

Orexins in the Regulation of the Hypothalamic-Pituitary-Adrenal Axis

RAFFAELLA SPINAZZI, PAOLA G. ANDREIS, GIAN PAOLO ROSSI, AND GASTONE G. NUSSDORFER

Departments of Human Anatomy and Physiology (R.S., P.G.A., G.G.N.) and Clinical and Experimental Medicine (G.P.R.), School of Medicine, University of Padua, Padua, Italy

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Address correspondence to: Dr. Gastone G. Nussdorfer, Department of Human Anatomy and Physiology, Section of Anatomy, Via Gabelli 65, I-35121 Padua, Italy. E-mail: gastone.nussdorfer@unipd.it

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Abstract—Orexin-A and orexin-B are hypothalamic peptides that act via two G protein-coupled receptors, named orexin type 1 and type 2 receptors (OX1-Rs and OX2-Rs). The most studied biological functions of orexins are the central control of feeding and sleep, but in the past few years findings that orexin system modulates the hypothalamic-pituitary-adrenal (HPA) axis, acting on both its central and peripheral branches, have accumulated. Orexins and their receptors are expressed in the hypothalamic paraventricular nucleus and median eminence and orexin receptors in pituitary corticotropes, adrenal cortex, and medulla. Whereas the effects of orexins on adrenal aldosterone secretion are doubtful, compelling evidence indicates that these peptides enhance glucocorticoid production in rats and humans. This effect involves a 2-fold mechanism: 1) stimu-

lation of the adrenocorticotropin-releasing hormone-mediated pituitary release of adrenocorticotropin, which in turn raises adrenal glucocorticoid secretion; and 2) direct stimulation of adrenocortical cells via OX1-Rs coupled to the adenylate cyclase-dependent cascade. The effects of orexins on catecholamine release from adrenal medulla are unclear and probably of minor relevance, but there are indications that orexins can stimulate *in vitro* secretion of human pheochromocytoma cells via OX2-Rs coupled to the phospholipase C-dependent cascade. Evidence is also available that orexins enhance the growth *in vitro* of adrenocortical cells, mainly acting via OX2-Rs. Moreover, findings suggest that the orexin system may favor HPA axis responses to stresses and play a role in the pathophysiology of cortisol-secreting adrenal adenomas.

I. Introduction

Orexins A and B are neuropeptides that were isolated from the rat hypothalamus by two independent groups of investigators in 1998 (De Lecea et al., 1998; Sakurai et al., 1998). They were originally named hypocretins because of their hypothalamic localization and their supposed similarity to secretin. Thereafter, their names were changed to orexins on the basis of their predominant expression in the hypothalamic feeding centers and their involvement in the control of food intake. Simultaneously with the discovery of orexins, two G protein-coupled receptors for these endogenous ligands have been identified by the same groups of investigators and called orexin type 1 and type 2 receptors (OX1-Rs¹ and OX2-Rs). Subsequent investigations showed that the orexin system also plays a role in the regulation of sleep/wakefulness, because orexin gene knockout mice and dogs with nonfunctional OX2-Rs because of a gene mutation display a narcolepsy-like condition (Chemelli et al., 1999; Lin et al., 1999), and narcoleptic humans lack orexin-containing neurons in the hypothalamus and orexins in the cerebrospinal fluid (Nishino et al., 2000; Peyron et al., 2000; Thannickal et al., 2000). Several excellent reviews (Meier, 1998; Wolf, 1998; De Lecea and Sutcliffe, 1999; Sakurai, 1999, 2005; Samson and Resch, 2000; Hungs and Mignot, 2001; Mignot, 2001; Siegel et al., 2001; Taheri and Bloom, 2001; Willie et al., 2001; Beuckmann and Yanagisawa, 2002; Kuk-

konen et al., 2002; Ferguson and Samson, 2003) on the involvement of the orexin system in the regulation of feeding and sleep are available.

Further studies suggested a possible role of the orexin system in the central stimulation of autonomic functions, including regulation of blood pressure and heart rate and neuroendocrine axes, as the hypothalamic-pituitary-adrenal (HPA) axis (reviewed by Samson and Resch, 2000; Taheri and Bloom, 2001; Kukkonen et al., 2002; Ferguson and Samson, 2003; Taylor and Samson, 2003; Jöhren et al., 2004). This last contention is of great interest, because compelling evidence has been accumulated that neuropeptides that are involved in the central regulation of feeding [e.g., leptin, neuropeptide Y (NPY), neuropeptide W, and cholecystokinin] also control the central branch of the HPA axis (Crawley and Corwin, 1994; Krysiak et al., 1999; Ahima and Flier, 2000; Watters et al., 2000; Baker et al., 2003; Malendowicz et al., 2003). Moreover, such peptides, acting via specific receptors, have been also found to exert direct regulatory control of the peripheral branch of the HPA axis (Bornstein et al., 1997; Glasow et al., 1998; Pralong et al., 1998; Glasow and Bornstein, 2000; Malendowicz et al., 2001c; Renshaw and Hinson, 2001; Hochól et al., 2004; Mazzocchi et al., 2004, 2005; Nussdorfer et al., 2005; Spinazzi et al., 2005a). Thus, after a brief account of the biology of orexin system, we will herein survey findings indicating that orexins, like the other peptides modulating food intake, are involved in the functional regulation of the adrenal gland under physiological and pathological conditions.

