REVIEW ARTICLE

DISCOVERY OF OREXINS/HYPOCRETINS: THEIR ROLES IN REGULATING THE HYPOTHALAMIC PITUITARY GONADAL AXIS

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INTRODUCTION

Orexin A and B, also named hypocretin 1 and 2, were identified in 1998 by two independent research groups led by Sutcliffe and Yanagisawa, respectively. Both peptides are derived from a common precursor containing 130 amino acids (aa), called prepro-orexin. Orexin A is a 33-aa peptide, while orexin B is a 28-aa peptide. Both peptides act on their targets via two G-protein coupled receptors (OX1 and OX2) which mobilize Ca++. Orexin A is highly conserved among human, pig, dog, rat and mouse whereas orexin B differs by 2 aa residues in rats and mouse from human. Diverse responses of orexin containing hypothalamic neurons include feeding behavior, sleep-wakefulness, neuroendocrine and, autonomic regulation and reproduction. Although, identified in the hypothalamus, expression of orexins and their receptors have been found in a variety of peripheral tissues. In the present review, we especially focus on the nomenclature and discovery of orexins, their localization in CNS and peripheral sites, genetics and structural constitution of orexins, their receptors, physiological actions, and roles in the regulation of hypothalamic-pituitary- gonadal axis.

Discovery and Nomenclature

The existence of an orexinergic system was established by the discoveries of two independent research groups. This in turn gave rise to a difference in nomenclature: "orexin" and "hypocretin". It is evident from literature that "Hypocretins" (also known as hypothalamic incretins) were named to reflect their hypothalamus associated tissue specificity and amino acid sequence similarity with the hormone secretin. On the contrary, "Orexin" has its roots in a Greek word, meaning "stimulators of appetite" and they were named for their role in the regulation of feeding [1-4].

"Hypocretins" were first isolated as neuropeptides from rat lateral hypothalamus using directional tag PCR subtraction cloning in 1996. In the subsequent year, information pertaining to gene cloning, origin, and distribution of these neuropeptides was revealed [1, 2, 5]. In 1998, De Lecea and Sutcliffe [4] speculated the existence of a rat hypothalamic mRNA species coding for a 130 amino acid precursor (named prepro-hypocretin). The precursor peptide was predicted to give rise to two distinct peptides; Hcrt-1 of 39 amino acids and Hcrt-2 of 29 amino acid [1, 6].

Orexins were also successfully isolated as a hypothalamic peptide by using reverse pharmacology approach in search of endogenous ligands for orphan G protein coupled receptors. The fractions that affected cytosolic Ca2+ levels were isolated, purified and analyzed. The peptide was found to stimulate the orphan receptor GCPR HFGAN72 (now OX1R/Hcrt1R) in transfectant cell lines [2,3]. Sakurai and his coworkers confirmed the length of precursor peptide (prepro-orexin) at 130 amino acids by cloning of cDNA [7].

Presently, confusion stems from choice of nomenclature to be used. The fact that both names give only partial information about the function/structure of Orexins/Hypocretins makes them somewhat misnomers. It was found that only Orexin-B and Secretin share noteworthy homology. However peptide sequences described by Sakuri et al [7] are now widely accepted as authentic and correct.

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In principle on the basis of their etymology, hypocretins (Hcrt-1 and Hcrt-2) and orexins (Orx-A and Orx-B) are two terms used interchangeably [1-4].

