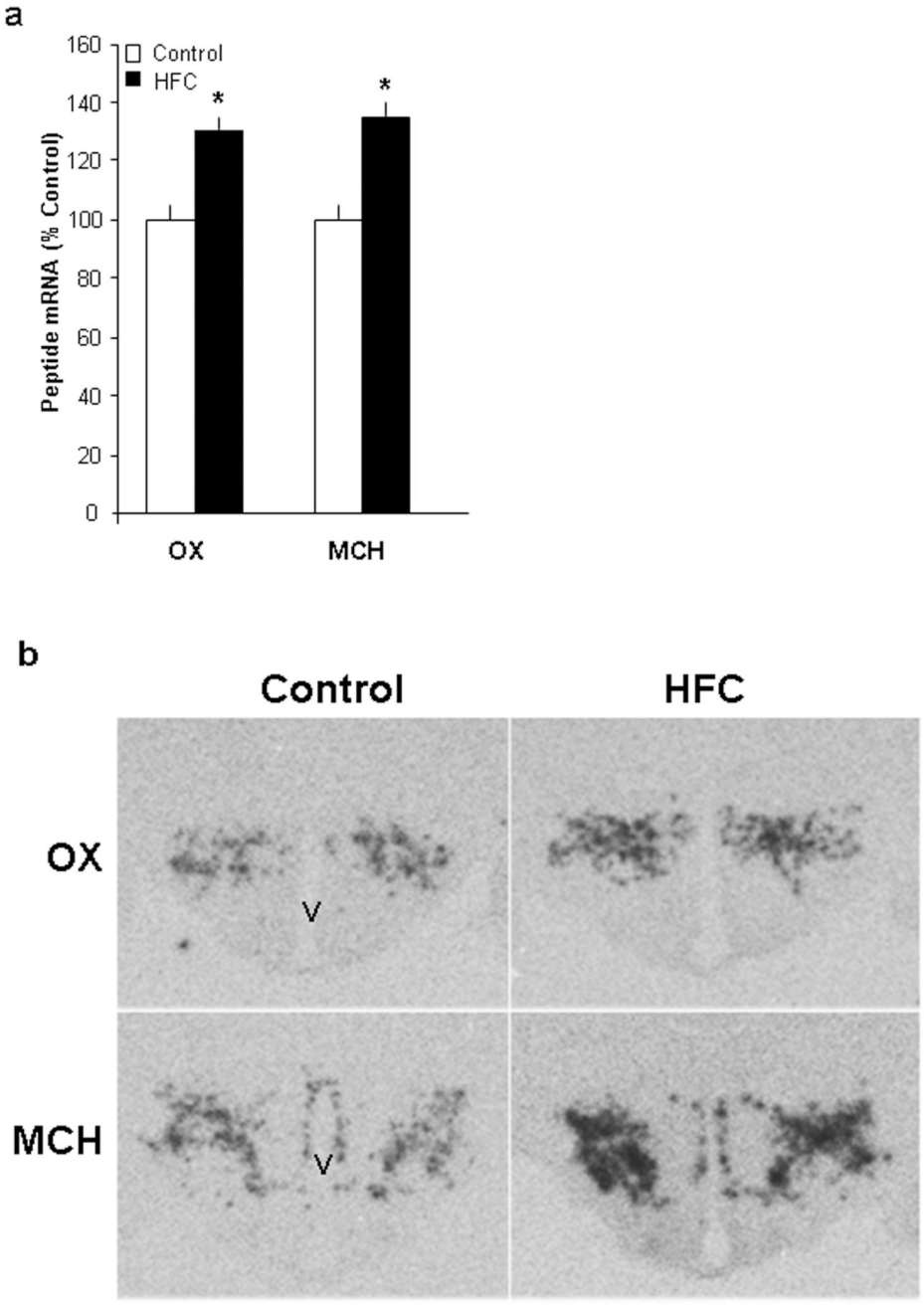

Fig. 2.