II. Biology of Orexins and Their Receptors

A. Orexin Biosynthesis

Orexins derive from the post-translational cleavage of a 130-131 amino acid precursor, the prepro (pp)-orexin, that displays an 83% amino acid identity between humans and rats. The human pp-orexin gene is located on chromosome 17q and consists of only two exons and one intron (Sakurai et al., 1999). After detachment of the

¹ Abbreviations: OX1-R, orexin type 1 receptor; OX2-R, orexin type 2 receptor; HPA, hypothalamic-pituitary-adrenal; NPY, neuropeptide Y; pp, prepro; CHO, Chinese hamster ovary; PLC, phospholipase C; IP₃, inositol triphosphate; PK, protein kinase; AC, adenylate cyclase; ICC, immunocytochemistry; PVN, paraventricular nucleus; RT, reverse transcription; PCR, polymerase chain reaction; ACTH, adrenocorticotropin hormone; CRH, corticotropin-releasing hormone; AVP, arginine-vasopressin; ZF, zona fasciculata; ZG, zona glomerulosa; ZR, zona reticularis; SQ-22536, 9-(tetrahydro-2'-furyl)adenine; H89, N-[2-(4-bromocinnamylamino)ethyl]-5-isoquinoline; U-73122, 1-[6-[[17β-methoxyestra-1,3,5(10)-trien-17-yl]amino]hexyl]-1H-pyrrole-2,5-dione; MAPK, mitogen-activated protein kinase; PD98059, 2'-amino-3'-methoxyflavone; SB203580, 4-(4-fluorophenyl)-2-(4-methylsulfinylphenyl)-5-(4-pyridyl)1H-imidazole; SB-334867, 1-(2-methylbenzoxanzol-6-yl)-3-(1,5)naphthyridin-4-yl-urea hydrochloride.

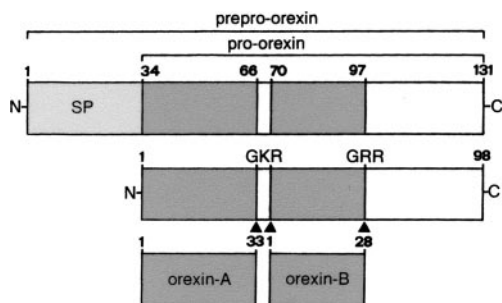


FIG. 1. Scheme illustrating the post-translational processing of pp-orexin. The arrowheads indicate the sites where, after removal of signal peptide (SP), pro-orexin is cleaved by prohormone convertase at sites with basic amino acid residues (GKR and GRR) to produce mature orexin-A and orexin-B.

N-terminal 33-amino acid residue signal peptide, pp-orexin (now pro-orexin) is cleaved by prohormone convertases to yield one molecule each of orexin-A and orexin-B (Fig. 1).

Orexin-A is a 33-amino acid peptide of ~3.5 kDa, which possesses an N-terminal pyroglutamyl residue, a C-terminal amidation, and two intrachain disulfide bridges between adjacent cysteine residues in the 6 and 12 and 7 and 14 positions (Fig. 2). The amino acid sequence of orexin-A is well preserved among mammalian species (Sakurai et al., 1998; Dyer et al., 1999). Mammalian orexin-B is a 28-amino acid peptide of ~2.9 kDa with C-terminal amidation. It is well preserved among mammalian species, and displays substantial identity in amino acid sequence of the C terminus with orexin-A (Fig. 2). Orexin-A is much more stable than

orexin-B (Kastin and Akerstrom, 1999), which explains why its tissue and blood concentrations are markedly higher than those of orexin-B. Moreover, orexin-A displays higher liposolubility than orexin-B, which makes it, in contrast with orexin-B, blood-brain barrier permeant (Kastin and Akerstrom, 1999).

B. Orexin Receptors and Their Signaling Mechanisms

OX1-Rs and OX2-Rs are seven transmembrane domain G protein-coupled receptors that are encoded by two genes located on chromosomes 1 and 6, respectively (Sakurai et al., 1998). Human OX1-Rs and OX2-Rs are 425 and 444 amino acids long, respectively, possess a 64% amino acid sequence identity, and are highly conserved among mammalian species (94% and 95% amino acid identity for OX1-Rs and OX2-Rs between humans and rats). The OX1-R is selective for orexin-A, whereas the OX2-R is nonselective for both orexins: affinities (EC_{50}) of orexin-A for OX1-Rs and OX2-Rs are 30 and 34 nM, and those of orexin-B are 2500 and 60 nM, respectively (De Lecea et al., 1998; Sakurai et al., 1998).

The bulk of evidence, obtained in a Chinese hamster ovary (CHO) cell line stably expressing OX1-Rs and/or OX2-Rs, indicates that activation of both receptors increases intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) (Smart et al., 1999; Kane et al., 2000; Lund et al., 2000; Holmqvist et al., 2001; Ammoun et al., 2003; Larsson et al., 2005). This effect ensues from the activation of phospholipase C (PLC), which in turn catalyzes the break-