Localization in CNS and Peripheral Sites

In the central nervous system (CNS), these peptides are found within the neurons of lateral hypothalamus and perifornical area [8, 9]. Some cells are also observed in posterior hypothalamus. From these regions, the cells project extensively to the neuroaxis excluding the cerebellum. The cerebral cortex, olfactory bulb, hippocampus, amygdala, septum, diagonal band of Broca, bed nucleus of stria terminalis, thalamus, midbrain and brainstem have particularly dense projections [10]. These peptides act as essential regulators of numerous physiological systems including neuroendocrine, autonomic, and cardiovascular. They have diverse physiological functions in circadian rhythms, sleep/arousal, appetite, pain modulation, stress reactions, reproductive and sexual behaviors, locomotor activity and energy metabolism [1-4, 11-13]. Orexins and their receptors are also found in the periphery e.g. testis [11,13], ovaries [11], kidneys, thyroid gland, adrenal glands [3,11], lungs [3], pancreas, gut [3, 13], and placenta [14]. Measurements of prepro-orexin mRNA and radio-immunoassay of orexin-A in rat testis showed that after the brain, it holds the second highest orexin expression levels reported so far [15]. Testicular expression levels of prepro-orexin gene were low (neonatal) to negligible (early juvenile) before initiation of puberty [15].

Genetics and Structural Constitution of Orexins

The human orexin gene resides on chromosome 17q21. The gene spans over a length of 1432 base pairs, including 2 exons and 1 intron [2, 3, 4, 7]. The exon 2 of the gene is completely responsible for the generation of the final peptide [3]. The mouse orexin gene was mapped to chromosome 11, at a region that indicated a conserved synteny with human chromosome 17q21-q24 [4].

When the preproorexin nucleotide sequences in mice and rats were compared, it was found that mouse preproorexin nucleotide sequence differed in 39 positions compared to rats. Among them, 19 variations were within the protein-coding region, yet only 7 of them seemed to be affecting the encoded protein sequence [4]. Mammalian preproorexin is derived from a 130-131 amino acid precursor [3, 6, 7, 16-18]. Human preproorexin is composed of 131 amino acids, in contrast to the 130 amino acid precursor in rats. However, in both cases, the first 33 amino acids of the precursor constitute the signal sequence [2]. The glycine residue at the C terminal is believed to act as a substrate for peptidyl glycinealpha-amidating monoxygenase, leaving an amide in both the mature peptides, namely, orexin A and B [3].

Orexin A has a molecular weight of 3562 Da and is composed of 33 amino acids. In addition to its pyroglutamyl residue at N-terminal and amide group at C-terminal, it also possesses two inter-chain disulphide bonds [2, 3, 19]. Orexin B comprises of 28 amino acids and has a molecular weight of 2937 Da. In contrast to orexin A, orexin B is a linear peptide with no inter-chain associations [2].

The resemblance of human orexin A to mouse, rat, bovine and porcine orexin A is worth mentioning. On the contrary, human orexin B has 1 and 2 amino acid substitutions compared to its porcine and rodent counterparts, respectively [2]. There happens to be a 46% sequence homology between human orexin A and B [2, 4]. Despite the numerous differences in preproorexin nucleotide sequences between mice and rats (as mentioned earlier), mature orexin A and orexin B were, surprisingly, found to be identical to each other [4].

Orexin Receptors

Orexin A and B exert their plethora of physiological effects by virtue of their receptors, namely, Orexin A receptor (Ox1R) and Orexin B receptor (Ox2R). On the basis of their mechanism of action, they are grouped under class A – G protein coupled receptors.

One of the primary effects after receptor-ligand binding is increased Ca2+ concentration [1, 3]. A multitude of studies have established that the genes of Ox1R and Ox2R are present on human chromosomes 1p33 and 6p11-q11, respectively [1, 5, 20, 21]. The genetic organization of both the receptors exhibits a marked similarity. In both cases, the gene transcript comprises of a 5'-untranslated region, followed by as many as seven exons separated by 6 introns. Orexin receptors possess as much as 28-31% homology when compared with other peptide receptors like NPY Y2, TRH receptor, NK2 tachykinin receptor and others. Whether or not these receptors belong to the same family of peptides is currently open to further research [2, 3]. The existence of mRNAs encoding for both the receptors in the brain is well established. The cytosolic and nuclear localization of Ox1R and Ox2R respectively was characterized by immuno-histochemical studies [2, 4]. Structurally, both Ox1R and Ox2R were found to have modestly long N and C terminals with average sized i3 loops. The length of mature Ox1R and Ox2R is 425 and 444 amino acids respectively, possessing a 64% homology between them. Initially, there have been two disagreements about their amino acid sequences: aa 280 in Ox1R could be either glycine or alanine whereas aa 308 in the sixth trans-membrane segment of Ox2R is either valine or isoleucine [3].Ox1R has a remarkably strong affinity for orexin A. However, its binding affinity to orexin B is approximately 100 to 1000 times less. Incongruously, Ox2R happens to possess a high affinity for both orexin A and orexin B [1, 2, 4].

