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REVIEW ARTICLE

Roles of the hypocretin/orexins in the regulation of sleep and wakefulness

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Abstract

Hypocretin/orexin is produced exclusively in the dorsal and lateral hypothalamus but its projection is widespread within the brain and plays important roles. In this paper, we review the independent discoveries of the hypocretin/orexin peptides, the neuroanatomy of this system, and the link to the sleep disorder narcolepsy that has led to the idea that this system plays a crucial role in the regulation of sleep and wakefulness.

Key words: hypocretin, orexin, narcolepsy, sleep, wakefulness

Discovery of the hypocretins and the orexins

Hypocretins 1 and 2 (Hcrt 1 and Hcrt 2) are hypothalamic neuropeptides derived from a single precursor molecule by proteolytic processing[1,2]. The mRNA encoding these peptides was originally identified in a directional tag PCR subtraction designed to enrich for hypothalamus-specific mRNAs[1]. Because the cell bodies expressing this gene were restricted to an area of the hypothalamus centered around the perifornical hypothalamus (PFH) and because of a weak homology to the gut peptide secretin, these molecules were called the "hypocretins."

Hypocretins were independently discovered by another group of investigators as ligands binding to cell lines expressing orphan G protein-coupled receptors[2]. The chemical isolation procedure that these investigators used allowed them to determine that the longer of these two peptides was 33 amino acids in length and to define the N-terminal pyroglutamyl residue, the intrachain disulfide links, and the expected C-terminal amidation of both peptides. Since intracerebroventricular injections of these peptides increased food intake in rats, these investigators called the peptides orexin-A and orexin-B. This paper also reported functional information on the receptors for the two peptides: the orexin-1 receptor (OX_1R) was shown to preferentially bind orexin-A over orexin-B, whereas the orexin-2 receptor (OX_2R) bound both peptides with similar affinity[2]. In the subsequent issue of *Cell,* Sakurai et al. confirmed the identity of the Hcrts and the orexins[3]. Thus, the *prepro-hypocretin* gene is identical to *prepro-orexin;* Hcrt 2 is identical to orexin-B; and Hcrt1 is equivalent to orexin-A. In this review, we use the terms "Hcrt 1/Ox-A" and "Hcrt2/Ox-B" to denote the two

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peptides and "H/O" to refer to the Hcrt/orexin-containing cells or to the *hcrt/orexin* gene. To refer to the receptors for these peptides, "HcrtR1/OX_1R" and "Hcrt/OX_2R" will be used.

Prepro-hypocretin mRNA is encoded by a gene localized to human chromosome 17q21[2] which consists of two exons and one intron. The first exon includes the 5'-untranslated region and the region that encodes the first 7 amino acid residues of the secretory signal sequence. Exon 2 encodes the remaining portion of the signal sequence and the precursor molecule from which Hcrt1/Ox-A and Hcrt2/Ox-B are proteolytically cleaved[4]. Very little is known about the mechanisms regulating the expression of *prepro-hypocretin* at present. A 3.2 kb fragment of the 5'-upstream region cloned from the human Hcrt gene is sufficient to direct the expression of *Escherichia coli* β-galactosidase gene in transgenic mice in the lateral hypothalamic area and adjacent regions[4]. We have demonstrated that a 450 bp of the 5'-flanking region of the Hcrt gene has the ability to promote expression of a reporter gene *in vitro* and that this expression is down regulated by the sleep promoting cytokine, α-interferon[5]. The promoters of the receptor genes have not yet been characterized.

Neuroanatomy of the hypocretin/orexin system

The distribution of H/O cells has been described both by *in situ* hybridization[1,2] and by immunohistochemistry using antibodies against Hcrt[6], Hcrt1/Ox-A[7-10] and Hcrt2/Ox-B[8,11]. H/O expression is restricted to a few thousand cells in the tuberal hypothalamus centered in the PFH, dorsomedial hypothalamic nuclei and the lateral hypothalamic areas, encompassing about 1 mm anterior-posteriorly in the rat brain. Cells labeled by antisera against Hcrt1/Ox-A or Hcrt2/Ox-B are medium sized (20-30 μm diameter) and multipolar to fusiform in shape. Hcrt1/Ox-A or Hcrt2/Ox-B seem to be co-localized in these cells[8].