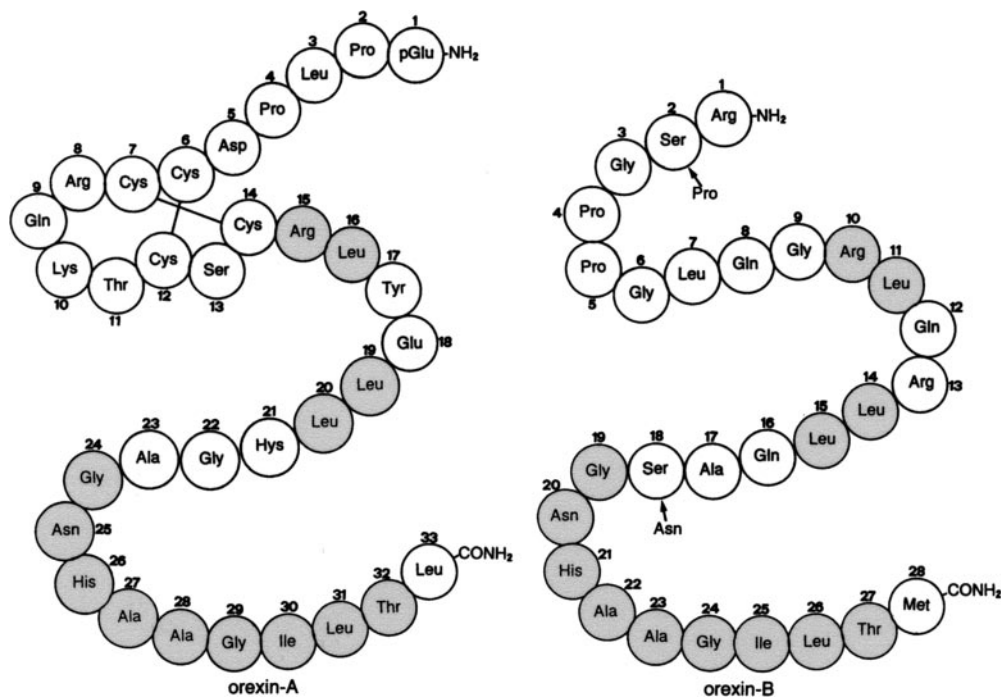


FIG. 2. Amino acid sequence of human orexin-A and orexin-B. Orexin-A possesses an N-terminal pyroglutamyl residue and two intramolecular disulfide bridges between cysteine residues in 6 and 12 and 7 and 14 positions. The orexin-A amino acid sequence is identical in humans, sheep, pigs, rats, and mice. Human orexin-B differs from that of pigs, rats, and mice for two amino acid substitutions in 2 and 18 positions (arrows). Identical amino acids between orexin-A and orexin-B are shadowed.

down of phosphatidylinositol to inositol triphosphate (IP_3) and diacylglycerol. Diacylglycerol activates protein kinase (PK) C and IP_3 enhances Ca^{2+} release from intracellular stores that in turn activates PKC. PKC and the increased $[Ca^{2+}]_i$ open voltage-gated Ca^{2+} channels, thereby stimulating Ca^{2+} influx and inducing cell membrane depolarization (Fig. 3). These effects of OX1-Rs and OX2-Rs are obtained by their coupling to excitatory G protein Gq/11. Findings indicate that OX2-Rs coupling to inhibitory G protein G_i/G_o may also cause K^+ efflux in neurons and cell membrane hyperpolarization (Fig. 3) (Willie et al., 2001; Beuckmann and Yanagisawa, 2002). It is also to be mentioned that there is evidence in human and rat adrenocortical cells that OX1-Rs are also coupled to the adenylate cyclase (AC)/PKA signaling pathway (see sections IV.B.2. and V.B.2.). Accordingly, the presence of putative phosphorylation sites for PKA has been reported in both OX1-Rs and OX2-Rs (Kreepipuu et al., 1999; Kukkonen et al., 2002), and OX1-Rs were found to stimulate AC in CHO cells via a low potency Gs coupling and a high potency PLC/PKC coupling (Holmqvist et al., 2005).

III. Expression and Function of Orexins and Their Receptors in the Central Branch of the Hypothalamic-Pituitary-Adrenal Axis

A. Expression of Orexins and Their Receptors

1. *Hypothalamus.* Orexins and their receptors were originally discovered in the rat and human brain. The huge mass of investigations dealing with the detection of pp-orexin and orexin receptor mRNA in the total hypothalamus homogenates have already been extensively reviewed (see section I.). Therefore, we will herein restrict discussion to survey studies on the hypothalamic distributions of orexin-positive neurons and OX1-Rs and OX2-Rs.

a. *Orexin expression.* In situ hybridization showed that pp-orexin mRNA expressing neurons were mainly present in the lateral hypothalamic area of both adult

(Lopez et al., 2000, 2001; Stricker-Krongrad and Beck, 2002) and newborn rats, in which expression increased between 15 and 20 days of age (Yamamoto et al., 2000). Immunocytochemistry (ICC) confirmed the main localization of orexin-positive neurons in the rat lateral hypothalamic area, especially at the level of the median eminence, with extensions to the perifornical nucleus and posterior hypothalamic area. From these neurons fibers project widely to the brain (cortex, olfactory bulb, thalamus, and brainstem) and other hypothalamic nuclei, including the arcuate nucleus, supraoptic nucleus, and paraventricular nucleus (PVN) (Van den Pol et al., 1998; Chen et al., 1999; Cutler et al., 1999; Date et al., 1999; Nambu et al., 1999; Funahashi et al., 2000; Cai et al., 2001; Kanenishi et al., 2004). The presence of orexin-A- and orexin-B-positive neurons in the lateral hypothalamic area, with orexinergic fibers throughout the entire hypothalamus, has been also demonstrated by ICC in Syrian, Siberian, and Djungarian hamsters (McGranaghan and Piggins, 2001; Khorrooshi and Klingenspor, 2005). Fasting- and insulin-induced hypoglycemia increased pp-orexin mRNA expression and orexin-A and orexin-B concentrations in the rat hypothalamus (Lopez et al., 2000; Cai et al., 2001; Karteris et al., 2005), and the i.c.v. administration of leptin, which lowers food intake and body weight, reversed the effects of fasting. By contrast, the i.p. injection of leptin was found to increase pp-orexin mRNA in the lateral hypothalamic area of newborn rats (Yamamoto et al., 2000). Adrenalectomy induced a 50% decrease in the pp-orexin mRNA in the rat hypothalamus, and dexamethasone administration abrogated this effect (Stricker-Krongrad and Beck, 2002). pp-Orexin mRNA has been shown to undergo a net rise during pregnancy in rats (Kanenishi et al., 2004), and the concentrations of both orexin-A and orexin-B increased in proestrus, which could contribute to luteinizing hormone and prolactin surges (Porkka-Heiskanen et al., 2004). Finally, an age-related (from 3 to 8 months) decrease in orexin-A, but not orexin-B,

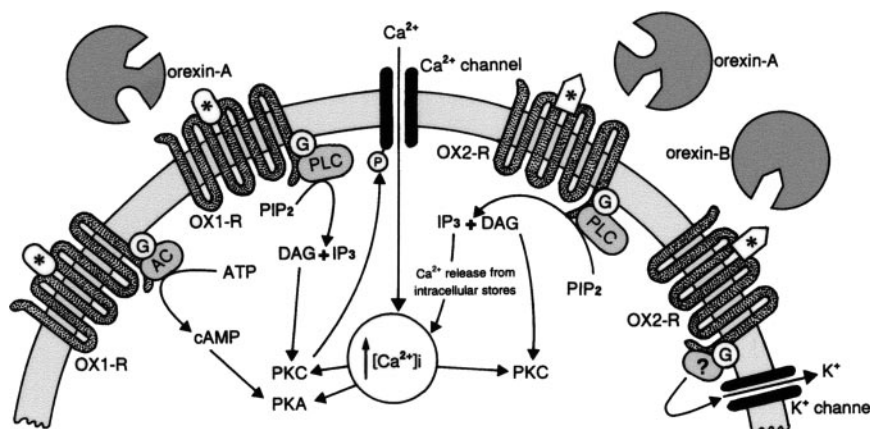


FIG. 3. Schematic drawing of the main signaling pathways of OX1-Rs and OX2-Rs upon activation by orexin-A and orexin-B, respectively. G, G protein; P, phosphorylation site; PIP2, phosphatidylinositol biphosphate. Other abbreviations are indicated in the text. Asterisks mark the orexin-recognizing sites of OX1-Rs and OX2-Rs.