Physiological Actions

Orexins are primarily excitatory neurotransmitters [1, 4]. They are found to increase neuronal activity via presynaptic effects (increased transmitter release) and via postsynaptic depolarization (block of K+ channels and activation of cation channels) [3]. At the cellular level, both Orexin A and B are well known for increasing calcium levels, as noted by fura-2 imaging [4]. Recombinantly expressed orexin receptor strongly elevated calcium levels in Chinese hamster ovary (CHO)-K1 cells [3]. It is proposed that this effect is mediated by the opening of plasma membrane calcium channels [4]. Orexin effects are completely blocked by protein kinase C (PKC) specific inhibitor (bisindolylmaleide), indicating G protein mediated activation of PKC leading to increased calcium conductance secondary to calcium channel phosphorylation [4]. Therefore, an activation sequence of the orexin receptor, as shown below in the (fig. 2), has been proposed [3].

Increased action potential frequency has been measured in hypothalamic neurons in response to orexin stimulation [3]. However, Orexin A suppresses the firing rate of glucose responsive neurons in rat ventro-medial hypothalamic nucleus [3]. Orexins may inhibit K+ channels, as indicated by the reduced after-hyperpolarization amplitude observed in rat locus ceruleus and guinea pig ileal submucosal ganglia [3]. Orexin stimulation has been observed to cause cAMP elevation in rat and human adrenal cortices putatively via OX1 receptors [3]. OX1 receptor stimulation also leads to the activation of mitogen-activated protein kinase (MAPK) pathways, supporting the idea that orexins are involved in cell growth, differentiation and survival cascades [3].

Orexins are implicated in the lowering of plasma prolactin and growth hormone levels and raising the levels of corticotrophin, cortisol, insulin and luteinizing hormone [1, 2]. A role for orexins in energy metabolism is suggested by their effects on the release of hormones including adrenal steroids, glucocorticoid, growth hormone, insulin and leptin [3]. Orexins enhance the CNS actions of excitatory action on serotonin, dopamine, histamine and acetylcholine. A facilitatory role on GABA and glutamate-mediated neurotransmission is also suggested, based on whole-cell patch-clamp recordings [1, 4].

The initial hypothesis of orexins as important modulators of food intake was based on the anatomical localization of

orexins in neurons concerned with regulation of appetite and satiety. Furthermore, fasting up-regulates orexins, as revealed by a study in which the concentration of orexin mRNA increased by 2.4 times in rats fasted for 48 hours [2, 4]. It is now known that an increase in orexin levels is related to an increase in food consumption and metabolic rate [4]. Although orexins increase food intake in the first 4 hours, they decrease food intake in the next 20 hours. Moreover, chronic administration of orexins does not lead to obesity [2]. These data clearly imply that although orexins markedly increase food intake initially, they have no effect on food intake in the long run. It may be that orexins are not as important as originally believed or that they may have a role in regulation in food intake only in certain circumstances [2, 4]. In experimental models where orexins were administered centrally, a noticeable increase in water consumption, stimulation of gastric acid secretion and augmentation in gut motility was observed [1, 3].

Orexins regulate hypothalamo-hypophyseal hormone secretion indirectly via neuronal circuits or may exert direct effects as well because of their localization in the median eminence [3]. Orexin-mediated increase in CRF, ACTH, corticosterone, aldosterone, vasopressin and epinephrine strongly suggest a regulatory role of orexins in mediating stress responses [3].