Although the H/O cells are a small population with restricted expression, their projections are widely distributed in the brain[6-10] and spinal cord[11]. Particularly abundant processes are found throughout the hypothalamus[6-9]. The densest extrahypothalamic input is to the locus coeruleus (LC)[6,12,13]. Other projection sites include the cerebral cortex, olfactory bulb, hippocampus, amygdala, septum, diagonal band of Broca, bed nucleus of the stria terminalis, thalamus, midbrain, brain stem, and spinal cord. H/O immunoreactivity has also been reported in the enteric nervous system and pancreas[14] and H/O mRNA expression has been detected in testes[2].

The localization of the H/O-containing cells was reminiscent of another peptide found in this region, melanin-concentrating hormone (MCH). Although the distributions of H/O and MCH cells overlap, these peptides are not present within the same cells[6,15,16]. The majority of H/O cells express leptin receptors and are immunoreactive for the transcription factor Stat-3[13,17]. Isolated H/O cells are galanin-immunoreactive[17]. An antiserum raised against ovine prolactin (oPRL) apparently labels every H/O-containing cell in the rat hypothalamus[18]. Since oPRL-like immunoreactivity co-localized with dynorphin B[19,20], bradykinin[20], and secretogranin Ⅱ[21], these latter substances undoubtedly co-localize with H/O.

The concentrations of Hcrt1/Ox-A and Hcrt2/Ox-B have been measured in microdissected rat brain areas by radioimmunoassay[22,23]. Hcrt1/Ox-A and Hcrt2/Ox-B were found to be abundant in the lateral and posterior hypothalamus, ventromedial hypothalamic (VMH), paraventricular thalamic and dorsal raphe nuclei (DRN), the periaqueductal gray and the LC. Moderate levels of the H/O peptides have also been measured in the median eminence, suprachiasmatic, arcuate, supraoptic, and paraventricular nuclei (PVN) of the hypothalamus, the substantia nigra, and the nucleus tractus solitarius. Low concentrations of Hcrt1/Ox-A and Hcrt2/Ox-B have been found in the cerebral cortex. In these studies, Hcrt2/Ox-B contents have always been 2-3 fold greater than the Hcrt1/Ox-A contents. Hcrt1/Ox-A has also been detected in cerebrospi-

nal fluid[24] and in plasma[25], but not in peripheral tissue or in the pineal and pituitary glands[26]. The Hcrt1/Ox-A distribution in hypothalamus apparently differs between males and females[26].

HcrtR1/OX_1R and HcrtR1/OX_2R mRNAs are differentially distributed throughout the brain. In the hypothalamus, high levels of HcrtR1/OX_1R mRNA were initially reported in the VMH while HcrtR2/OX_2R mRNA was found to be highly expressed in the PVN[27]. More recent studies[28,29] have shown moderate to high levels found throughout the hypothalamus although some nuclei, such as the arcuate, mammillary and tuberomammillary nuclei (TMN), apparently express only the HcrtR2/OX_2R. High levels of HcrtR1/OX_1R mRNA have also been reported in the tenia tecta, the bed nucleus of the stria terminalis, the CA1 and CA2 fields of the hippocampus, the amygdalohippocampal area, the lateral dorsal tegmental and pedunculopontine nuclei (LDT and PPT) and the LC. HcrtR2/OX_2R has mainly been found in olfactory tubercule, shell of the nucleus accumbens, the lateral and medial septal nuclei, central medial thalamic nuclei, the subthalamic and anterior pretectal nucleus, and the CA3 region of the hippocampus. In the cerebral cortex, layers Ⅲ, Ⅴ and Ⅵ also express HcrtR1/OX_1R mRNA whereas the HcrtR2/OX_2R mRNA is found in layers Ⅱ and Ⅵ. Some brain regions such as the paraventricular thalamic nucleus, the DRN and ventral tegmental area (VTA) express the mRNA for both receptors in roughly equal abundance. The expression of H/O receptors has also been reported in the adrenal gland[30], enteric nervous system, and pancreas[14].