Expression of OX and MCH mRNA in the PFLH of HFC and control rats, immediately after 5 days of high-fat diet consumption, as measured by radiolabeled ISH (Experiment 2). (a) The data (mean ± SEM), presented as percent of controls, show a significant increase in OX and MCH mRNA levels in HFC compared to control animals (* $p<0.05$). (b) Photomicrographs illustrate the increase in OX and MCH expression in the PFLH of HFC rats.

a

		Experiment 2: High-Fat Diet (cells/um^2 x10^{-4})	
		Control	HFC
OX	PF	4.14 ± 0.07	4.87 ± 0.05*
	LH	2.43 ± 0.29	2.52 ± 0.24
MCH	PF	5.03 ± 0.28	6.40 ± 0.13*
	LH	5.04 ± 0.27	6.54 ± 0.10*

Values are means ± SEM; * p<0.05 for comparisons between HFC and control rats

b

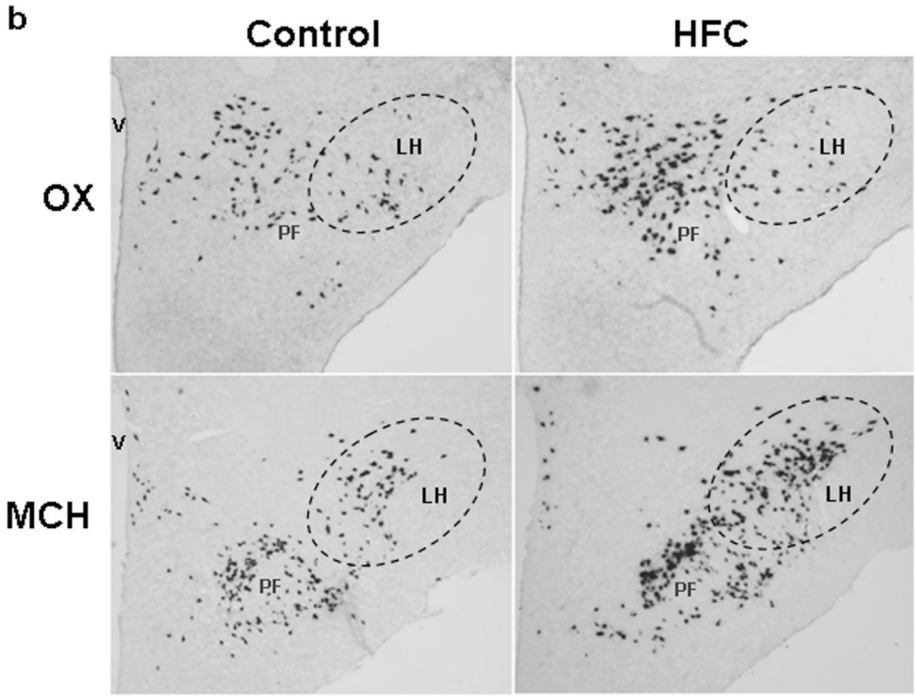

Fig. 3.

Expression of OX and MCH mRNA in the PF vs LH regions of the HFC and control rats following 5 days of high-fat diet consumption, as measured via digoxigenin-labeled ISH (Experiment 2). (a) The data (mean ± SEM), expressed as cell density, show a significant increase in expression of OX in the PF, but not LH region, and of MCH in both PF and LH regions of HFC compared to control animals (* p<0.05). (b) Photomicrographs illustrate the increased OX and MCH expression in the PFLH of HFC rats.