The role of orexins in increasing mean arterial blood pressure and heart rate is well documented [1, 4]. In rats, central administration of orexin A increases arousal while reducing REM sleep and prolonging the latency to the first occurrence of REM sleep [2]. Since orexin- containing, descending axonal projections are found at all levels of the spinal cord, orexins' role in the modulation of sensation and pain has also been hypothesized [4].

Role of Orexins in Modulation of Hypothalamic – Pituitary - Gonadal Axis

The distribution of orexin immuno-reactive fibers overlaps luteinizing hormone-releasing hormone (LHRH) neuronal system in the septo-preoptic area and the arcuate nucleus- median eminence region, raising the possibility of involvement of orexins in the regulation of pituitary LH secretion by influencing LHRH release. Shye Pu et al [22] showed that LH secretion is stimulated by intra-cerebroventricular injection of orexin A or orexin B in a dose and time- related fashion in estradiol benzoate (EB) and progesterone pretreated ovariectomized rats .The effect of ICV-injected orexins depended on the status of ovarian steroids [22]. Furthermore, whereas in ovariectomised (OVX) rats treated with 17β-estradiol and progesterone, ICV orexin increased plasma LH levels, in untreated OVX rats orexin decreased plasma LH levels. One explanation for this bimodal action of orexins on LH release is a possible regulation of orexin receptors by estrogens [12, 23]. The LH response to orexin A in the hypothalamus also appears to be site specific [24, 25] and mediated through Ox1R, which are located on the gonadotrophin releasing hormone cells [26]. It has thus been shown that orexin A-stimulated LH-releasing hormone from the hypothalamus is dependant upon the steroid milieu [27].

In sheep, orexinergic neurons are mainly distributed to the dorso-medial hypothalamic nucleus, lateral hypothalamic area, and zona incerta and perifornical area. Furthermore, a significant number of GnRH cells had orexin immuno-reactive terminals in close contact. These findings suggest an integral role for orexins in the regulation of GnRH cells. Orexin receptor mRNA have also been localized in hypothalamic areas which are involved in neuroendocrine functions [28]

Orexin-A simulates GnRH release from rat hypothalamic explants of male and female rats and hypothalamic neurons express OX1R and are directly contacted by orexin fibers [13]. A number of conflicting studies are reported as far as the role of orexins on LH secretion is concerned [12, 27, 29]. In one setting, orexin-B stimulated LH release from pre-pubertal gilt pituitary cells in primary culture. The cells were challenged with

GnRH, hGRF, orexin B individually or in combinations with GnRH and GRF. In another setting, Orexin-B failed to alter the basal and stimulatory effects of GnRH on LH secretion in pituitary cells from adult males and randomly cycling female rats. This apparent contradiction in results could be attributed to species or age differences [30].

Estrogen priming suggested that on the day of pro-estrus the effect of orexins on LH secretion would be stimulatory. Orexin secretion appears to be vital for the appearance of LH and prolactin surges, as antibody to orexin A abolishes the steroid-induced LH and prolactin surges in ovariectomised rats [24, 31]. Thus, an increase in orexin secretion may be an integral part of the hormonal secretion cascade which precedes ovulation. Hypothalamic concentrations at different phases of the estrous cycle were measured by radioimmunoassay and found to be lowest late in the day of proestrus. During the same period, the orexin concentration in other parts of the brain was highest. It is speculated that hypothalamus releases orexin during this time, hence decreasing its concentration [27].

