Histol Histopathol (2001) 16: 713-717

http://www.ehu.es/histol-histopathol

Histology and Histopathology

Cellular and Molecular Biology

Effects of orexins A and B on the secretory and proliferative activity of immature and regenerating rat adrenal glands

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Summary. Orexins A and B are two hypothalamic peptides, involved in the central regulation of feeding, which act through two receptor subtypes, named OX_1R and OX_2R. OX_{1R} is selective for orexin-A, and OX_2R binds both orexins. We have investigated the effects of three subcutaneous injections of 10 nmol/kg body weight of orexins on the secretion and proliferative activity of immature (20-day-old) and regenerating rat adrenal cortex. The presence of both OX_1R and OX_2R mRNAs has been detected by reverse transcription-polymerase chain reaction in adult, immature and regenerating adrenals. Orexin-A increased corticosterone plasma concentration in immature rats, but not in animals with regenerating adrenals. Both orexins raised metaphase index (‰ of metaphase-arrested cells) in immature rat adrenals, orexin-B being more effective than orexin-A. In contrast, both orexins equipotently lowered adrenal metaphase index at day 5 (but not day 8) of adrenal regeneration. We conclude that orexins (1) stimulate secretion and proliferative activity of immature rat adrenals, acting through OX_1R and OX_2R, respectively; and (2) do not affect secretion, but inhibit proliferative activity of regenerating adrenals, mainly via the activation of OX_2R.

Key words: Orexins, Orexin receptors, Steroid secretion, Cell proliferation, Adrenal cortex, Adrenal regeneration

Introduction

Orexins A and B are two hypothalamic peptides, which originate from the post-translational proteolytic cleavage of a common precursor, called prepro-orexin. Orexins act through two subtypes of G protein-coupled receptors, named OX_1R and OX_2R. OX_1R is selective for orexin-A, while OX2R is non-selective for both orexins (for review, see De Lecea et al., 1998; Sakurai et al., 1998).

Hypothalamic prepro-orexin mRNA was found to increase after 48 h of fasting (Sakurai et al., 1998), and orexins were shown to be mainly produced in neurons located in the hypothalamic areas implicated in the control of food intake (Bernardis and Bellinger, 1996; van den Pol et al., 1998). Hence, orexins have been included in that group of neuropeptides, such as neuropeptide Y (NPY) and leptin, involved in the central regulation of feeding and energy homeostasis (Rohner-Jeanrenaud et a., 1996; Kalra, 1997; Elias et al., 1998; Spinedi and Gaillard, 1998; Wolf, 1998). This contention is also supported by findings indicating that orexin-immunoreactive nerve terminals originating from the lateral hypothalamus make synaptic contact with neurons of the arcuate nucleus, which not only express NPY, but are also provided with leptin receptors (Horvath et al., 1999).

There is evidence that NPY, leptin and orexins control the function of the pituitary-adrenocortical axis in the rat (Bornstein et al., 1997; Malendowicz et al., 1997,1998, 1999b; Nussdorfer and Gottardo, 1998). Since NPY and leptin were also found to enhance adrenocortical growth (Nussdorfer and Gottardo, 1998; Malendowicz et al., 1999a), we have investigated the effect of orexins A and B on the secretory and proliferative activity of immature and regenerating rat adrenal cortex.

Materials and methods

Reagents and animals

Orexins were purchased from Peninsula Laboratories (St. Helene, UK), vincristin from Gedeon-Richter (Budapest, Hungary), and all other reagents from Sigma Chemical Co. (St. Louis, MO, USA).

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Adult (2-month-old) female Wistar rats and their offspring (20-day-old), bred in our laboratory, were kept under a 12:12 h light-dark cycle (illumination onset at 8.00 a.m.) at 23 °C, and were maintained on a standard diet with tap water *ad libitum*. Under ether anaesthesia, the left adrenal gland of adult rats was enucleated, and the contralateral gland was removed. All adult female rats were in diestrus phase, as determined by cytologic evaluation of vaginal epithelium. Operated rats were given 0.9% NaCl to drink, and were sacrificed 5 or 8 days after surgery.

