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Leptin Regulation of Prepro-orexin and Orexin Receptor mRNA Levels in the Hypothalamus

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The aim of this study was to determine the effects of leptin treatment on prepro-orexin and orexin receptor expression in the rat hypothalamus. Adult male rats, food-deprived for 48 and 72 h, were treated one time with vehicle or leptin (10 μg, icv). Prepro-orexin mRNA content was measured by semiquantitative RT-PCR, Northern blot, and *in situ* hybridization; orexin receptor 1 and 2 mRNA content was quantified by Northern blot and/or semiquantitative RT-PCR. Our results indicate that leptin inhibits a fasting-induced increase in prepro-orexin mRNA and orexin receptor 1 mRNA levels in the rat hypothalamus, while orexin receptor 2 mRNA levels were unchanged in all situations evaluated. These data provide direct evidence for an additional mechanism of adaptation of the hypothalamus to food deprivation and for a new effect of leptin in the regulation of food intake.

Key Words: **prepro-orexin; orexin receptors; hypothalamus; leptin; fasting; feeding; obesity; RT-PCR; *in situ* hybridization; Northern blot.**

The hypothalamus plays a central role in the integrated control of feeding and energy homeostasis. Two novel hypothalamic neuropeptides: the orexins A and B (OX-A and OX-B) have recently been identified and shown to play a role in the regulation of food intake (1). Both of them derived from a common precursor, the prepro-orexin (prepro-OX, also called prehyprocretin), which is synthesized in neurones within and around the lateral and posterior hypothalamus in the adult rat brain (1–4). The orexin-containing neurones project to multiple sites of the central nervous system (5, 6) These peptides bind and activate two closely related G-protein coupled receptors, called orexin receptor 1 (OX_1R), and orexin receptor-2 (OX_2R) (1). Several evidences suggests the involvement of the orexin in the regulation of feeding, so, the central administration of orexins increases food intake (1), and the levels of prepro-OX mRNA are clearly influenced by the nutritional status of the animal, being up-regulated upon fasting (1).

Leptin, the product of the ob gene, is a hormone mainly synthesized in the adipose tissue, and which is important in the regulation of food intake, energy expenditure and adiposity (7). Recently, it has been reported that orexin-containing neurones in the lateral hypothalamic area expressed leptin receptors (8), although the particular subtype and therefore the signaling competence of these receptors is uncertain. Moreover, chronic treatment with leptin (ip) decreased OX-A concentration in the lateral hypothalamus (9). These findings suggest that leptin may influence food intake by regulating orexin-gene expression, among other mechanisms.

To evaluate this possible functional linkage between leptin and orexins, we determined the effects of leptin administration (icv) on prepro-OX mRNA levels, as well as on OX_1R- and OX_2R-gene expression in the rat hypothalamus.

MATERIAL AND METHODS

Animals. Adult male Sprague–Dawley rats (200–250 g) were housed on a 14-h light (8:00 to 22:00), 10-h dark cycle, in a temperature and humidity controlled room. Animals were allowed free access to standard laboratory pellets of rat chow and tap water.

Implantation of intracerebroventricular cannulae and leptin treatment. Chronic intracerebroventricular (icv) cannulae were implanted under sodium pentobarbital anaesthesia (50 mg/kg, ip), as described previously (10). Animals were used for the experiment a week later, thereafter, the rats continued to have food available *ad libitum* (Fed rats), or were deprived of food for 48 or 72 h (Fast 48 h, and Fast 72 h). After this, rats received a single administration either vehicle (5 μl of distilled water) or leptin (10 μg per rat, dissolved in 5 μl of distilled water), kindly supplied by Eli Lilly, for 6 h as described previously (10); during this time rats were main-