However, orexins may act indirectly via β-endorphin pathway. β-Endorphin (β-END) belongs to a family of endogenous opioid peptides that suppress GnRH secretion. Orexin neurons project into the arcuate nucleus (ARC) and innervate POMC neurons, the precursors of β-END. Naloxone, a specific opioid antagonist, was used to test whether β-END is involved in orexin's mechanism of action. Orexin-A significantly reduced the mean LH concentration and the pulse frequency, but co-administration of naloxone reversed this effect. This suggests that orexin-A may suppress GnRH secretion via β-END to a considerable degree. On the other hand, orexin-B significantly reduced the mean LH concentration and the pulse frequency but naloxone did not block the effect. Hence, β-END may not be involved in the suppression of GnRH secretion by orexin B [23]. The ability of orexins to either increase or decrease the magnitude of LH response to GnRH suggests the presence of orexin receptors on gonadotrophs [30]. The occurrence of Ox1R and Ox2R on somatotrophs and corticotrophs has already been proven [32]. Orexin receptors were detected by in situ hybridization histochemistry in the rat pituitary and by immuno-histochemistry in the human pituitary [33]. Therefore, direct stimulation of LH secretion by Orexin-B from anterior pituitary glands via receptors on gonadotrophs seems plausible [9, 11, 12, 34].

Expression of Orexins

Sexually dimorphic expression of prepro-orexin mRNA in the rat hypothalamus was shown using qualitative real time PCR. Significantly higher levels of prepro-orexin mRNA in the hypothalamus of female rats compared to male rats were found [35]. It was also shown that prepro-orexin and orexin receptor mRNAs are differentially expressed in peripheral tissues of male and female rats. OX1R mRNA levels in the pituitary were much higher in male than in female rats [11]. The effect of 17β-estradiol and testosterone in female and male rats, respectively, on prepro-orexin and orexin receptor mRNA expression was investigated. It was concluded that the gonadal steroids differentially regulate pituitary OX1 receptors and adrenal OX2 receptors in male and female rats and may contribute to specific sex-dependant neuroendocrine and endocrine actions of orexins [12].

Despite the initial contention that orexins are exclusively produced and act in the brain; studies in rats revealed that prepro-orexin mRNA and OX1R [11], but not OX2R are expressed in the testis .Whereas only OX1R and OX2R, not prepro-orexin mRNA was found in human testis [36]. In vitro slice preparation and in vivo studies in rats showed that Orexin-A stimulated basal testosterone secretion. These observations support previous findings on the expression of orexin binding sites and their immunoreactivity in the testis [13]. This study has reported expression of significant amount of prepro-orexin mRNA and OX1R gene in

