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Hypothalamic Hypocretin/Orexin and Neuropeptide Y: Divergent Interaction with Energy Depletion and Leptin

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The aim of this study was to measure the effects of chronic leptin treatment on two orexigenic peptides present in the hypothalamus namely hypocretin/orexin and neuropeptide Y (NPY). For this purpose, recombinant murine leptin (0.2 mg/rat/day) or saline were injected intraperitoneally in Long-Evans rats for 7 consecutive days. Food intake (−8%; $p < 0.002$) and body weight gain (23.7 ± 1 vs 31.5 ± 1.3 g; $p < 0.003$) were significantly lower in leptin-treated rats than the saline-treated rats. NPY concentrations did not change significantly in any of the microdissected brain areas including the arcuate and paraventricular nuclei. Orexin A concentration in the lateral hypothalamus was significantly decreased by the leptin treatment (−68%; $p < 0.01$). A smaller decrease (−46%; $p < 0.04$) was also noted in saline-treated rats pairfed to the level of the leptin-treated rats. We conclude that orexin/hypocretin could be considered as a new relay for leptin in the central nervous system. Its variation in case of lower energy supply observed in pairfed rats could constitute an alerting system for the brain and therefore considered as the first step in the establishment of defense mechanisms against energy depletion.

Since its discovery in 1994 (1), the ob protein or leptin has received considerable attention (review in 2). It is secreted by the adipose tissue in relation to the feeding state and is well correlated with body weight and adiposity. Therefore, it signals to the brain the need for energy intake necessary to maintain the stability of body weight. It inhibits food intake and stimulates energy expenditure when it is injected either intraperitoneally (I.P.) or centrally. Its inhibitory action on food intake is thought to be mediated through the inhibition of the most potent orexigenic peptide found in the hypothalamus, neuropeptide Y (NPY) (3).

NPY is, however, not the sole peptide involved in the action of leptin for several reasons. First, leptin still inhibits food intake in the NPY knockout mice (4). Second, it does not modify acutely the NPY release in the paraventricular nucleus (5) where this peptide is active (6) and where leptin receptors have been detected (7). Several other hypothalamic peptides, including neurotensin, corticotropin-releasing hormone, cholecystokinin, pro-opiomelanocortin and glucagon-like peptide 1, may be the partners of leptin for regulating feeding behavior (8-15). All of these peptides inhibit food intake and their synergy of action with leptin has been shown.

Recently, a new peptide family named orexins (OX) has been discovered (15, 16). Two peptides have been identified, orexin A and orexin B. Both orexin A and B stimulate food intake when they are injected in brain ventricles (15) but there is a controversy on the orexigenic effect of orexin B (17). They are synthesized in a distinct population of neurons of the lateral hypothalamus (15, 16, 18-20). These neurons project their efferences to multiple areas involved in the regulation of feeding behavior including the arcuate, paraventricular and ventromedian nuclei (19, 21). Two types of receptors OX_1-R and OX_2-R have been detected with a predominance of OX_1-R in the ventromedian nucleus and of OX_2-R in the paraventricular nucleus (22).

The presence of leptin receptors in these areas (7) as well as that of NPY in terminals surrounding and in close relationship with orexin neurons in the lateral hypothalamus (18) let us hypothesize that leptin biological action might be also linked to this new class of orexigenic peptides through an arcuate nucleus-lateral hypothalamus pathway. That is why we decided to determine the effects of chronic leptin injections on orexin A content in the lateral hypothalamus. We completed our study by measuring NPY in the same conditions.

MATERIALS AND METHODS

Protocol. Male Long-Evans rats (225-250 g) were obtained from Centre d'Elevage R. Janvier (Le Genest St Isle, France). They were placed in individual wire cages in an air-conditioned room with a 12 h light/12 h dark cycle (lights on at 7 AM). They were fed on a

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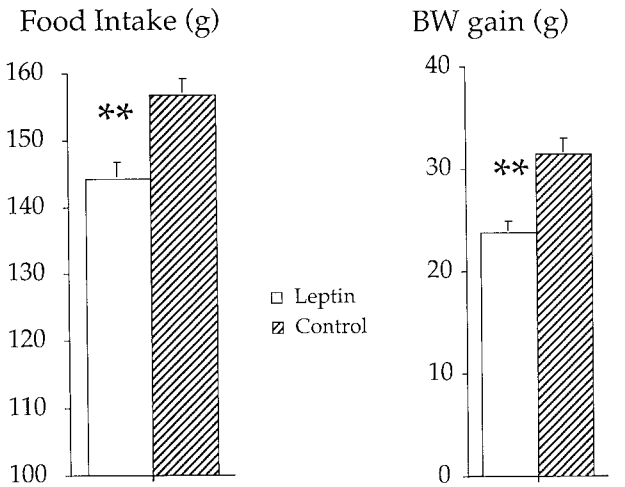

FIG. 1. Cumulated food intake and body weight gain (mean ± S.E.M.) of Long-Evans rats either treated with leptin (n = 12) or saline (n = 12) for 7 consecutive days. **: $p < 0.01$.

standard lab chow (A04, UAR, Villemoisson sur Orge, France) ad libitum with tap water to drink.