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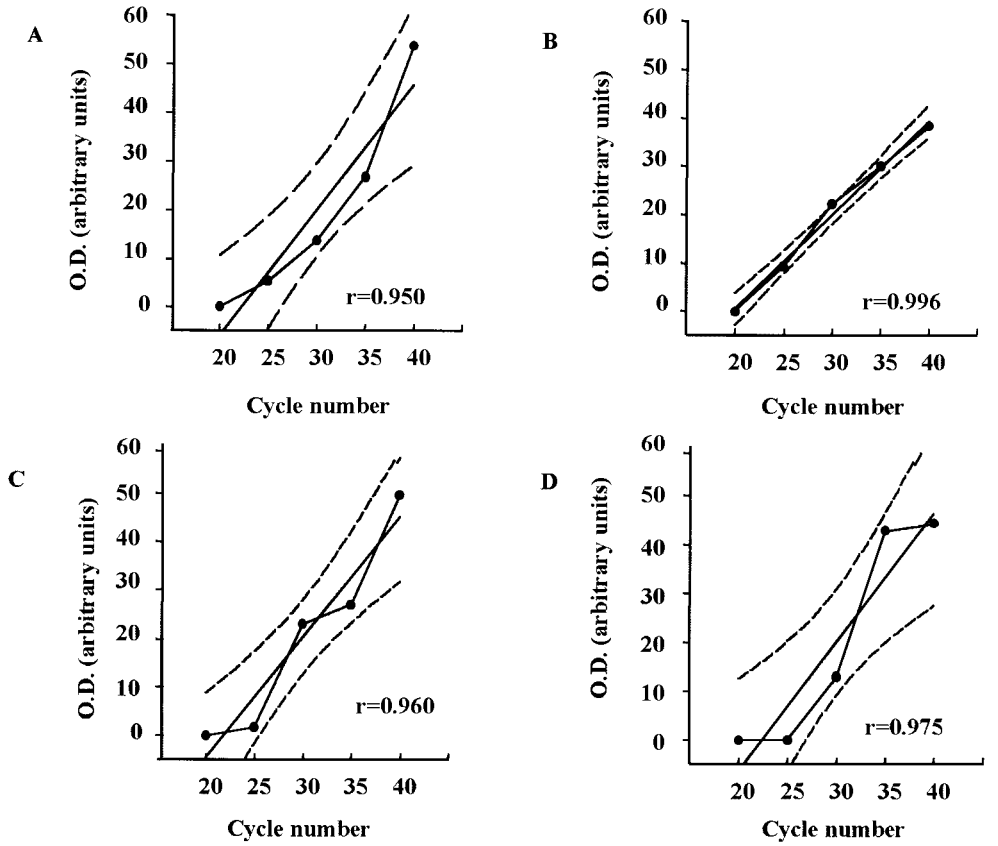

FIG. 1. Amplification plots for (A) HPRT, (B) Prepro-OX (C) OX_1R, and (D) OX_2R. Samples were amplified for 15, 20, 25, 30, 35, and 40 cycles. The reaction was linear over this range for all PCR-products. (*r*, correlation coefficient of the linear range).

tained in the same conditions (fed or fast) than after treatment. All experiments were conducted during the beginning of the lights-on phase (10:00). We used 11–13 animals in each group.

Hypothalamic dissection and RNA isolation. To isolate the hypothalamus, animals were decapitated and their brains removed rapidly. The hypothalamus, defined by the posterior margin of the optic chiasm and the anterior margin of the mamillary bodies to the depth of approximately 2 mm, was dissected out and frozen immediately into dried ice. Total RNA was isolated using Trizol Reagent (Gibco-BRL, Life Technologies, U.S.A.). The content of prepro-OX mRNA and OX_1R and OX_2R mRNA was analyzed in each hypothalamus individually.

RT-PCR. The RT and PCRs were carried out as described elsewhere (11). To assure that the PCR was performed in a linear range, samples were amplified for 15, 20, 25, 30, 35 and 40 cycles. As shown in Fig. 1 the reactions were linear under these conditions. Therefore, we used 28 cycles of amplification in all our experimental samples. Amplified products were quantitated by densitometry using a digital imaging system (Molecular Analyst, Bio-Rad, Hercules, CA) (12).

Northern blot. Northern blot analysis was carried out as described previously (13) using 20 μg of total RNA. Antisense riboprobes labeled with ^{32}P-CTP were used to detect prepro-OX and OX_1R mRNA levels.

In situ hybridization. For *in situ* hybridization coronal hypothalamic sections (15 μm) were cut on a cryostat, and immediately stored at −80°C until hybridization. An antisense riboprobe for prepro-OX, labeled with [^{32}P]CTP was used, and the hybridization was performed as reported (14). Prepro-OX sense riboprobe was used as negative control. To compare anatomically similar regions, slides were matched according to the rat brain atlas of Paxinos and Watson (15). Sections from at least three animals per experimental group were processed together, and were always apposed to the same autoradiographic film. Independent experiments were repeated at least three times. All sections were scanned and the specific hybridization signals were quantitated by densitometry using digital imaging system (Molecular Analyst, Bio-Rad). All sections were assessed bilaterally. The background pixel density for each section was measured, and the specific signal was corrected subtracting this background value.