concentration in the rat hypothalamus has been demonstrated (Porkka-Heiskanen et al., 2004).

b. Orexin receptor expression. In situ hybridization and ICC showed that in the rat OX1-R expression was largely restricted to the ventromedial and dorsomedial nuclei and OX2-R expression to the lateral hypothalamic area, PVN, and arcuate, mammillary and tuberomammillary nuclei (Trivedi et al., 1998; Lu et al., 2000; Marcus et al., 2001). Cluderay et al. (2002) confirmed the distribution of the OX2-Rs but were unable to detect the expression of OX1-R mRNA. In contrast, Hervieu et al. (2001) did not find OX2-R expression but reported the presence of OX1-R mRNA in the above-mentioned nuclei expressing the OX2-R. Overall, these conflicting findings cast doubts about the specificity of the probes used. In situ hybridization demonstrated that the hypothalamic distribution of orexin receptors in the male sheep reflected that occurring in the rat, with the following exceptions: OX1-R mRNA was present in the lateral hypothalamic area, arcuate nucleus, and PVN and both OX1-R and OX2-R mRNA in the median eminence (Zhang et al., 2005). Food deprivation has been reported to up-regulate the expression of OX1-Rs and OX2-Rs in the rat hypothalamus, as well as to increase their coupling to the AC- and PLC-dependent cascades (Karteris et al., 2005).

2. Pituitary Gland. No expression of orexins, as mRNA and proteins, was found in the human pituitary gland (Arihara et al., 2000). Likewise, there is evidence that this may occur in the gland of other mammalian species.

Reverse transcription (RT)-polymerase chain reaction (PCR) and ICC demonstrated the expression of both OX1-Rs and OX2-Rs in the pituitary gland of rats (Date et al., 2000; Jöhren et al., 2001, 2003), sheep (Zhang et al., 2005), and humans (Blanco et al., 2001). According to Blanco et al. (2001), the OX1-R was present in acidophils, where it was coexpressed with GH, and the OX2-R in the pars intermedia and basophils, where it was coexpressed with ACTH. In the rat pituitary, OX1-R expression prevailed over that of OX2-R and was higher in males than in females (Jöhren et al., 2001). Gonadectomy increased OX1-R expression in both female and male rats, the effect being abrogated by the administration of 17β -estradiol and testosterone, respectively (Jöhren et al., 2003).

B. Effects of Orexins

The hypothalamic nuclei involved in the secretion of adrenocorticotropin-releasing hormone (CRH) and arginine-vasopressin (AVP) contain orexin-positive neurons and are provided with orexin receptors (see section III.A.1.), and pituitary gland possesses orexin receptors (see section III.A.2.). Here, we will survey findings indicating that orexin system plays a role in the control of AVP, CRH, and ACTH release, at least in the rat.

Orexin-A and orexin-B were found to enhance *c-fos* mRNA expression (Kuru et al., 2000), and orexin-A has been reported to raise CRH and AVP mRNA levels in the PVN (Al-Barazanji et al., 2001; Brunton and Russell, 2003). Orexin-A stimulated CRH release from hypothalamic explants (Russell et al., 2001), and its application to hypothalamic slices resulted in depolarization and an increase in spike frequency of PVN neurons (Samson et al., 2002). Moreover, the i.c.v. administration of orexin-A increased plasma AVP concentration in rabbits (Matsumura et al., 2001). The CRH-stimulating action of orexin-A appears to be, at least partly, mediated by NPY via the Y1 receptor subtype, because orexin-A increased not only CRH but also NPY release from hypothalamic explants and its stimulating effect on CRH release was blocked by an Y1 receptor antagonist (Russell et al., 2001). Accordingly, the orexin-induced surge in the blood level of corticosterone (see section IV.B.2.) was blocked by pretreatment with a NPY antagonist or NPY antiserum (Jaszberényi et al., 2001). However, more recent findings did not confirm the involvement of NPY in the orexin-induced stimulation of HPA axis in rats (Moreno et al., 2005). In fact, the destruction of the arcuate nucleus and hypothalamic NPY-ergic activity by neonatal monosodium L-glutamate treatment was found to impair food-intake response to i.c.v. orexin-A but to spare glucocorticoid response.

Orexins enhance the pituitary release of ACTH, as shown by the rapid rise in ACTH blood level after their i.c.v. administration (see section IV.B.2.), and the effect appears to be indirectly mediated by the stimulation of hypothalamic production of CRH and AVP, the main physiological ACTH secretagogues (reviewed in Engelmann et al., 2004). In fact, orexins neither affected basal ACTH release from cultured rat pituitary cells (Samson and Taylor, 2001) nor raised pro-opiomelanocortin mRNA expression in the pituitary gland (Al-Barazanji et al., 2001). However, orexin-A and orexin-B were found to prevent submaximally CRH-stimulated ACTH release by cultured pituitary cells, minimal and maximal effective concentrations being 10^{-10} and 10^{-7} M, respectively (Samson and Taylor, 2001). Orexin-A (10^{-7} M) did not affect either basal or CRH-stimulated cyclic adenosine 3',5' monophosphate (cAMP) production, and its inhibitory action on CRH-induced ACTH release was reversed by the PKC inhibitor calphostin C, but not pertussis toxin. Samson and Taylor (2001) concluded that this effect of orexins is mediated by OX1-Rs coupled to the PKC-dependent cascade. It remains to be ascertained whether these findings are indicative of the existence of a local counterregulatory mechanism aimed at quenching an exceedingly high CRH-mediated ACTH response to orexins.