After a week of adaptation to these conditions, they were injected either with leptin (n = 12) or saline (n = 12) for 7 consecutive days. Recombinant murine leptin kindly provided by Dr F. Cumin (Novartis, Basel, Switzerland) was injected at the dose of 0.2 mg/rat/day. Injections were done I.P. during the half hour that preceded the dark phase. Twelve additional rats injected with saline were pairfed to the level of the leptin-treated rats. Body weight and food intake were measured during the experimental period.

At the end of the experiment, the rats were killed by decapitation three hours after the beginning of the light phase that followed the last injection. Food was withdrawn at the dark/light transition. The brains were sampled and stored at −80°C until processed for peptide content determination.

Peptide determinations. Several hypothalamic and extra-hypothalamic areas were microdissected as previously described (23). The following areas were sampled: the paraventricular (PVN), arcuate (ARC), suprachiasmatic (SCN), dorsomedian (DMN) and ventromedian (VMN) nuclei, the lateral hypothalamus (LH) and the nucleus accumbens (ACC). Parietal cortex (CX) served as control area. Bilateral tissue samples were immediately placed in 500 μl cold extraction solution (HCl 0.2N Iniprol/EDTA) and stored −80°C until extraction and assay.

Orexin A was measured in the lateral hypothalamus with a specific radioimmunoassay developed in our laboratory. Orexin A antibodies (kindly given by Dr Stricker-Krongrad, Millenium Pharmaceuticals, Cambridge, USA) were produced in rabbits and fully (100%) cross-react with human, mouse and rat orexin A. They do not cross-react at all with orexin A (16-33)-amide, human orexin B, human agouti-related protein (83-132)-amide, α-MSH or leptin.

Standard orexin A or lyophilized unknown sample were reconstituted with assay buffer: 0.04 M phosphate buffer pH 7.4 containing bovine serum albumin (fraction V, Sigma Chemicals, La Verpillière, France), aprotinin (4000 IU/ml, Antagosan, Laboratoires Hoechst, Paris) and sodium azide (Merck, Darmstadt).

One hundred microliters of antiserum diluted in assay buffer and one hundred microliters of standard or unknown sample were preincubated for 24 hours at 4°C. Then, one hundred microliters of ^{125}I-labeled orexin A (10 000-12000 CPM; specific activity 5400Ci/mmol) were added and incubated for a further 24 hours. Bound and free fraction were separated by the addition of five hundred microliters of a solution of 2% charcoal (Norit A, Kodak, Rochester, NY) and 0.2% Dextran (T70, Pharmacia, Uppsala, Sweden) in assay buffer. Bound fraction was measured in a gamma counter coupled to a microcomputer (MDA 312 system, Kontron, Velizy, France) for the plotting of the standard curve and the calculation of the results. In these conditions, maximal binding was 49.9 ± 4.7%. A 50% decrease of the bound activity (IC50) was obtained with a concentration of 4.76 ± 0.34 ng/ml orexin A. Non specific binding varied between 8 and 11%.

NPY was also measured by RIA as previously published (23).

Statistics. Results are given as mean ± SEM. They were compared by two-tailed unpaired Student's t test or variance analysis followed by PLSD Fisher test when necessary. A probability of less than 5% was considered significant.

RESULTS

Food intake and body weight changes are shown in Fig. 1. Repeated leptin injections induces a significant decrease in food intake which was evident already after the first injection. Over the week, leptin-treated rats ate about 8% less food than the saline-treated rats ($p < 0.002$). They also gained less weight than the saline-treated rats (23.7 ± 1 vs 31.5 ± 1.3 g; $p < 0.003$).

NPY concentrations in the different hypothalamic and extra-hypothalamic nuclei are shown in Fig. 2. NPY concentrations did not change significantly in any of these areas.

Orexin A concentrations in the lateral hypothalamus is shown in Fig. 3. There was a significant effect of treatment ($p < 0.025$). Orexin A in the control rats was significantly higher than in pair-fed rats (+117%; $p = 0.033$) and than in leptin-treated rats (+215%; $p < 0.01$).

