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Modulation of hypothalamic hypocretin/orexin mRNA expression by glucocorticoids

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Abstract

The orexins are peptides which were recently isolated from the rat hypothlamus. They play a role in energy homeostasis and regulation of feeding as well as in other functions such as the sleep–wake cycle. The involvement of glucocorticoids in stress processes as well as in body weight regulation is well known. In the present paper, we investigated the role of glucocorticoids on hypocretin (Hcrt)/orexin (OX) pathway in Sprague–Dawley rats. We confirmed by in situ hybridization that prepro-Hcrt/OX mRNA expression is restricted to the lateral hypothalamus area with extension to the perifornical nucleus and the posterior hypothalamic area. Lateral hypothalamic prepro-Hcrt/OX mRNA expression was decreased by 50% after adrenalectomy (99.8 ± 5.0 vs 49.2 ± 4.4 nCi/g, $p < 0.01$). Peripheral glucocorticoid treatment (dexamethasone) restored its expression to normal levels (105.4 ± 6.1 nCi/g). The present data provide direct evidence that Hcrt/OX expression in the lateral hypothalamus is modulated by the glucocorticoids status. As the Hcrt/Ox system is closely interactive with the corticotropin-releasing hormone and neuropeptide Y systems, we propose that hypocretin/orexins peptides constitute a very sensitive key relay for mediating both stress and feeding behavior.

Keywords: Neuropeptides; Dexamethasone; Pituitary; Adrenalectomy; Stress

The precursor of hypocretin (Hcrt)/orexin (OX) peptides is encoded by a mRNA restricted to neuronal cell bodies of the lateral hypothalamus (LH) [1,2]. There is some evidence that Hcrt/OX peptides are involved in the regulation of food intake [3–6] and energy metabolism [3,7–9]. However, the broad distribution of Hcrt/OX peptides immunoreactivity [10–14] in the rodent brain indicates that Hcrt/OX peptides are implicated in the central regulation of other functions.

One candidate function is stress. It therefore implicates the activation of the hypothalamic-pituitary-adrenal axis (HPA) which is also observed in the development of obesity in rodents [15–21]. Recent evidence suggests that orexins stimulate corticosterone release when injected in the brain ventricles [22], suggesting a role in the activation of the HPA. This hypothesis is supported by the fact that neuropeptide Y and corticotropin-releasing-hormone, two peptides also involved in the regulation of feeding and in the stimulation of corticosterone release, are closely interacting with Hcrt/OX effects on food intake [23]. For these reasons, we hypothesized that in addition to stress, Hcrt/OX peptides might participate in the neuro-endocrine disturbances associated with obesity and we investigated the regulation of Hcrt/OX pathway by glucocorticoids in rats.

Materials and methods

Sham and bilaterally adrenalectomized (ADX) male Sprague–Dawley rats (body weight 250 g, IFFA CREDO, France) were individually housed in plastic cages with a stainless steel cover in a temperature-controlled room (22 ± 2 °C) on a 12:12 h light/dark cycle (light on at 06h00). They were allowed ad libitum access to tap water supplemented with 9 g/L NaCl and standard rat laboratory chow diet. Three experimental groups were made ($n = 8$ per group), a sham-operated control group treated with vehicle, an adrenalectomized group treated with vehicle (ADX) and an adrenalectomized group

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treated with dexamethasone (DEX) at a dose of 20 μg/kg/day injected subcutaneously for 5 days. Rats were anaesthetized in the middle of the light cycle by carbon dioxide inhalation and killed by decapitation. The brain was quickly removed and frozen with powdered dry ice. Cryostat sections were cut at 12 μm thickness through the hypothalamus region, mounted on Superfrost-Plus slides and stored at −80 °C. Riboprobes for in situ hybridization were prepared by polymerase-chain reaction (PCR) amplification of a rat brain cDNA library (Clontech). PCR primers were selected from the prepro-Hcrt/OX sequence (GenBank Accession No. V18866) (5′-AGGGATGG TGATG ATGA-3′ (upper) primer sequence; 5′-TTAACCCTCACTAAAGG G-3′ (lower) primer sequence). Products from the PCR were sequenced. We used a 500-bases riboprobe recognizing 87% of the encoding region of the entire prepro-Hcrt/OX mRNA. Antisense and sense riboprobes were generated by in vitro transcription using T7 and T3 RNA polymerases. Sections were air dried for 20 min and then incubated with ice cold 4% PFA (paraformaldehyde)/1× PBS (phosphate-buffered saline) for 10 min. Slides were washed with 1× PBS twice 5 min each, incubated with 0.25% acetic anhydride/1 M triethanolamine for 10 min, washed with PBS for 5 min and dehydrated with 70%, 80%, 95%, and 100% ethanol at 1 min each. Sections were incubated with chloroform for 5 min, rehydrated with 100% and 95% ethanol, then air dried. Hybridization was performed with ^{35}S-radiolabeled (5×10^7 cpm/ml) riboprobes in the presence of 50% formamide, 10% dextran sulfate, 1× Denhardt's solution, 600 mM NaCl, 10 mM DTT, 0.25% SDS, and 100 μg/ml tRNA for 18 h at 55 °C. After hybridization, slides were washed with 10 mM Tris–HCl (pH 7.6)/500 mM NaCl/1 mM EDTA (TNE) for 10 min, incubated in 40 μg/ml RNase A in TNE at 37 °C for 30 min, washed in TNE for 10 min, incubated once in 2× SSC (saline sodium citrate) at 60 °C for 30 min, once in 0.1× SSC at 65 °C for 30 min, 0.1× SSC at 65 °C for 30 min, and dehydrated with 50%, 70%, 80%, 95%, and 100% ethanol. Controls for the in situ hybridization experiments included the use of a sense probe that showed no signal above background levels. For each animal, four to six full coronal sections were used from the region of the hypothalamus that includes the lateral hypothalamus. All brain slices were concurrently prepared for hybridization and autoradiographed on film. Film autoradiographs were digitized and hybridization signals were determined by quantitative densitometry using radioactive microscales and the Ultimage/Pro image analysis software (Graftek, Mirmande, France).