Reverse transcription (RT)-polymerase chain reaction (PCR)

Adult, immature and regenerating adrenals were collected, and adult and immature glands were enucleated to eliminate adrenal medulla. RNA was extracted with the guanidium isothiocyanate method, and total RNA was reversed transcribed to cDNA, as detailed earlier (Mazzocchi et al., 2001). The 5' and 3' primers used for OX_1R and OX_2R mRNAs were selected according to Lopez et al. (1999). In a Delphi 100 Thermal Cycler (Oracle Biosystem, MJ Research Inc., Waterston, MA, USA), we used a denaturation step at 95 °C for 1 min, an annealing step at 57 °C for 1 min, and an extension step at 72 °C for 1 min for a total of 38 cycles. An additional extension step at 72 °C for 7 min was then carried out (Mazzocchi et al., 2001). The sizes of the amplification products were: OX1R, 328 bp; and OX2R 226 bp. To rule out the possibility of amplifying genomic DNA, in some experiment PCR was performed without prior RT of the RNA. Detection of the PCR amplification products was first carried out by size fractionation on 2% agarose-gel electrophoresis. After purification using the QIA quick PCR purification kit (Qiagen, Hilden, Germany), PCR products were identified by sequencing on an Alf sequencer (Pharmacia Biotech, Freiburg, Germany).

Animal treatment

Groups of adult rats with adrenal regeneration, and immature animals (n=12) were given three subcutaneous injections (28, 16 and 4 h before being sacrificed) of 0.2 ml 0.9% NaCl where 10 nmol/kg body weight of orexin-A or orexin-B were disolved. Control rats were injected with the saline vehicle. Six animals in each group received an intraperitoneal injection of 0.1 mg/100 g vincristine, 180 min before the autopsy. Rats were decapitated at 11.00 a.m., and their trunk blood was collected in the presence of EDTA (1 mg/ml). Plasma was separated and stored at -30 °C. Adrenal glands from immature rats and regenerating adrenals were promptly removed.

Corticosterone assay

Corticosterone was extracted from plasma and purified by HPLC (Neri et al., 1993), and its concentration was measured by radioimmune assay, as previously described (Malendowicz et al., 1996). Intra- and interassay variation coefficients were 7.1% and 9.0%, respectively.

Cell proliferation

Adrenals were fixed in Bouin's solution for 24 h, embedded in paraffin and sectioned at 5-6 μm. Sections were stained with hematoxylin and eosin. The metaphase index (‰ of metaphase-arrested cells) was calculated at 400, by counting 5,000 cells in the subcapsular zona glomerulosa or zona fasciculata of immature adrenals, and in the regenerating adrenal parenchyma.

Statistics

Data were expressed as means SEM (n = 6). The statistical comparison was realized by ANOVA, followed by the Multiple Range Test of Duncan. p values < 0.05 were considered significant.

Results

RT-PCR demonstrated the presence of sizeable levels of OX_1R and OX_2R mRNAs in adult, immature and regenerating rat adrenal cortex, the expression of both receptors being rather weak in the glands at day 5 of regeneration (Fig. 1).

Orexin-A, but not orexin-B, increased (by about 45%) corticosterone plasma concentration in immature rats (Fig. 2, upper panel). Corticosterone blood level was higher (about 60%) at day 8 than at day 5 of adrenal regeneration, and neither orexin-A nor orexin-B affected it (Fig. 2, lower panels). Similar resutls were obtained when rats were sacrificed 2 h after the last injection of

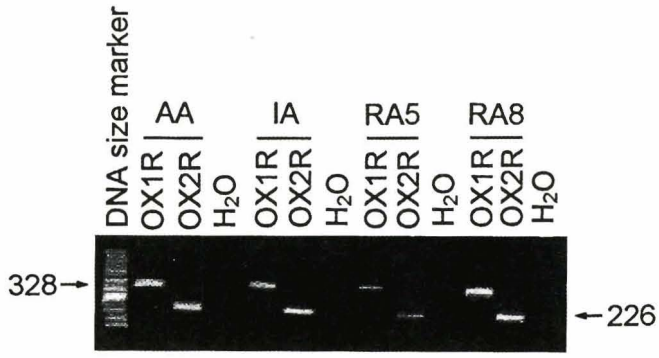

Fig. 1. Ethidium bromide-stained 2% agarose gel showing cDNA amplified with rat OX1R- and OX2R-specific primers from RNA of adult (AA) and immature adrenal cortex (IA), and adrenocortical tissue at day 5 (RA5) and 8 of regeneration (RA8). Lane 1 was loaded with 200 ng of a size marker (Marker VIII, Roche Molecular Biochemicals). The amplified fragments were of the expected size, which was 328 bp for OX1R and 226 bp for OX2R. No amplification with water instead of RNA, as a negative control, is shown in lanes 4, 7 and 10.

orexins, instead of 4 h (data not shown).