Statistical analysis. RT-PCR and *in situ* data was analyzed by ordinary parametric ANOVA followed by post hoc Bonferroni test. For all test significance was set at the $P < 0.05$ level.

RESULTS

Fasting Increases Prepro-OX mRNA Levels and OX_1R Content in the Rat Hypothalamus

Rats deprived of food for 48 h exhibited a significant increase in prepro-OX mRNA versus normally fed rats as assessed by either RT-PCR, *in situ* hybridization and Northern blot (Figs. 2–4). In contrast OX_1R and OX_2R mRNA levels were not changed under these experimental condition (Figs. 2 and 4). In rats that were food-restricted for 72 h, prepro-OX mRNA were further increased (Figs. 2–4), and OX_1R mRNA levels were now higher than the levels observed in fed rats (Figs. 2

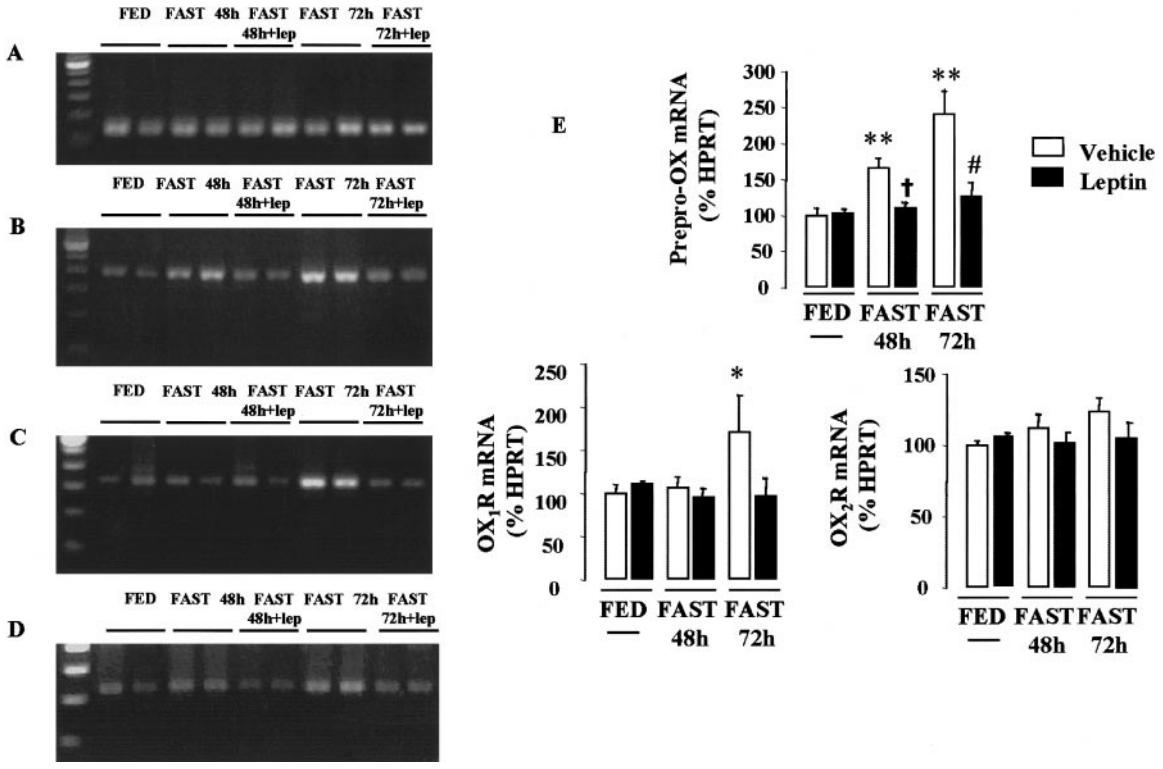

FIG. 2. (A) Representative RT-PCR detection of (A) HPRT, (B) prepro-OX, (C) OX_1R and (D) OX_2R mRNA levels in the hypothalamus of fed rats, rats deprived of food and treated with vehicle for 48 hours (Fast 48 h) or 72 h (Fast 72 h), or treated with leptin (Fast 48 h + lep; Fast 72 h + lep). (E) Prepro-OX, OX_1R and OX_2R mRNA levels (means ± SEM) in the hypothalamus of fed rats, rats deprived of food for 48 h, rats deprived of food for 48 h and treated with leptin, rats deprived of food for 72 h, and rats deprived of food for 72 h and treated with leptin. Data (mean ± SEM) were expressed in relation to HPRT levels. *$P < 0.05$ vs FED; **$P < 0.001$ vs FED; †$P < 0.01$ vs FAST 48 h; #$P < 0.01$ vs FAST 72 h.

and 4). In contrast, no significant changes were observed in OX_2R mRNA content (Fig. 2).