Results

Fig. 1 shows the central distribution of prepro-Hcrt/OX mRNA using in situ hybridization in rat brain coronal section. In control rat, a strong hybridization signal was observed in the lateral hypothalamus with extension into the perifornical nucleus and the posterior hypothalamic area. In ADX rats, the signal persisted in the lateral hypothalamus, but less intense, with fewer hybridization signals in the perifornical nucleus and the posterior hypothalamic area. Almost all hybridization signals were restored in the ADX rats treated with DEX. In all cases no hybridization signals were observed in the dorsomedian and ventromedian nuclei or the arcuate nucleus or outside the hypothalamus.

Quantification of the hybridization signal indicated a 50% decrease ($p < 0.01$) of prepro-Hcrt/OX mRNA in the ADX rat when compared to the control rat (Fig. 2). Glucocorticoids therapy restored normal levels of prepro-Hcrt/OX mRNA.

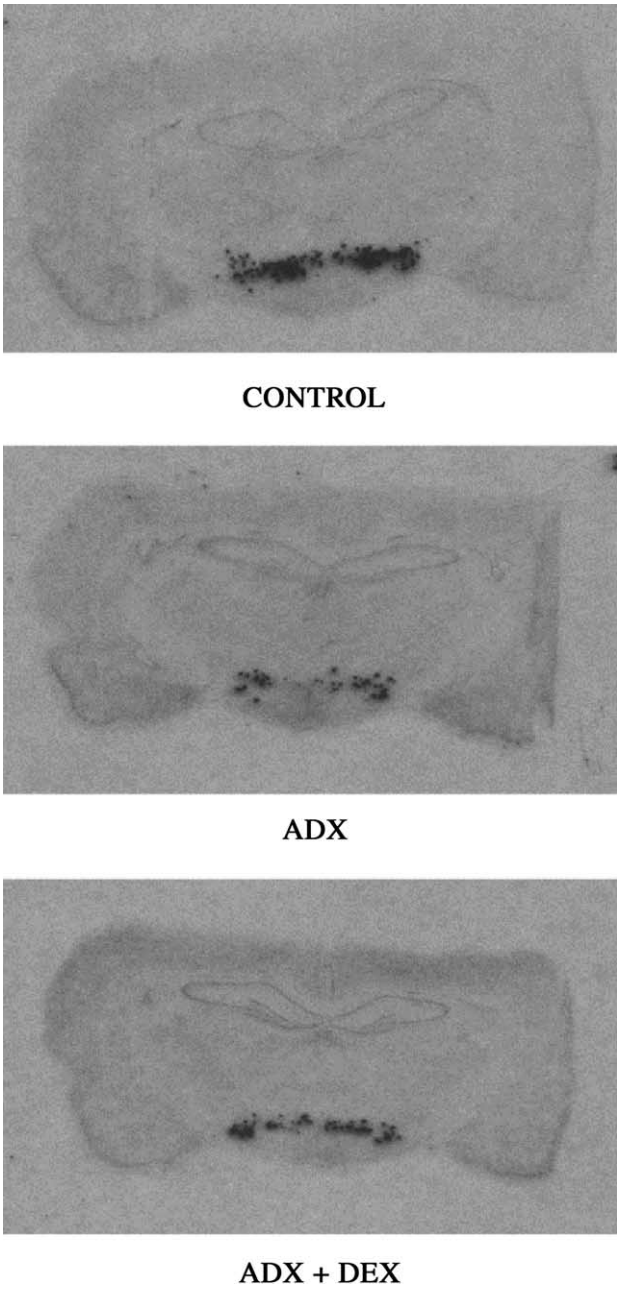

Fig. 1. Hypothalamic distribution of prepro-Hcrt/OX message in control, adrenalectomized (ADX), and dexamethasone-treated adrenalectomized (ADX + DEX) rats (12 μm brain coronal sections).