Both orexins significantly raised metaphase index in the zona glomerulosa of immature rat adrenals, the effect of orexin-B being more intense than that of orexin-A (2.7-fold versus 1.5-fold increase) (Fig. 3, upper panel). The metaphase index of the zona fasciculata was negligible and unaffected by orexins (data not shown). The metaphase index was markedly higher (about 2-fold) at day 5 than at day 8 of adrenal regeneration. Both orexins equipotently evoked a slight decrease (about 25%) in the metaphase index at day 5, but not at day 8 of regeneration (Fig. 3, lower panels).

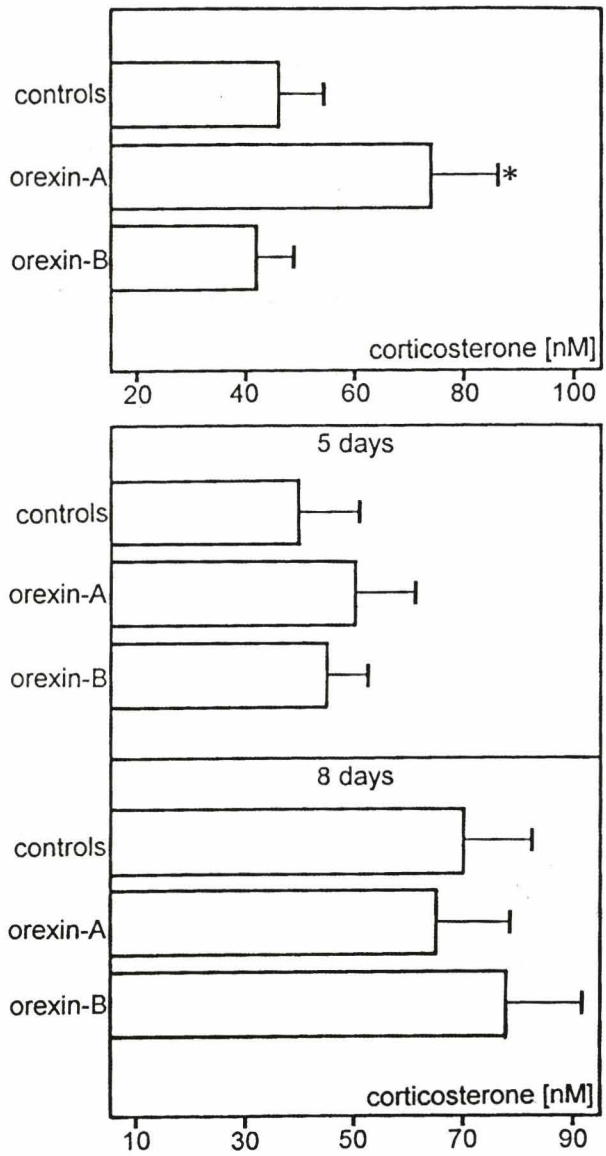

Fig. 2. Effects of orexins on plasma corticosterone concentration in immature rats (upper panel) and rats with regenerating adrenal cortex (lower panels). Bars are means SEM (n = 6). *: p<0.01 from the respective control group.

Discussion

Previous studies have shown that both OX_1R and OX_2R are expressed in rat adrenal medulla (Lopez et al., 1999), and human adrenal cortex and medulla (Mazzocchi et al., 2001). Our RT-PCR findings confirm the presence of the specific mRNAs for both receptor subtypes in adult, immature and regenerating rat adrenal glands, thereby making likely the possibility that the herein described effects of orexins may be due, at least in part, to their direct action on adrenocortical cells.

Evidence has been provided that the *bolus* administration of orexin-A, but not orexin-B, enhances the level of circulating corticosterone in adult rats, by