DISCUSSION

In the present experiment, we tried to further characterize the links that exist between periphery and the central nervous system for the regulation of energy homeostasis and body weight. Leptin was chosen as a

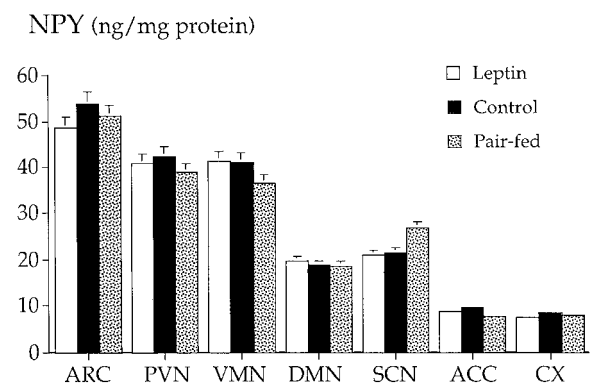

FIG. 2. Neuropeptide Y (NPY) concentrations (mean ± S.E.M.) in different brain areas of Long-Evans rats either treated with leptin (n = 12) or saline (n = 12) and in saline-treated pairfed rats (n = 12). PVN, paraventricular nucleus; ARC, arcuate nucleus; SCN, suprachiasmatic nucleus; DMN, dorsomedian nucleus; VMN, ventromedian nucleus; ACC, nucleus accumbens; CX, parietal cortex.

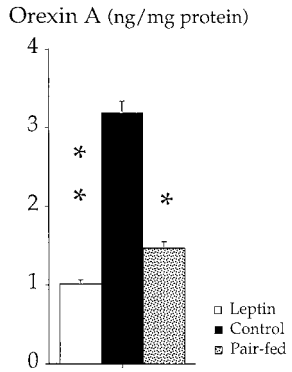

FIG. 3. Orexin A concentrations (mean ± S.E.M.) in the lateral hypothalamus of Long-Evans rats either treated with leptin (n = 12) or saline (n = 12) and in saline-treated pairfed rats (n = 12). *: $p < 0.04$; **: $p < 0.01$.

major signal produced by the adipose tissue to inform the brain about the energy stores (2). We measured its influence on two orexigenic peptides, NPY and hypocretin/orexin when it was injected peripherally for 7 consecutive days. Its relationship with NPY, the most potent stimulator of food intake has been largely studied (3). However, it is not fully understood with hypocretin/orexin, a new class of orexigenic peptides found in the lateral hypothalamus (15, 16). Neuroanatomical observations can partly answer to this question. There are now a small number of papers that show that the hypocretin/orexin system is linked to the leptin-NPY system in the hypothalamus (18-22, 24). Hypocretin/orexin neurons are indeed innervated by NPY boutons (19, 24). On the other hand, hypocretin/orexin neurons project to the NPY neurons in the arcuate nucleus (18, 21) which themselves contain leptin receptors (7). Hypocretin/orexin fibers are also present in the paraventricular nucleus where orexin receptors have been detected (22) and where ICV orexin injections induce c-fos expression (21). The paraventricular nuclei and arcuate nuclei form the main pathway for the regulation of food intake by NPY as shown by several experiments including hypothalamic injections, diet manipulations and release measurements (6, 25-28).

As expected, chronic leptin injection induced a slight diminution (8%) of food intake as well as a lowering of the body weight gain. The doses used were not large enough to induce a body weight loss. They were however sufficient to induce a large decrease (68%) in hypothalamic orexin. To our knowledge, this paper is the first to measure hypocretin/orexin content and variations in the rat lateral hypothalamus. Orexin/hypocretin could therefore be considered as a new relay for leptin in the central nervous system. This might be possible through the presence of the long form of the leptin receptor in the lateral hypothalamus (29, 30). Its relationship with leptin is probably complex because as for leptin (2), it plays a role in energy control (17) and likely in other metabolic pathways as indicated by the wide dissemination of its fibers in the brain (19, 21).

The orexin decrease was however not entirely due to the leptin effect per se as a smaller (46%) diminution was observed in the pairfed animals. A part of the hypocretin/orexin decrease was then secondary to the decreased food intake and therefore possibly related to the activation of other regulatory factors. This decreased food intake was however not sufficient to induce any change in NPY concentrations whatever the brain area considered. This is likely related also to the absence of weight loss in the leptin-treated rats. A large food restriction or inhibition with pronounced effects on body weight as in fasting conditions (25, 26) is probably necessary to activate the NPY system. To explain the variations of hypocretin/orexin levels in these conditions, we hypothesized that this decrease might reflect an increased transport and liberation at targets outside the lateral hypothalamus. It could constitute an alerting system in case of lower energy supply and therefore considered as the first step in the establishment of defense mechanisms against energy depletion. To support this hypothesis, measurements of hypocretin/orexin mRNA expression by in situ hybridization as well as its release by push-pull perfusion are actually planed in our laboratory.

The addition of this peptide to the long list of neuropeptides in relation with peripheral factors such as leptin and insulin would still complicate the understanding of the regulation of feeding behavior but might also shed new lights to unravel these complex mechanisms (31).

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