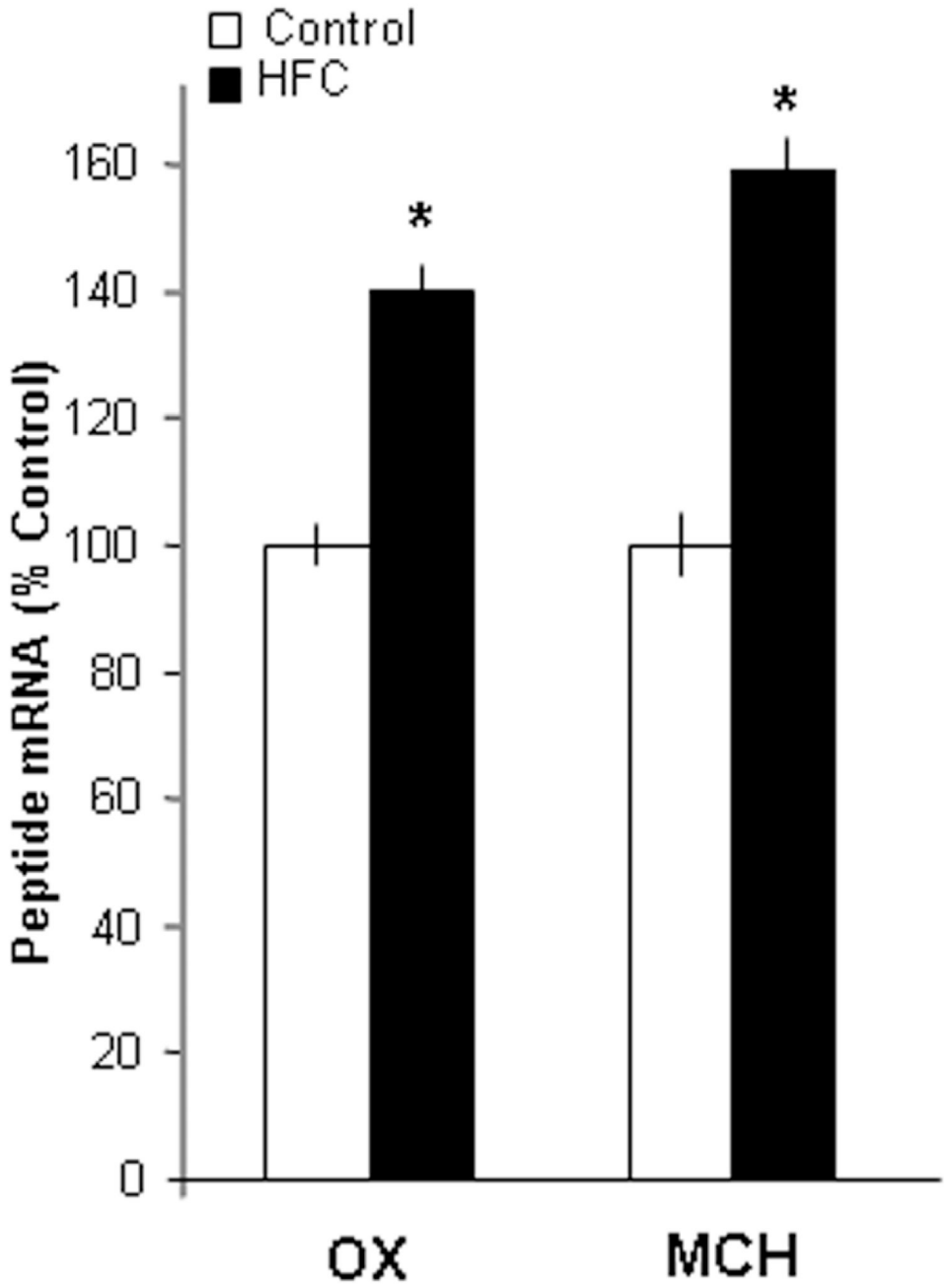

Fig. 4.

Expression of OX and MCH in the PFLH of HFC and control rats 2 weeks after being placed on a chow diet, as measured by qRT-PCR (Experiment 3). The data (mean ± SEM) revealed a significant increase in expression of OX and MCH in the PFLH of HFC compared to control rats (* $p<0.001$).