Discussion

In the present report, we investigated the regulation of Hcrt/OX pathway by glucocorticoids by measuring Hcrt/OX brain expression in the presence or absence of corticosterone. Firstly, we confirmed that prepro-Hcrt/OX mRNA expression is restricted to the lateral hypothalamus area with extension to the perifornical nucleus and the posterior hypothalamic area [1,2]. This pattern of expression is consistent with a role of Hcrt/OX peptides in the control of food intake [3–6] and body weight

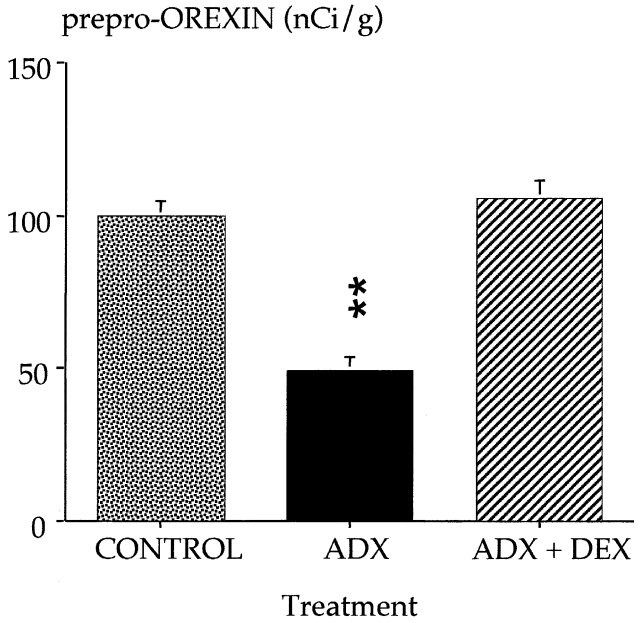

Fig. 2. Hypothalamic expression levels of prepro-Hcrt/OX message measured by quantitative in situ hybridization in control (CONTROL), adrenalectomized (ADX), and dexamethasone-treated adrenalectomized (ADX + DEX) rats (means ± SEM, $n = 8$, $^{**}p < 0.01$ vs control rat).

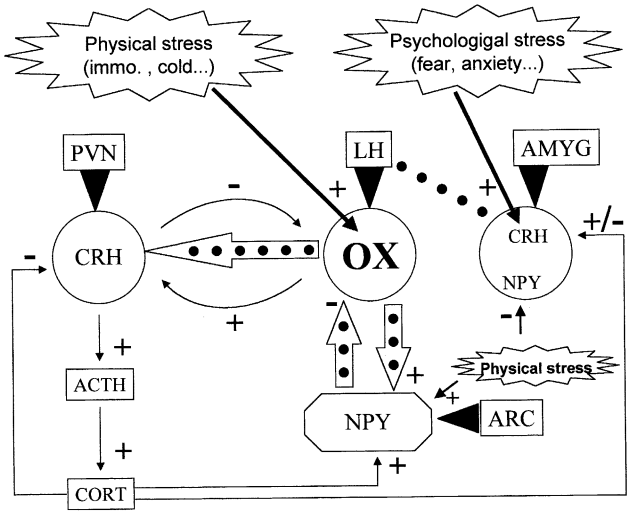

Fig. 3. Schematic representation of possible mechanisms mediating the physiologiacal effects of orexins (OX), neuropeptide Y (NPY), and corticotropin-releasing hormone (CRH) in feeding behavior and stress. Black points represent anatonomical connections between areas and/or neuronal populations. The arrows surrounding the points indicate the sense of projections (when known). ARC: arcuate nucleus; PVN: paraventricular nucleus; LH: lateral hypothalamus; AMYG: amygdala; ACTH: adrenocorticotropin hormone; CORT: corticosterone.

[7–9]. Secondly, we observed a marked decrease (−50%) of the prepro-Hcrt/OX message level in ADX rats, which was restored to normal levels by peripheral treatment with DEX. Orexins are therefore involved in the regulation of the HPA axis and could therefore play a role in body weight regulation as well as in stress.