Leptin Reverses the Fasting-Induced Increase on Prepro-OX and OX_1R mRNAs in the Rat Hypothalamus

Since food deprivation is associated with a marked decrease in serum leptin levels (7) and leptin receptors are expressed in orexin producing neurones (8), we next assessed whether the effects of food-deprivation on prepro-OX and OX_1R mRNA levels would be reversed by exogenous leptin administration. We found that leptin administration reversed the stimulatory effect of food-deprivation on both prepro-OX and OX_1R mRNA levels (Figs. 2–4). In contrast OX_2R mRNA levels were not modified by leptin (Fig. 2). However, leptin treatment have no effects on prepro-OX and OX_1R mRNA content in fed rats (Figs. 2–4).

DISCUSSION

Previous data regarding the possible interaction between leptin and orexins have failed to reach an unanimous conclusion. The findings that prepro-OX mRNA levels were decreased in *ob*/*ob* and *db*/*db* mice (16) led to some authors to conclude that the effects of leptin on food intake were not mediated by orexins. On the other hand, it has been recently published that systemic (intraperitoneal) administration of leptin to Siberian hamsters does not induce significant changes in hypothalamic prepro-OX mRNA levels (17). On the contrary, others have found elevated orexin concentrations in the brains of Zucker rats (18), while experiments carried out in normal rats have shown the presence of leptin receptors in orexin-containing neurones (8). All together these data suggest a regulatory role of leptin on orexin-producing neurones (8, 9).

Our data, showing a stimulatory effect of food-deprivation on the hypothalamic prepro-OX mRNA levels that was reversed by leptin administration, provide new evidence that leptin regulates orexin gene expression in food restricted rats. However, leptin treatment did not change prepro-OX mRNA content in *ad libitum* fed rats. Whether the opposite effects on prepro-OX mRNA levels found in *ob*/*ob* and *db*/*db* mice, in comparison to our data, are due to the different experimental animals used, rats vs mice, or to the fact that these genetically obese mice exhibit many other endocrine