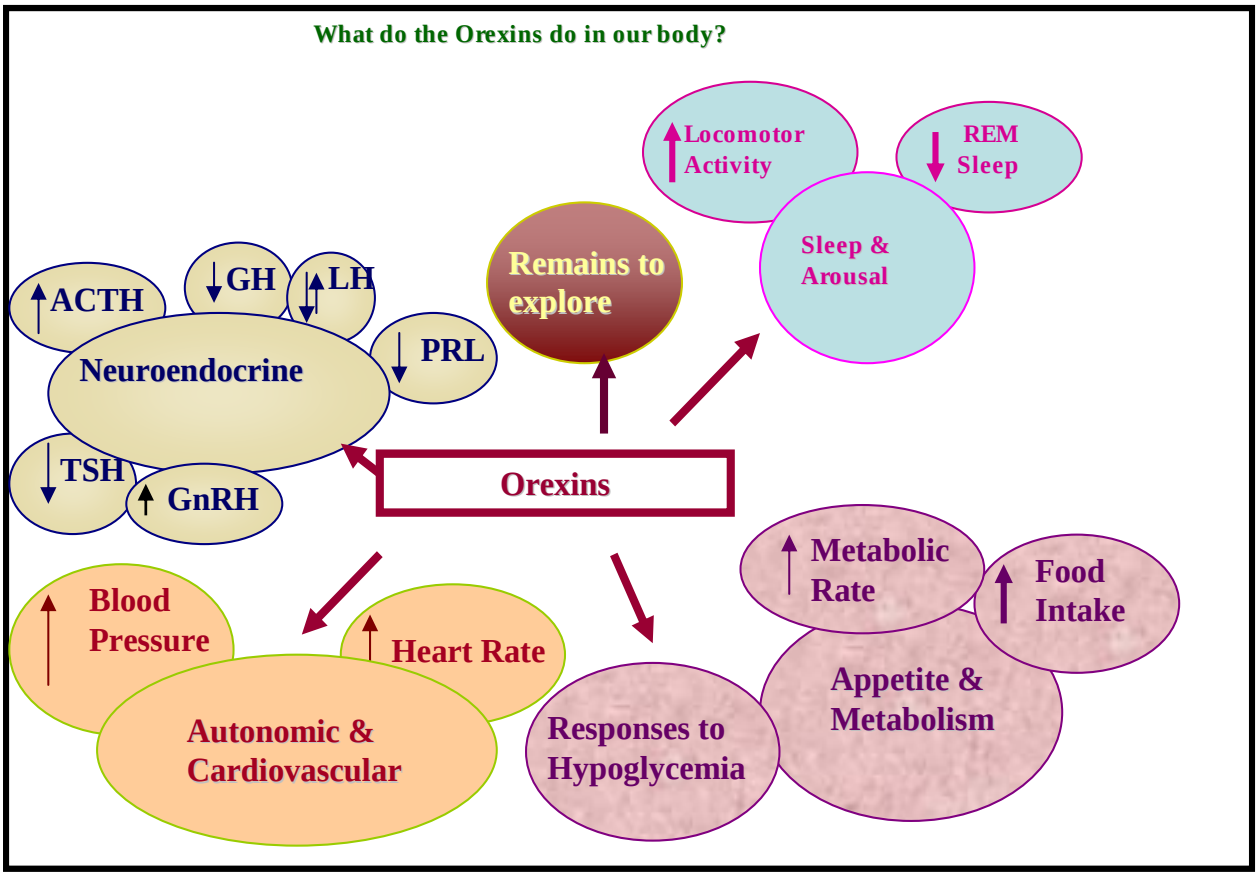

Fig. 1: The rate of orexin on different metabolic pathway.

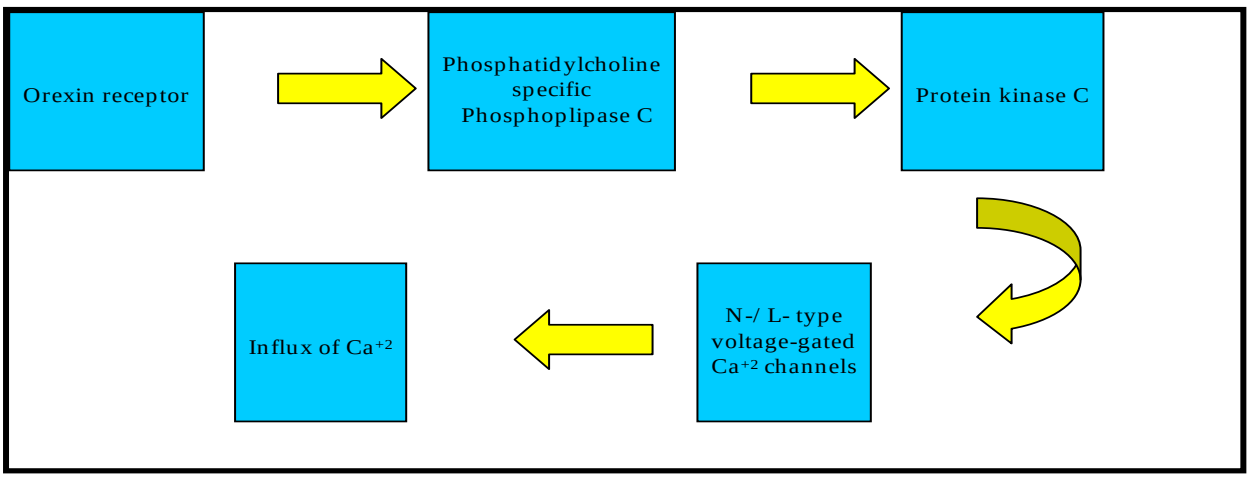

Fig. 2: Proposed mechanism of action of Orexins, a diagram showing its signal transduction pathway.