Corticotropin-releasing-hormone (CRH) is the main hypothalamic peptide involved in the HPA axis. It is the most sensitive neuropeptide in sensing glucocorticoids status. Adrenalectomy induces and dexamethasone reduces the expression of CRH in the paraventricular nucleus [24–26], creating a feedback loop in the HPA axis. Besides its recognized action on pituitary ACTH release, CRH is important in mediating physiological responses to stress and induces inhibition of food intake [27]. It has some important relationships with the orexin system. Firstly on an anatomical basis, Hcrt/OX neurons located in the LH are sending projections to the paraventricular nucleus (PVN) [10,11,13,28,29]. CRH neurons in the amygdala are also sending projections to the lateral hypothalamus [30]. Secondly on a functional basis, blockade of CRH through an antagonist or an antiserum favors the orexigenic effects of orexin [23]. When injected in the brain ventricles, orexin induces ACTH [31] and corticosterone release [22,31,32]. This effect is observed after central injections only [31]. According to this information and our present results, we propose that Hcrt/OX in the LH and CRH in the PVN might be linked in a regulatory loop (cf. Fig. 3). Increase in CRH in the PVN due to the lack of retro-control through glucocorticoids in ADX would decrease Hcrt/OX expression in the LH whereas establishment of normal glucocorticoid status through DEX treatment and consequently the decrease of CRH expression would stimulate Hcrt/OX expression. On the other hand, an augmentation of Hcrt/OX levels as that induced by ICV injection would stimulate CRH expression in the PVN which in turn will activate the HPA axis. This is supported by recent data indicating that physical stressors such as immobilization or cold which stimulates PVN CRH neurons and activates the HPA axis also induces increase prepro-OX mRNA levels in rats [22,33,34]. However, stress is not only mediated by the PVN. Other brain areas such as the amygdala and the bed nucleus of the stria terminals are involved [35]. The former area appears to mediate more likely psychological stress. Glucocorticoids do no regulate CRH in the amygdala as a manner as in the PVN, as at high doses, they can upregulate CRH mRNA expression [37]. The efferent CRH projections from the amygdala to the LH have not been characterized in terms of type of neuronal populations. Therefore, the interaction of the Hcrt/OX with the amygdala in relation with stressful events remains to be determined.

Hcrt/OX neurons located in the LH are also sending projections to the arcuate nuclei [10,11,13,28,29] where they make direct synaptic contact with neuropeptide Y (NPY) neurons [16]. On the other hand, NPY exists in nerve terminals in close relationship to Hcrt/OX perikarya [9,38]. This indicates a functional relationship

between Hcrt/OX and NPY. This is confirmed by the ability of some NPY receptor antagonists to suppress the orexin-induced food intake [4,39,40]. In addition, NPY directly interacts with CRH in the PVN through the projections of ARC NPY neurons to CRH neurons [28,42] and through its presence in the amygdala. Once again, there are discrepancies in the regulation of NPY expression by stressors as restraint stress diminish it in the amygdala and increase it in the ARC [43,44]. So, hypocretins/orexins, CRH and NPY form a complex interactive network. These peptides can modulate anxiety and feeding depending on the activated brain area [45–47].

Besides the intra-hypothalamic relationship between the three peptides, each peptide might be differently sensitive to peripheral factors. It is well established that NPY brain levels are sensitive to the glucocorticoid status and accordingly its effects on food intake and body weight are dependent of glucocorticoids [48]. Adrenalectomy induces either a decrease [49] or no change in NPY peptide or expression in the ARC [50,51]. However, it seems that this might be more related to the concomittant and ADX-associated changes in insulin levels rather than to the decrease in corticosterone levels [43]. On the other hand, chronic administration of glucocorticoids directly upregulates prepro-NPY mRNA levels in the arcuate nucleus of the rat [52] and increases hypothalamic NPY secretion in mice [19]. Moreover, acute DEX treatment induces a large augmentation of NPY directly in the LH [53]. It is therefore possible that the high NPY levels in the ARC down-regulate Hcrt/OX in the LH to diminish the CRH tone and the HPA activation (cf. Fig. 3). So, NPY might influence PVN CRH either positively through a direct way with the projections of the ARC NPY neurons to the CRH PVN neurons or negatively and indirectly through the hypocretin/orexin system. The activation of the HPA will result from the sum of these opposite actions. The opposite status of NPY and Hcrt/OX in genetic models of obesity (Zucker rat, ob/ob mouse) where expressions of the 2 peptides are, respectively, up- and down-regulated, [7,9,38] support this hypothesis. The decrease in the Hcrt/OX tone is, however, not sufficient to normalize the HPA axis in these obese animals. Additional studies such as the determination of the Hcrt/OX status in NPY and CRH knockout mice might help to better understand the NPY–CRH–orexin relationships.

In conclusion, our findings provide direct evidence that hypocretin/orexin peptides constitute a very sensitive key relay for mediating both stress and feeding behavior. Other factors either peripheral such as leptin [54] or central such as pro-opio-melanocortins [55] influence also the hypocretin/orexin system making possible to finely tune metabolic and environmental changes.

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