The findings reviewed above have been obtained in the rat, but indirect evidence suggests that the endogenous orexin system is involved in the central regulation of the HPA axis in humans also. In fact, narcoleptic

(orexin-deficient) patients displayed blunted ACTH and cortisol secretion (Kok et al., 2002).

IV. Expression and Function of Orexins and Their Receptors in the Peripheral Branch of the Hypothalamic-Pituitary-Adrenal Axis

A. Expression of Orexins and Their Receptors

1. *Orexin Expression.* Low pp-orexin mRNA expression has been detected by RT-PCR in human adrenal glands (Nakabayashi et al., 2003), and the presence of pp-orexin and orexin-A proteins has been demonstrated by Western blotting in both adult and fetal (2nd trimester) human glands (Karteris et al., 2001; Randeve et al., 2001), but more recent studies did not confirm these findings (Spinazzi et al., 2005b). No orexin expression was found in the rat adrenal glands (Lopez et al., 1999, 2001; Karteris et al., 2005). Collectively, these findings suggest that the activation of adrenal OX1-Rs and OX2-Rs could be mediated by circulating orexins (see section VI.).

2. *Orexin Receptor Expression*

a. *Human adrenal gland.* Blanco et al. (2002) reported ICC evidence that adrenal cortex and medulla were exclusively provided with OX1-Rs and OX2-Rs, respectively. In contrast, combined RT-PCR, fluorescent in situ hybridization, ICC, and Western blot findings consistently showed that adult and fetal adrenal cortex exclusively expressed the OX2-R (Karteris et al., 2001; Randeve et al., 2001). Mazzocchi et al. (2001b) reported rather different RT-PCR findings: both OX1-R and OX2-R mRNAs were expressed in zona fasciculata (ZF)-zona reticularis (ZR) and adrenal medulla and only OX1-R mRNA in zona glomerulosa (ZG). Subsequent studies confirmed the expression of both receptor subtypes in freshly dispersed and cultured human adrenocortical cells (Ziolkowska et al., 2005), and real-time PCR and Western blotting demonstrated that the levels of expression of OX1-Rs and OX2-Rs did not differ significantly (Spinazzi et al., 2005b).

b. *Rat adrenal gland.* RT-PCR allowed the detection of both OX1-R and OX2-R mRNAs in adrenal homogenates (Lopez et al., 1999, 2001; Malendowicz et al., 2001a) and freshly dispersed ZF/ZR cells (Spinazzi et al., 2005c). In contrast, no expression of OX1-Rs (Nanmoku et al., 2000) or very low expression of OX1-Rs compared with OX2-Rs have been reported (Jöhren et al., 2001, 2003). Of interest, fluorescent in situ hybridization showed OX2-R mRNA in the ZG and ZR, but not the ZF and adrenal medulla (Jöhren et al., 2001). OX2-R expression displayed an evident sex dimorphism, being higher in males than in females (Jöhren et al., 2001). Accordingly, ovariectomy increased it in females and orchietomy decreased it in males, the effects being hampered by 17β -estradiol and testosterone replacement, respectively (Jöhren et al., 2003). In contrast with the hypothalamus (see section III.A.1.), food deprivation

was found to down-regulate OX1-R and OX2-R mRNA expression in the rat adrenal cortex, as well as to markedly reduce orexin receptor coupling to G_q , G_s , and G_o and to increase coupling to G_i with the ensuing impairment of cAMP and IP_3 responses to orexin-A (Karteris et al., 2005). These data suggest that hypothalamic, but not adrenal orexin receptors may mediate the fasting-induced surge in glucocorticoid blood levels in rats (Dallman et al., 1999).

c. *Sheep and pig adrenal gland.* The sheep adrenal cortex was found to contain only OX1-R mRNA (Zhang et al., 2005). The exclusive expression of OX1-Rs has been also reported in cultured pig adrenocortical and adrenomedullary cells, the expression being exceedingly higher in the former than in the latter (Nanmoku et al., 2002).

B. Effects of Orexins

1. *Aldosterone Secretion.* Despite orexin receptors having been demonstrated in the ZG (see section IV.A.2.), the bulk of evidence indicates that orexins do not affect aldosterone secretion from dispersed rat and human ZG cells (Malendowicz et al., 1999a; Mazzocchi et al., 2001b). However, cultured pig adrenocortical cells (actually a mixture of ZG and ZF/ZR cells) displayed a sizable aldosterone response (60% increase) to 10^{-7} / 10^{-6} M orexin-A (Nanmoku et al., 2002), but it must be recalled that the physiology of cultured cells may not completely reflect that of freshly dispersed cells.

In contrast, findings that orexin administration increases aldosterone plasma concentration in rats are available. Acute s.c. injection of orexins (10 nmol/kg) raised within 60 to 120 min the level of circulating aldosterone (Malendowicz et al., 1999b; Nowak et al., 2000), and chronic orexin administration (daily s.c. injections of 20 nmol/kg for 7 days) increased it by approximately 70% (Malendowicz et al., 2001a). Conceivably, the in vivo aldosterone secretagogue effect of orexins is indirectly mediated by their stimulating action on the hypothalamic release of CRH and AVP (see section III.B.), which not only stimulate pituitary ACTH secretion but also directly enhance the secretory activity of ZG cells (reviewed in Nussdorfer, 1996). Alternatively, it could be also possible that in vivo administration of orexins may alter plasma angiotensin II or K^+ levels.