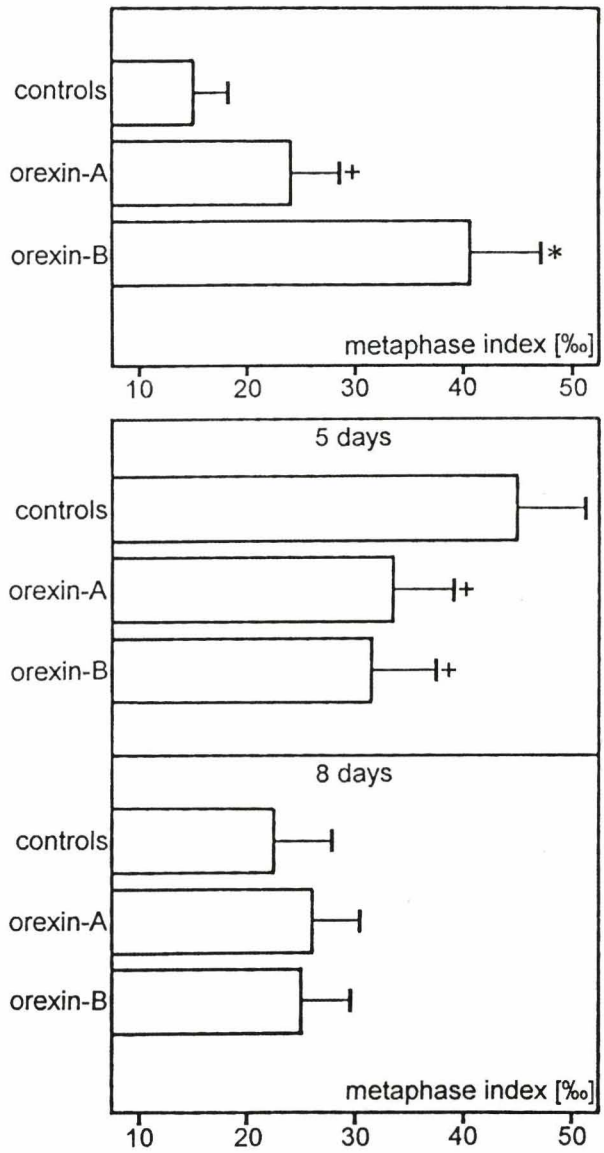

Fig. 3. Effects of orexins on the mitotic index of immature (upper panel) and regenerating rat adrenal cortex (lower panels). Bars are means SEM (n = 6). +: p<0.05 and *: p<0.01, from the respective control value.

stimulating pituitary ACTH release and by acting directly on adrenocortical cells (Malendowicz et al., 1999b). Our present findings confirm that this also occurs in immature rats. These authors also showed that orexin-B is much more potent than orexin-A in enhancing proliferative activity of adrenal zona glomerulosa, the "cambium" layer involved in the adrenal enlargement during maturation (for review, see Nussdorfer, 1986).

Orexin-A has high affinity for both OX_1R and OX_2R, while orexin-B possesses a 10-fold higher affinity for OX_2R than OX_1R (De Lecea et al., 1998; Sakurai et al., 1998). On these grounds, it may be concluded that in immature rat adrenals, the secretagogue effect of orexins is exclusively mediated by OX_1R, while the proliferogenic action mainly involves the activation of OX_2R.

Completely different results have been obtained in rats with regenerating rat adrenals, where orexins do not affect corticosterone secretion, but lower adrenal proliferative activity. The lack of any secretagogue action of orexins may reflect a maximal stimulation of the regenerating tissue. In fact, the low levels of circulating glucocorticoids, ensuing from the reduced mass of regenerating adrenocortical tissue, conceivably release hypothalamo-pituitary axis from the negative feedback regulating ACTH release (Nussdorfer, 1986). It is to be noted that the antiproliferogenic action of orexins manifests itself only at day 5 of regeneration, when dedifferentiated remanant subcapsular cells display a high rate of mitotic activity coupled with a low rate of steroid secretion (Taki and Nickerson, 1985; Nussdorfer, 1986). No histological signs of cell necrosis of apoptosis were observed, which seems to exclude that the effect was due to a cytotoxic action of orexins at this earlier stage of regeneration. Orexins A and B are equipotenent in their antiproliferogenic action, thereby suggesting the involvement of OX_2R.

Hence, our study seems to suggest that OX2R activation mediates opposite effects on the proliferative activity of immature and regenerating rat adrenals. This puzzling finding may be tentatively explained by taking into account that these are two completely different models of adrenocortical growth. Immature adrenals undergo an almost exclusively ACTH-dependent growth, while adrenal regeneration is only partially dependent upon pituitary ACTH release; regenerating cells resembling the actively proliferating poorly differentiated cells forming adrenal cortex during embryogenesis (for review, see Engeland et al., 1996; Engeland and Levay-Young, 1999). In light of these considerations, it could be hypothesized that the orexin-induced activation of OX_2R, which is the subtype mainly expressed in the hypothalamic nucleus paraventricularis (Trivedi et al., 1998), although leading to the increase in the release of ACTH by pituitary corticotropes, may inhibit the neural pathways mediating adrenal growth during regeneration (Dallman, 1985). The balance of these two opposite actions could result in the depression of the proliferative activity of regenerating adrenals. An alternative explanation could stem from the recent finding that orexins suppress catecholamine synthesis and secretion from cultured PC12 cells probably via receptors distinct from the classic OXR1 and OXR2 (Nanmoku et al., 2000). On these grounds, the possibility that orexins exert their antiproliferogenic effect on regenerating adrenals acting through this novel receptor subtype cannot be excluded and await to be investigated.

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Accepted March 14, 2001