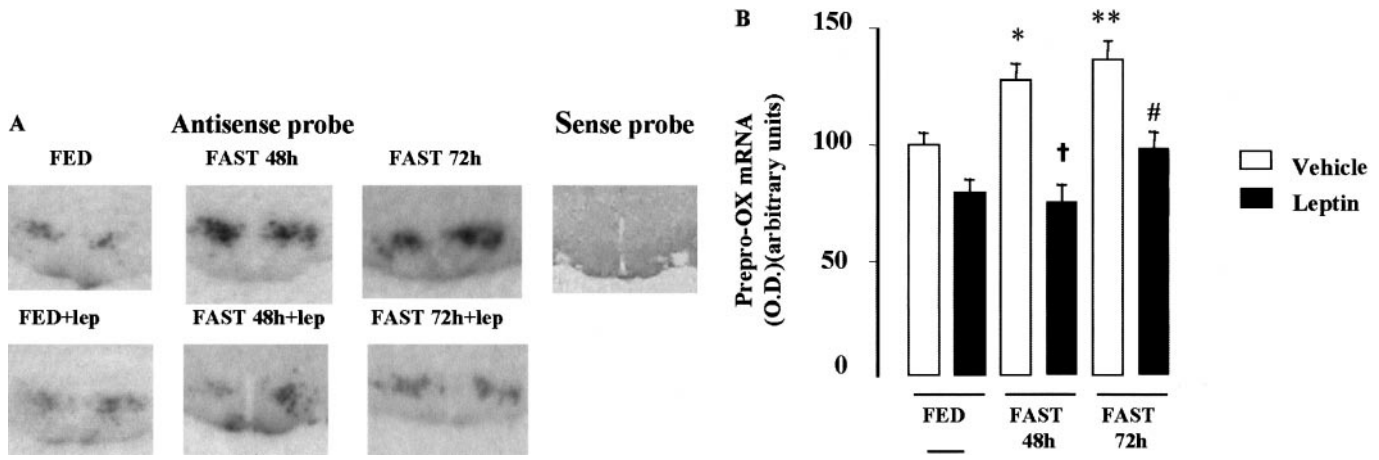

FIG. 3. (A) Autoradiographic images of representative brain coronal sections at the level of the hypothalamus incubated with ^{35}S-labeled antisense and sense prepro-OX RNA probes in the described experimental groups. Prepro-OX mRNA is found in the lateral hypothalamus (B) Prepro-OX mRNA levels (mean ± SEM) were expressed in relation to mRNA levels of vehicle treated rats. *$P < 0.05$ vs FED; **$P < 0.01$ vs FED, †$P < 0.01$ vs FAST 48 h; #$P < 0.05$ vs FAST 72 h.

and metabolic alterations that could influence prepro-OX gene expression remains to be established.

In addition to the reported effects of food-deprivation and leptin on prepro-OX mRNA content, we assessed in the same experimental model the levels of OX_1R and OX_2R mRNA obtained by RT-PCR and/or Northern blot. Our data provide the first evidence that OX_1R mRNA levels are up-regulated by food-deprivation in the rat hypothalamus, an effect that is reversed after exogenous leptin administration. On the contrary, OX_2R mRNA levels were unchanged in the same experimental setting. These data indicate a different regulation of both types of receptors, which is also supported by their different anatomical distribution within the hypothalamus: OX_1R is present mostly in the ventromedial nucleus, and OX_2R is localized in the paraventricular nucleus (19), indicating that these two receptors may influence different functions. Indeed, recent data suggest that the OX_2R is involved in the sleep control. Thus, it was found that knock-out mice lacking the prepro-OX gene exhibited sleep control abnormalities that resembling narcolepsy (20). In keeping with this observation, using the well-characterized canine models of narcolepsy, namely a breed of Doberman and Labrador retriever, two different deletions in the transcripts of the OX_2R were found (21). Taken together these data indicate that the interaction of orexins with the OX_2R appears to be a key signaling pathway in sleep regulation. On the other hand, taking into account that we found that OX_1R is influenced by nutritional status, it is tempting to suggest that the effects of orexins on food intake are mainly influenced

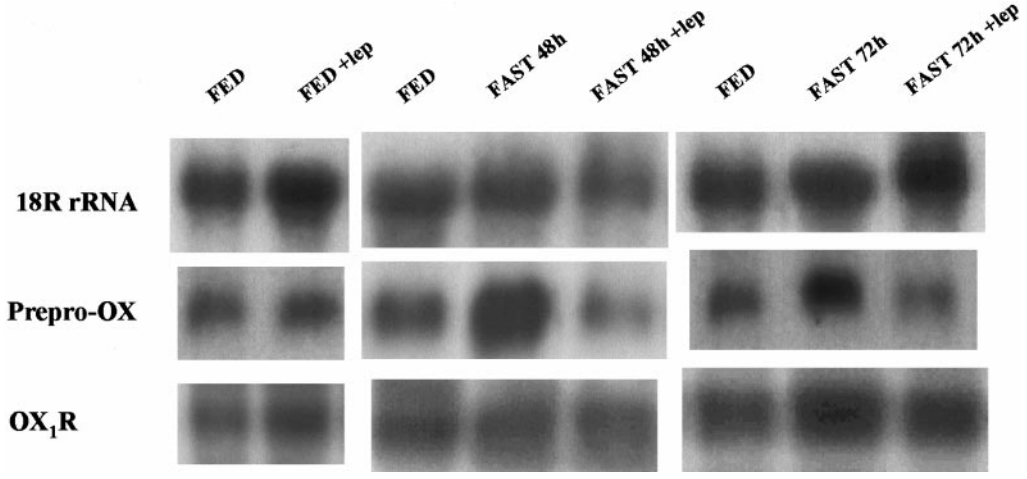

FIG. 4. Northern blot analysis of hypothalamic prepro-OX and OX_1R mRNA expression in the described experimental groups. The membranes was rehybridized with 18S rRNA as an internal reference for the amount of RNA loaded.

through the OX_1R. Further studies with specific agonist and antagonist drugs of both receptor subtypes should help to clarify this issue.

In summary, our data show that prepro-OX mRNA and OX_1R but not OX_2R mRNA levels in the hypothalamus are up-regulated by food-deprivation, an effect that can be reversed by exogenous leptin administration. These data provide direct evidence of an additional mechanism of adaptation of the hypothalamus to food-deprivation and of the action of leptin in the regulation of food intake.

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