2. *Glucocorticoid Secretion.* Compelling evidence indicates that orexins increase glucocorticoid secretion from inner adrenocortical layers, via a 2-fold mechanism: 1) stimulation of CRH/AVP-mediated release of ACTH (see section III.B.); and 2) direct stimulation of ZF/ZR cells, which are provided with orexin receptors (see section IV.A.2.).

a. *Indirect mechanism.* In vivo studies showed that the systemic administration of orexin-A acutely raised ACTH and corticosterone blood levels in the rat (Malendowicz et al., 1999b; Nowak et al., 2000). Orexin-B was ineffective, which accords well with the notion that

orexin-A rapidly crosses the blood-brain barrier by simple diffusion, whereas orexin-B is rapidly degraded in the blood (see section II.A.). Orexins are much more effective when they are administered i.c.v. Orexin-A was found to enhance both ACTH and corticosterone blood levels, with the corticosterone response being slightly delayed (Ida et al., 2000; Jaszberényi et al., 2000; Kuru et al., 2000; Al-Barazanji et al., 2001; Russell et al., 2001; Brunton and Russell, 2003). Orexin-B was less effective than orexin-A (Jaszberényi et al., 2000, 2001; Kuru et al., 2000), thereby suggesting that the effect was mediated by both OX1-Rs and OX2-Rs (see section II.B.). The ACTH and corticosterone responses to orexin-A were blunted in pregnant rats (Brunton and Russell, 2003), a finding not apparently in keeping with the reported enhanced expression of pp-orexin during pregnancy (see section III.A.1.). The contention that the *in vivo* glucocorticoid secretagogue action of orexins is indirectly mediated by their hypothalamic effects is confirmed by the demonstration that pretreatment with the CRH antagonist α -helical CRH(9-41) blocked orexin-A-evoked surges in ACTH and corticosterone plasma concentrations in rats (Ida et al., 2000; Samson et al., 2002).

b. Direct mechanism. The bulk of evidence indicates that orexins enhance glucocorticoid secretion acting via OX1-Rs located on ZF/ZR cells despite the prevalence of OX2-Rs in adrenal glands described by some groups of investigators (see section IV.A.2.). Although earlier studies showed that both orexins stimulate corticosterone production (Malendowicz et al., 1999a), subsequent investigations consistently showed that only orexin-A concentration dependently increased basal corticosterone or cortisol secretion from dispersed or cultured rat and human ZF/ZR cells (Mazzocchi et al., 2001b; Spinazzi et al., 2005b). Minimal and maximal effective concentrations were 10^{-8} and $10^{-7}/10^{-6}$ M in rats and 10^{-10} and 10^{-8} M in humans. The ineffectiveness of orexin-B, which exclusively binds OX2-Rs (see section II.B.), ruled out the involvement of this receptor subtype in the direct glucocorticoid secretagogue action of orexins. This contention has been directly supported by the demonstration that the blockade of OX1-Rs by specific antibodies abolished the secretory response of both cultured rat and human ZF/ZR cells to orexin-A, whereas the immunoneutralization of OX2-Rs was ineffective (Ziolkowska et al., 2005).

Orexin-A did not maximally affect ACTH-stimulated glucocorticoid secretion from dispersed ZF/ZR cells, but enhanced the glucocorticoid response to maximal effective concentrations of angiotensin II and endothelin-1 (Malendowicz et al., 1999a; Mazzocchi et al., 2001b), which are agonists mainly activating PLC/PKA cascade (reviewed in Nussdorfer et al., 1999). This finding suggests that orexin-A and ACTH share a common signaling mechanism, i.e., the activation of the AC-dependent cascade. This contention was supported by the demonstration that 1) orexin-A enhanced cAMP but not IP₃

production by ZF/ZR cells (Malendowicz et al., 1999a; Mazzocchi et al., 2001b) and 2) the glucocorticoid secretagogue effect of orexin-A was abolished by either the AC inhibitors SQ-22536 or the PKA inhibitor H89 and unaffected by the PLC and PKC inhibitors U-73122 and calphostin-C (Spinazzi et al., 2005b). Randeve et al. (2001) reported that orexin-A (from 10^{-11} to 10^{-7} M) increased not only cAMP, but also IP₃ production from human adrenal membranes, but it must be noted that such preparations were conceivably obtained from the entire adrenal gland, i.e., including both cortex and medulla.

3. Adrenocortical Cell Growth. The presence of OX2-Rs in adrenocortical cells not coupled to any secretory effect of orexins may suggest the alternative possibilities that either they are silent receptors or mediate a growth effect of these peptides. The latter appears to be the most convincing possibility in light of the following findings.

The systemic administration of orexins was found to increase the proliferation index in immature rat adrenal cortex and to lower it in enucleated glands at day 5 but not at day 8 of regeneration (Malendowicz et al., 2001b). Orexin-A (24-h exposure) increased the proliferation rate of cultured rat adrenocortical cells, but the effect was inversely correlated with the peptide concentration (maximum at 10^{-10} M, and minimum at 10^{-6} M). In contrast, orexin-B (10^{-8} or 10^{-6} M) decreased the proliferation rate. OX1-R immunoblockade reversed the proliferogenic effect of orexin-A, causing a small but significant decrease in growth. OX2-R immunoneutralization magnified the proliferogenic effect of orexin-A and abrogated the antiproliferogenic action of orexin-B (Spinazzi et al., 2005c). The proliferogenic effect of orexin-A (in the presence of OX2-R blockade) was annulled by the mitogen-activated protein kinase (MAPK) p42/p44 inhibitor PD98059, and the antiproliferogenic action of orexin-B was abrogated by the MAPK p38 inhibitor SB203580 (Spinazzi et al., 2005c). Thus, it was concluded that OX1-Rs and OX2-Rs mediate the proliferogenic and antiproliferogenic actions of orexins on rat adrenocortical cells through the activation of the MAPK p42/p44 and p38 cascades, respectively.

Different results were obtained in cultured human adrenocortical cells, in which both orexins (10^{-8} M) enhanced proliferative activity (Spinazzi et al., 2005b). Because orexin-B was more effective than orexin-A, it has been suggested that OX2-Rs mediate the growth-promoting action of orexins. Further investigations must be carried out to solve these discrepancies between rats and humans, also in light of findings showing an OX1-R-mediated antigrowth (proapoptotic) effect of orexins in human colon cancer- and neuroblastoma-derived cell lines as well as in CHO OX1-R-transfected cells (Rouet-Benzineb et al., 2004).