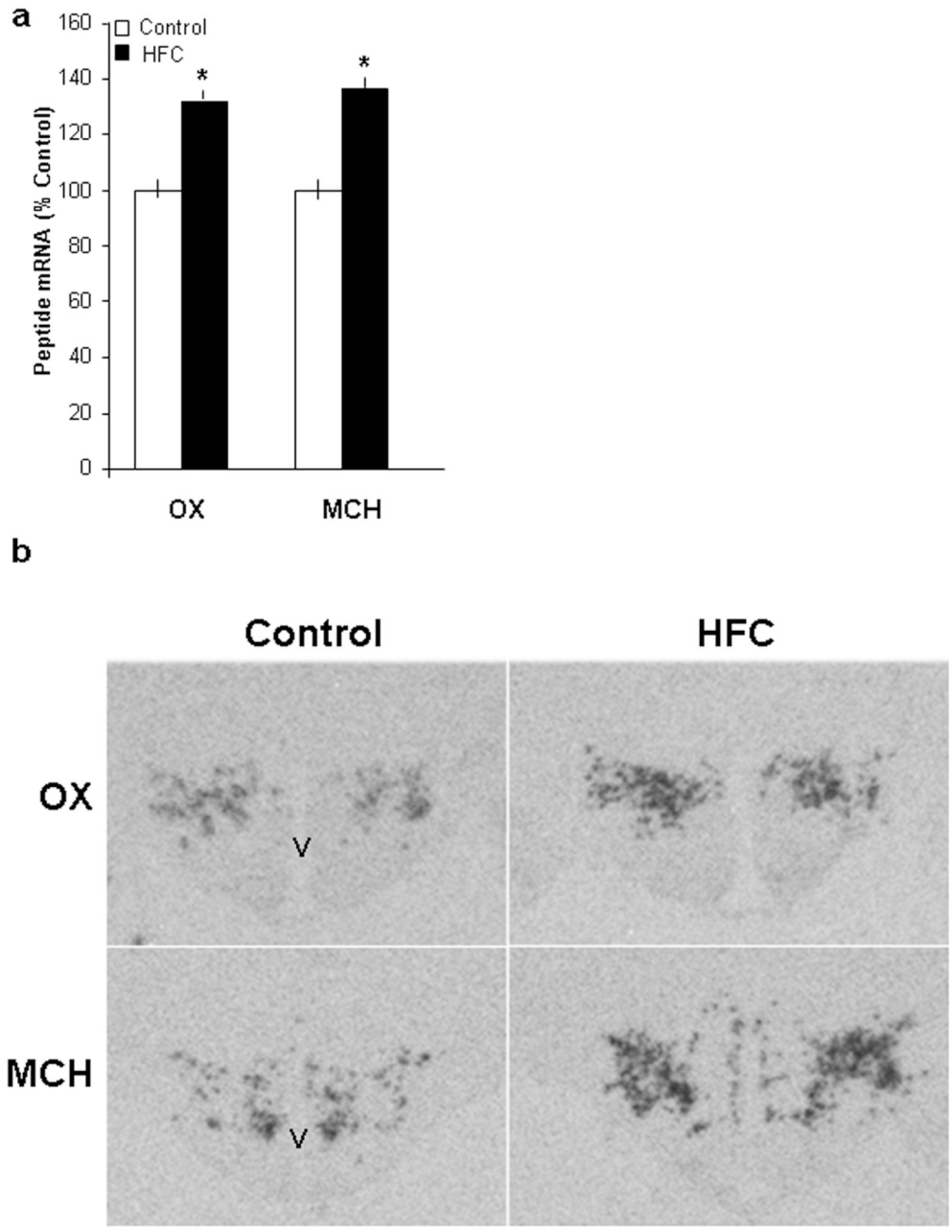

Fig. 5.

Expression of OX and MCH mRNA in the PFLH of HFC and control rats 2 weeks after being placed on a chow diet, as measured by radiolabeled ISH (Experiment 4). (a) The data (mean ± SEM), presented as percent of control animals, show a significant increase in expression of OX and MCH in HFC compared to control animals (* $p<0.05$). (b) Photomicrographs illustrate the increase in OX and MCH mRNA levels in the PFLH of HFC rats.

Table 1

Measures of caloric intake, weight gain, locomotor activity, anxiety and serum levels of triglyceride, insulin and leptin in high-fat consumers (HFC) and control animals. The rats were sacrificed immediately after the 5 days of high-fat diet access (Experiment 1) or following 2 weeks of chow consumption (Experiment 3).

		Experiment 1		Experiment 3	
Measure	**Diet**	**Control**	**HFC**	**Control**	**HFC**
Caloric Intake (kcal/day)	Chow	89 ± 5.2	92 ± 4.3	85 ± 5.3	90 ± 4.6
	High-fat	96 ± 5.7	124 ± 3.5*#	92 ± 4.7	120 ± 3.9*#
	Chow	---	---	90 ± 3.5	93 ± 5.2
Weight Gain (g/day)	Chow	7.2 ± 1.1	7.8 ± 1.3	6.9 ± 1.4	7.2 ± 1.2
	High-fat	9.8 ± 2.1	11.1 ± 1.5#	9.1 ± 1.7	10.7 ± 1.5#
	Chow	---	---	7.1 ± 0.9	7.5 ± 1.1
Activity (lines crossed)		---	---	22.3 ± 4.1	38.3 ± 1.6*
Anxiety (seconds)	Time in Middle	---	---	161 ± 25	124 ± 15
	Time in Periphery	---	---	139 ± 18	176 ± 22
Triglycerides (mg/dl)		110 ± 5.3	169 ± 7.5*	101 ± 6.3	118 ± 4.9
Insulin (ng/ml)		1.6 ± 0.6	2.1 ± 0.5	1.3 ± 0.3	1.7 ± 0.4
Leptin (ng/ml)		4.7 ± 0.7	5.5 ± 1.3	4.2 ± 0.5	4.4 ± 0.7

Values are means ± SEM;

* $p<0.05$ for comparisons between HFC and control rats;

# $p<0.05$ for comparisons between high-fat and chow diets.