the rat testis but not OX2R mRNA throughout postnatal development. Peak values were detected in neonatal and puberty to early adult periods. This study reported that the seminiferous tubules but not the Leydig cells are the major source of testicular expression of OX1R gene. These authors did not rule out the possibility of low expression of OX1R gene in the Leydig cells. Emmanouil Karteris et al [36] have reported conflicting results from Barreiro et al [13]. They have shown that Orexins activate the PLC pathway by inducing IP3 production, OX-B being more potent. These results contradict current information in the literature. Moreover testes used in this study were taken from patients having cryptorchidism.

Sun et al 2003 demonstrated that central and peripheral orexin-A immunoreactivities were down- and up-regulated respectively in pregnant rats [37]. The change in circulating

levels of orexin-A and leptin were consistent in pregnant women and rats. Serum levels of orexin-A and leptin correlated positively with fetal gestational age in pregnant women. Higher levels of orexin-A and leptin were being observed in mid- and late- pregnancy than non-pregnant women. These findings are consistent with observations of Garcia et al [38]. On the other hand, some data indicate that preproorexin mRNA expression during late pregnancy is lower than on day 1 post-partum in rats [39]. However, Wang et al. 2003 did not study the difference in prepro-orexin mRNA expression in virgin and pregnant rats, and did not observe orexin-A immunoreactivity [39]. Previous studies on prepro-orexin mRNA expression during pregnancy are confusing, indicating opposite results. One group [38] reported low, while Kanenishi et al. [40] reported high levels of prepro-orexin mRNA in rat brain. Perhaps this discrepancy can be explained by the different methods used in the experiments. Sun et al. 2003 observed different orexin-A immunoreactivity in the hypothalamus during lactation [41], indicating that hypothalamic orexin-A may have different roles in the hyperphagia induced by pregnancy and lactation.

CONCLUSION

Orexins were discovered only a few years ago, and more than one thousand papers have been published on these neuropeptides. These studies demonstrate the anatomic architecture of the orexinergic system as well as the comprehensive role of orexins in the regulation of peripheral functions, either through central control or direct interaction with peripheral target tissues. Recently the discovery of orexins releasing endocrine cells in the hypothalamic pituitary gonadal axis also indicates that orexins may play a role in regulation of reproduction.

More studies have been done on Orexin A than on B, reasons for this are differences in metabolic onset and duration speeds related to the molecular structures. The structure of Orexin A is more complex than that of orexin B, and orexin A may therefore be resistant to inactivating peptidases. Another factor is the difference in the affinities to their receptors. Orexin A binds to both OX1R and OX2R, whereas orexin B binds mainly to OX2R.

These small peptides and their receptors hold great promise and have attracted the attention of pharmaceutical companies wishing to exploit new areas for development of therapeutic agents. On one hand, orexins have great potential to be used as appetite enhancer to pharmacologically affect food ingestion in patients with anorexia nervosa; a disease notoriously resistant to therapeutic manipulation, or the anorexia associated with many disease states, including AIDS wasting syndrome. On the other hand, analogs could be devised that might inhibit the action of orexin so as to be of possible benefit in obesity. However, before effective treatments can be implemented, pathways and transmitters involved in different physiological functions must be resolved. Likewise, increased understanding of the orexin system will require development of novel chemicals acting on orexin receptors as potential targets for other disorders like narcolepsy and cardiovascular disease.

Several open questions remain about the actual roles of orexins on hypothalamic-pituitary gonadal axis. Future studies should focus on elucidating further the potential effects of orexins on reproductive hormones, their signal transduction pathways and also their roles in other peripheral functions. Another important issue to resolve about orexins is their effects on hypothalamic pituitary gonadal axis under fasting conditions. Model systems based on cultured purified cells and orexin or orexin receptor knock-out mice provide important information on orexins effects, signaling, and mechanism of action.

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