4. Catecholamine Secretion. Contrasting findings on the effects of orexins on adrenal-medulla secretion of

various mammalian species exist. Using the rat pheochromocytoma-derived PC12 cell line, Nanmoku et al. (2000) showed that both orexin-A and orexin-B (10^{-7} M) decreased either basal or pituitary adenylate cyclase-activating polypeptide-stimulated tyrosine hydroxylase expression, cAMP release, and dopamine secretion. Because PC12 cells displayed equal binding capacity for both orexins, these investigators suggested that orexins inhibited catecholamine synthesis via OX2-Rs negatively coupled to the AC-dependent cascade.

Orexin-A (10^{-10} M) was found to enhance TH activity and catecholamine synthesis (but not release) in cultured bovine adrenomedullary cells that expressed only OX1-R mRNA (Kawada et al., 2003). The effect was blocked by the PKC inhibitor staurosporin, making likely the possibility that orexins stimulate catecholamine synthesis in bovine adrenal medulla via OX1-Rs coupled to the PKC pathway.

Orexin-A (10^{-6} M) increased epinephrine and norepinephrine release from cultured pig adrenomedullary cells that possessed low OX1-R expression (Nanmoku et al., 2002). Orexin-A was also found to lower both basal and pituitary adenylate cyclase-activating polypeptide-stimulated cAMP production, thereby suggesting that orexins stimulate catecholamine secretion from pig adrenal medulla via OX1-Rs negatively coupled to the AC-dependent cascade.

Finally, human adrenal medulla slices did not exhibit any appreciable secretory response to orexins, despite the fact that they express both OX1-R and OX2-R mRNAs (Mazzocchi et al., 2001b) and that orexins exert a relevant catecholamine secretagogue action on pheochromocytoma slices (see section V.C.2.).

V. Involvement of Orexins in the Pathophysiology of the Hypothalamic-Pituitary-Adrenal Axis

A. Stressful Conditions

Evidence suggests that orexins may modulate HPA axis response to different types of stress in the rat. The systemic administration of orexin-A and orexin-B (10 nmol/kg) magnified ACTH and glucocorticoid response to cold stress. Conversely, the response to ether stress was enhanced only by orexin-B (Nowak et al., 2000). Immobilization stress increased pp-orexin mRNA expression in the lateral hypothalamic area in juvenile rats (2 month-old), and cold stress evoked the same effect in adult animals (Ida et al., 2000).

These findings could indicate that orexins act as prostress agents, which is of great interest in light of the possible antistress effect of leptin (Bornstein, 1997; Heiman et al., 1997). The possibility of interactions between leptin and orexins at the hypothalamic level are supported by the demonstration that 1) neurons provided with leptin receptors innervated hypothalamic orexin-positive neurons (Funahashi et al., 2000), 2) lep-

tin receptors were expressed in some orexin-positive neurons (Horvath et al., 1999), and 3) systemic leptin administration enhances pp-orexin mRNA in newborn rat hypothalamus (Yamamoto et al., 2000).

B. Adrenocortical Tumors

1. Orexin and Orexin Receptor Expression. Earlier studies did not report pp-orexin mRNA and orexin-A immunoreactivity in adrenocortical tumors (Arihara et al., 2000), but more recent investigations detected pp-orexin mRNA in 75% of cortisol-secreting adenomas, and measurable concentrations of orexin-A (12 ± 4 fmol/mg), but not orexin-B, in 70% of these tumors (Spinazzi et al., 2005b). ICC demonstrated that adrenocortical adenomas were provided with OX1-Rs, but not OX2-Rs (Blanco et al., 2002). Using real-time PCR and Western blotting, Spinazzi et al. (2005b) not only showed that all cortisol-secreting adenomas examined possessed both OX1-Rs and OX2-Rs but also that their mRNA was significantly up-regulated compared with that in normal adrenal cortex (114% and 63% higher).

2. Effects of Orexins on Secretion and Growth. Orexin-A concentration dependently increased basal cortisol production from freshly dispersed adenomatous cells, minimal and maximal effective concentrations being 10^{-10} and 10^{-8} M, respectively. Orexin-B was ineffective, which is consistent with the exclusive involvement of OX1-Rs (Spinazzi et al., 2005b). OX1-Rs were conceivably coupled to the AC/PKA cascade, because specific inhibitors of AC and PKA, but not of PLC and PKC, abolished the cortisol secretagogue effect of 10^{-8} M orexin-A. Spinazzi et al. (2005b) also demonstrated that both orexins (10^{-8} M) significantly enhanced the proliferative activity of cultured cortisol-secreting adenoma cells, thereby suggesting the involvement of OX2-Rs (see section IV.B.3.).

3. Conclusions. The effectiveness of orexins in eliciting both secretory and growth responses was markedly higher in cortisol-secreting adenomas than in normal adrenal cortex: the efficacy (percent increase elicited by the maximal effective concentration) of the secretagogue and proliferogenic effects of orexins were approximately 2-fold and 3- to 4-fold higher in adenomas (Spinazzi et al., 2005b). This observation accords well with the reported up-regulation of OX1-Rs and OX2-Rs in adenomas, and, along with the ability of adenomas to synthesize orexin-A, suggests that endogenous orexin system may be involved, through an autocrine-paracrine mechanism, in the pathogenesis of cortisol-secreting adrenal tumors.

C. Pheochromocytomas

1. Orexin and Orexin Receptor Expression. Although pp-orexin mRNA and orexin-A protein have been detected in ganglioneuroblastomas and neuroblastomas, they were not found in pheochromocytomas (Arihara et al., 2000). At variance, the expression of OX2-Rs but not of OX1-Rs, as mRNA and protein, has been demon-

strated in all benign human pheochromocytomas so far examined (Mazzocchi et al., 2001a; Blanco et al., 2002).

2. *Effects of Orexins on Catecholamine Secretion.* Mazzocchi et al. (2001a) showed that both orexin-A and orexin-B equipotently enhanced catecholamine secretion from pheochromocytoma quarters, with the maximal effective concentration being 10^{-8} M. Orexins (10^{-8} M) stimulated IP_3 , but not cAMP, production from tumor slices, and catecholamine secretory response to orexins was abrogated by the inhibitors of PLC and PKC and unaffected by the inhibitors of AC and PKA. Taken together, these findings suggested that orexins stimulate pheochromocytoma secretion via OX2-Rs coupled to the PLC/PKC signaling cascade.