The hypocretin/orexins and food intake regulation

As indicated above, H/O expression is restricted to the PFH, an area classically associated with the regulation of food intake. Since the original description of the orexigenic effects of the H/O peptides[2], other investigators have confirmed that administration of Hcrt1/Ox-A into the lateral ventricles to fed rats stimulated food consumption[31-33]. Fasting has been shown to upregulate *prepro-H/O* mRNA levels[2]. Injection of neutralizing anti Hcrt-antibodies significantly suppressed feeding in fasted rats[34]. Peripheral administration of a novel HcrtR1 antagonist inhibited natural and stimulated feeding for several days[35].

The H/O neurons are in close proximity to the cells that contain MCH, a potent promoter of food consumption[36,37]. The composite distribution of the HcrtR1/OX_1R and HcrtR2/OX_2R mRNAs summarized above strongly resembles the distribution of MCH receptor mRNAs[38,39], with the highest receptor levels for both systems being present within the hypothalamus, the LC, and the hippocampal formation. Despite the similarities between these two systems, H/O-and MCH-containing neurons apparently differ in their response to stimuli. H/O cells increase firing upon application of glucose[40] whereas MCH cells are insensitive to deoxyglucose[41]. Administration of agouti-related protein (AGRP) increases MCH but not *H/O* gene expression[42], suggesting that the promoters of these genes respond to different signals that regulate food intake. Nonetheless, the similarity in the localization of the cell bodies for these two peptide systems and the overlapping distributions of their receptors strongly suggests an interaction between the two systems in some homeostatic functions[39].

As indicated above, the widespread anatomical projections of the H/O neurons suggest that they may be involved in multiple functions. In addition to food intake regulation, some of the functions in which this system has been implicated include neuroendocrine[43-47], cardiovascular[48,49], water balance[50], and gastrointestinal[51] control.

The hypocretin/orexins and the sleep disorder narcolepsy

Two independent lines of research converged in 1999 to implicate the H/O system in the sleep disorder narcolepsy. Narcolepsy is a neurological

disorder that specifically affects the generation and organization of sleep[52]. Narcoleptic patients have persistent abnormal daytime sleepiness and fragmented sleep at night[52-54]. The pathognomic symptom of narcolepsy is cataplexy, the sudden loss of skeletal muscle tone for periods that can vary from seconds to minutes that is usually precipitated by strong emotion[52,54]. Other symptoms include sleep paralysis, hypnogogic hallucinations and sleep onset rapid eye movement (REM) periods: while normal individuals begin their rest with non-REM sleep, narcoleptic patients go directly into REM sleep, often accompanied by dreamlike hallucinations and muscle tone paralysis[54]. Because of these traits, it has been hypothesized that the symptoms of narcolepsy result from the malfunction of some aspects of the REM sleep control mechanism[55].

A genetic component for narcolepsy has been established in both humans and dogs. In narcoleptic dogs, the *canarc-1* mutation is transmitted in Doberman pinschers and Labrador retrievers as an autosomal recessive trait with full penetrance. The first link between the H/O system and narcolepsy was identification of *canarc-1* as a deletion mutation in the *HcrtR2/OX$_2$R* gene which results in a truncated, nonfunctional protein[56]. In a remarkable convergence, *H/O* null mutant mice were found to exhibit periods of "behavioral arrest" that strongly resemble the cataplectic attacks and sleep-onset REM periods characteristic of narcolepsy in other species[57]. These mice also have an altered sleep architecture, as evidenced by increased levels of both REM and non-REM sleep, short latency REM periods, and decreased sleep bout lengths, primarily during the dark (active) period. Together, these two papers indicate that dysfunction of either the H/O ligand or one of its receptors can result in narcolepsy.

Although mutations in genes of the hypocretin system are directly responsible for the etiology of narcolepsy in animals, human narcolepsy appears to be different and, in most cases, seems to be transmitted as a multifactorial trait. Genetic studies suggest a relationship between human narcolepsy and the immune system[58-60]. Across ethnic groups, expression of the MHC class Ⅱ allele HLA-DQB1*0602 confers increased susceptibility to narcolepsy[22]. Studies of familial narcolepsy also demonstrate that environmental factors acting on a specific genetic background may contribute to susceptibility to narcolepsy[61]. The high degree of discordance in identical twins further implicates environmental influences. Nonetheless, at least one human narcoleptic bears a mutation in the *H/O* gene[62] but no receptor mutations have been reported to date. The likely cause of narcolepsy in the majority of human cases was recently described. An abnormality in H/O neurotransmission in narcoleptic humans was first suggested by undetectable levels of Hcrt1/Ox-A in cerebrospinal fluid (CSF) from 7 of 9 narcoleptic patients[24]. Subsequent studies show that human narcolepsy is associated with loss of H/O-containing cell bodies in the PFH. In postmortem analyses, *H/O* mRNA was undetectable in two narcoleptic brains, although mRNA for MCH was readily detectable in both controls and narcoleptics[62]. Immunohistochemistry showed an 85-95% reduction in the number of H/O-containing cells in narcoleptic brains with no evident change in the number of MCH cells[63]. These studies suggest that degeneration of the H/O cells may be the cause of human narcolepsy. Increased staining for glial fibrillary acid protein in the PFH combined with the previously described genetic link with the HLA system suggests that the H/O cells may degenerate by an autoimmune mechanism[63].

The hypocretin/orexins and sleep/wake regulation

The involvement of H/O system in the pathophysiology of narcolepsy suggests a role for this system in 'normal' sleep/wake regulation. The region of the posterior hypothalamus (PH) containing the H/O cells has long been implicated in arousal state control. Subsequent to World War I, a viral pandemic resulted in *encephalitis lethar-*

gica in which patients experienced persistent sleepiness that was attributed to injury of the PH and rostral midbrain[64]. Experimental lesions of the posterior lateral hypothalamus also causes persistent hypersomnia in monkeys[65], rats[66] and cats[67]. The H/O cells in the PH project to the monoaminergic and cholinergic groups historically implicated in arousal state regulation. Intracerebroventricular injections of Hcrt1/Ox-A into rats at light onset (the major sleep period) increases arousal and locomotor activity accompanied by a marked reduction in REM sleep and deep slow wave sleep[68]. A diurnal variation in Hcrt1/Ox-A immunoreactivity has been reported in the pons and in the preoptic/anterior hypothalamic region and in *prepro-hcrt* mRNA in the hypothalamus[26], further supporting involvement of the H/O system in arousal state regulation. On the other hand, *prepro-hcrt* mRNA levels were unaffected in either rats and mice by a period of sleep deprivation sufficient to cause elevation of EEG delta power activity during recovery sleep after sleep deprivation[69].

Any influence of the H/O system on sleep and wakefulness is likely to be manifest through projections of the H/O cells onto the monoaminergic and cholinergic cell groups[70]. In addition to intense innervation of the LC mentioned above[6], numerous synaptic contacts have been observed between H/O boutons and noradrenergic cells of the LC[13]. H/O terminals are also in close proximity to histaminergic cells of the TMN, serotonergic cells of the DRN and cholinergic cells in both the pons (LDT and PPT) and basal forebrain (diagonal band of Broca and the medial septum)[57]. These cholinergic areas are thought to be involved in the EEG desynchronization characteristic of waking and REM sleep.

Diagnosis/drug treatment of narcolepsy

Current treatment for narcoleptic patients employ monoaminergic amphetamine-like and antidepressant compounds[71]. These compounds have many side effects and only partially control the condition. The loss of H/O neurons in the hypothalamus[62,63], reduction of Hcrt1/Ox-A levels in the CSF of narcoleptic patients[24], and the existence of a mutation in the *H/O* gene of a child with severe symptoms[62] strongly supports a central role for H/O deficiency in the human narcolepsy and suggests several possibilities for improved diagnosis and treatments. First, monitoring CSF Hcrt1/Ox-A levels will be useful for diagnosis of narcolepsy. Second, supplementation of H/O transmission with receptor agonists will likely be an effective treatment strategy, at least for the sleepiness aspects of narcolepsy since, fortunately, human narcoleptics do not have the receptor mutation found in narcoleptic canines[62]. Conversely, selective antagonists for H/O receptors might be also useful for sleep promotion, since H/O system seems to be important for maintenance of wakefulness. These compounds should be stable *in vivo* to have a long lasting effect and also, should cross blood-brain barrier so that the drug can be given peripherally. The first orexin receptor antagonist for insomnia treatment was recently reported[72] and more compounds will follow in the near future. Regulation of the activity of the H/O neurons is another potential therapeutic avenue.

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