VI. Concluding Remarks

The preceding sections of this review have shown that in the 7 years elapsed from their discovery findings suggesting that orexins can play a potentially important role in the regulation of the function of the HPA axis have accumulated. However, several problems remain to be addressed, and we shall now take the opportunity to mention some issues that surely await further investigative effort.

Is the regulatory role of orexin on adrenal secretion relevant under physiological conditions? The bulk of evidence indicates that orexins stimulate CRH-ACTH secretion. The existence of orexinergic fibers reaching the PVN, which is provided with orexin receptors may easily account for the stimulating effect of orexin on CRH and AVP release but surely not for the activation of orexin receptors in the pituitary gland (see section III.B.). According to Nussdorfer (1996), if the tissue content of a regulatory peptide is 100 fmol/g, its 30% release will produce a local concentration of approximately 10^{-9} M. The orexin-A concentrations in rat and human hypothalamus range between 200 and 20 fmol/mg (Arihara et al., 2000; Spinazzi et al., 2005b); hence they could give rise to local orexin-A levels of approximately 2×10^{-6} and 2×10^{-7} M, that, via the hypothalamopituitary portal blood, could be capable of activating the OX1-Rs and OX2-Rs found in the pituitary gland. The physiological relevance of this central effect of orexins is indirectly confirmed by the blunted ACTH secretion occurring in hypothalamic orexin-deficient narcoleptic patients (Kok et al., 2002). Consistent experimental findings showed that orexins may enhance glucocorticoid secretion acting not only on the central branch of the HPA axis but also directly on adrenocortical cells that are provided with orexin receptors. However, the relevance of the latter mechanism of action of orexins remains questionable: in fact, 1) the level of circulating orexin-A in rat and humans does not exceed 15 and 2 pmol/l (Arihara et al., 2001; Jöhren et al., 2001, 2003); 2) minimal effective concentrations of orexin-A in eliciting in vitro glucocorticoid secretagogue effect are approximately 10^{-8} and

10^{-10} M in rats and humans (see section IV.B.2.); and 3) no measurable concentrations of orexins are present in normal adrenal glands (see section IV.A.1.), thereby ruling out the possibility of the involvement of an auto-crine-paracrine mechanism of action. However, because fasting and hypoglycemia increase orexin concentrations in the hypothalamus (Sakurai et al., 1998; Cai et al., 1999, 2001; Karteris et al., 2005) and hypoglycemia enhances pancreatic release of orexins (Ouedraogo et al., 2003), it cannot be excluded that in these conditions the blood concentrations of orexins could reach levels capable of directly stimulating the secretion of adrenocortical cells. However, this possibility apparently conflicts the recent demonstration that fasting down-regulates orexin receptor expression in the rat adrenal glands (Karteris et al., 2005) (see section IV.A.2.). In this connection, we want to recall that leptin, which decreases food intake and body weight (Remesar et al., 1997; Mantzoros and Moschos, 1998; Ahima and Flier, 2000) and interacts with the orexin system at the hypothalamic level (see section V.A.), displays increased plasma concentrations in obese and hyperglycemic patients with Cushing's syndrome (Leal-Cerro et al., 1996; Blum et al., 1997; Weise et al., 1999). Because leptin inhibits glucocorticoid secretion (Bornstein et al., 1997; Glasow et al., 1998; Pralong et al., 1998; Glasow and Bornstein, 2000), the possibility awaits to be explored whether orexins together with leptin may take part in counter-regulatory mechanisms, which, through the modulation of glucocorticoid secretion, are involved in the maintenance of glycemia and body weight homeostasis. Surely, in vivo investigations on this topic could take advantage of the use of animals in which endogenous orexin system is stably suppressed (e.g., orexin gene knockout animals). So far immunoblockade of orexin receptors with specific antibodies has been used in in vitro experiments (Spinazzi et al., 2005c; Ziolkowska et al., 2005), but this approach is unfeasible in long-term in vivo experiments. Furthermore, at present, only a selective not yet commercially available OX1-R nonpeptide antagonist (SB-334867) has been synthesized (Porter et al., 2001; Smart et al., 2001).

Are there functional interrelationships between adrenal and gonadal orexin systems? Orexin immunoreactivity has been detected in the rat testis (Mitsuma et al., 2000), orexin receptors were found in rat and human testis (Jöhren et al., 2001; Barreiro et al., 2004; Karteris et al., 2004), and orexin-A has been reported to enhance testosterone secretion both in vivo and in vitro in the rat (Barreiro et al., 2004). Although in the rat hypothalamus orexin expression is increased in proestrus and during pregnancy (see section III.A.1.), orexin receptor expression in adrenals appears to be down-regulated by estrogens and up-regulated by androgens (see section IV.A.2.). Further experiments are needed to ascertain whether the testis orexin system and gonadal hormones

may account for the gender-related differences in orexin system expression in the HPA axis (see section IV.A.2.).

Is the orexin system involved in the pathophysiology of adrenal tumors? Cortisol-secreting adrenal adenomas were found not only to possess up-regulated orexin receptors but also to contain sizable amounts of orexin-A (12 fmol/mg), which could give rise to local concentrations (approximately 10^{-7} M) well above those able to elicit in vitro secretory and proliferative responses (see section V.B.). Despite the fact that the effects of orexin on catecholamine production from adrenal medulla are doubtful (see section IV.B.4.), orexins have been shown to stimulate catecholamine secretion from human pheochromocytomas (see section V.C.2.). Unfortunately, neither the orexin content of pheochromocytomas nor its possible effect on tumor cell growth has been studied. Additional investigations must be carried out to explore the appealing possibility that the orexin system plays a relevant role in the control of secretion and growth of adrenal tumors.

The answers to these questions, along with the development of potent and selective antagonists of orexin receptors, not only will increase our understanding of the physiology of the HPA axis but also could open new perspectives in the therapy of many diseases coupled to dysregulation of adrenal function.

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