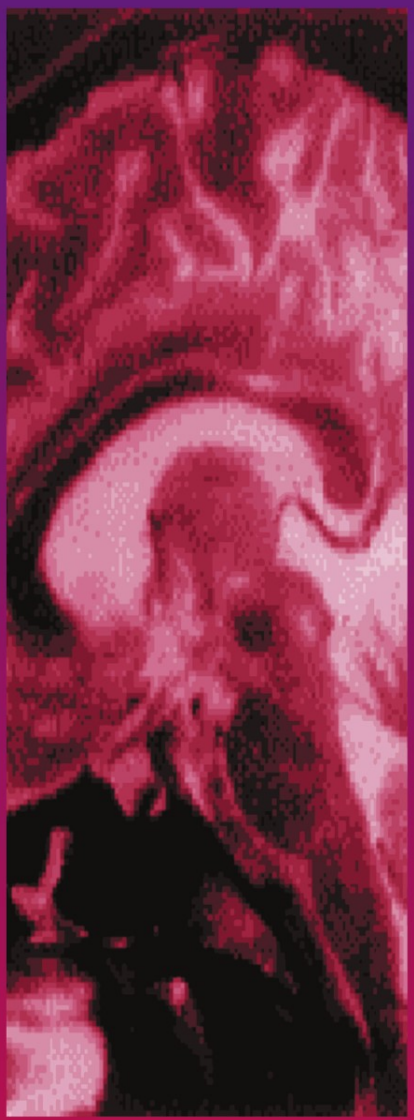
The Orexin/Hypocretin System

Physiology and Pathophysiology



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The Orexin/Hypocretin System

Contemporary Clinical Neuroscience

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PREFACE

Orexin/hypocretin research began in 1998, as a result of the discovery of a new hypothalamic neuropeptide. In 1999, it was found that mutations in the orexin/hypocretin-related genes caused a sleep disorder (narcolepsy) in dogs and mice. These findings were soon followed by the discoveries of orexin/hypocretin ligand deficiency in human narcolepsy.

The finding of the major pathophysiological mechanisms of human narcolepsy resulted in its reclassification as a neurological, not a psychiatric, disorder. The importance of early diagnosis and initiation of treatment for human narcolepsy has been repeatedly emphasized because the disease typically starts around puberty (when social and school influences become important). Orexin/hypocretin deficiency in narcolepsy subjects can be detected clinically in cerebrospinal fluid (CSF) orexin/hypocretin measures (low CSF orexin/hypocretin levels are strongly associated with narcolepsy–cataplexy among various neurologic and sleep disorders). Thus, the CSF orexin/hypocretin measurements are expected to be included as a diagnostic test for narcolepsy–cataplexy in the second revision of international diagnostic criteria (ICSD). This positive diagnostic test is very useful for establishing an early diagnosis for narcolepsy–cataplexy, and many patients will likely receive immediate benefits. Cerebrospinal orexin/hypocretin measurements are also informative for the nosological classification of hypersomnia. Because orexin/hypocretin deficiency is observed in most human narcolepsy–cataplexy, orexin/hypocretin replacement therapy is now a promising new choice for the treatment of human narcolepsy, and research in this area is actively in progress.

The Orexin/Hypocretin System: Physiology and Pathophysiology examines these exciting discoveries and presents new findings such as ligand replacement and gene therapies in animal models of narcolepsy. The next important step for narcolepsy research is to discover the pathological mechanisms for the loss of orexin/hypocretin neurons in humans. This information is critical to prevention or cure of the disease, and another breakthrough in this area is expected in the not too distant future.

How the orexin/hypocretin system physiologically regulates sleep and wakefulness remains largely unknown. It is not fully understood how and why the symptoms of narcolepsy occur when orexin/hypocretin neurotransmission is impaired. Sleep is a complex physiological phenomenon, and multiple systems are involved in its regulation. Because we were reluctant to request just one author to cover the roles of orexin/hypocretin in sleep regulation, we invited several contributors who are working in this field to freely discuss their opinions; as a result we could not avoid significant overlaps among these chapters. Because we did not instruct the authors to unify their hypotheses, controversies may also exist. However, there is room for readers to actively participate in these debates and to carry out the experiments to prove or disprove these hypotheses.

The orexin/hypocretin system is also of exceptional interest in neuroscience research. In addition to its involvement in vigilance control and narcolepsy, the system likely regulates various hypothalamic functions such as neuroendocrine functions, stress reactions, and autonomic functions necessary for human survival. Numerous researchers have initiated multidisciplinary approaches in order to understand the various aspects of the physiological functions of the orexin/hypocretin system. In the same way, narcolepsy is a useful disease model for understanding the link between vigilance control with other fundamental hypothalamic functions, such as regulation of feeding behavior and autonomic function. Similarly, clinical applications of orexin/hypocretin agonists and antagonists for various diseases are suggested.

We introduced several experimental methods for orexin/hypocretin research and discussed the use and limitations of these methods that are useful for the multidisciplinary approaches in the orexin/hypocretin research field, as well as for other neuropeptidergic systems.

Finally, we would like to emphasize that rapid, significant success in narcolepsy research has not been achieved without careful observations in the appropriate animal models of the disease. These approaches, which are used to study narcolepsy, will now encourage researchers to initiate genetic linkage and positional cloning experiments, as well as to generate various genetically engineered animal models. A link between orexin/hypocretin ligand deficiency and narcolepsy in orexin/hypocretin knockout mice could not have been made without excellent scientific acumen combined with a modicum of luck. Tenacious efforts by researchers, together with the application of modern technologies, made these breakthroughs possible in a timely manner.

Living in a post-genome era, the success of the orexin/hypocretin story is driving many researchers to search novel bioactive peptides and their receptors for further discoveries in physiology, and these are likely to lead to novel opportunities for clinical treatments. Orexins/hypocretins are one of the first endogenous ligands discovered for orphan G protein–coupled receptors. Since 1995, about 70 ligands and/or orphan receptors have been identified or re-recognized. There are still about 100 orphan receptors, and the search for the endogenous ligands for these receptors is actively in progress. It is therefore possible that the abnormal function of some of the unknown ligands and uncharacterized receptors are directly involved in the etiology and/or pathophysiology of several neurologic and psychiatric disorders. The study of the orexin/hypocretin system is a good example of what can be achieved.

We would like to acknowledge Dr. Shuji Hoshino (Hoshino Surgical Clinic, Hiroshima) for the generous gift to support the project, and Edward Tuan for his editorial and clerical assistance.

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I
History

1

History and Overview of Orexin/Hypocretin Research

From Orphan GPCR to Integrative Physiology

Michihiro Mieda, PhD, and Takeshi Sakurai, MD, PhD

1. INTRODUCTION

Since its discovery in 1998, the field of orexin/hypocretin biology has grown rapidly. In the last 6 yr, more than 900 articles on orexin/hypocretin research have been published. Information on the role of the orexin/hypocretin system in narcolepsy–cataplexy has had a huge impact on the study of sleep and wakefulness. Scientists are now using a multidisciplinary approach to understand various aspects of the physiological functions of orexins/hypocretins in order to apply orexin/hypocretin biology to the diagnosis and treatment of sleep-related disorders.

2. DISCOVERY OF OREXIN/HYPOCRETIN

Identification of orexin/hypocretin peptides in 1998 was the result of a beautiful convergence of two independent research groups utilizing completely different methodologies. de Lecea et al. *(1)* utilized a molecular biological technique to isolate a series of cDNA clones that are expressed in the hypothalamus but not in the cerebellum and the hippocampus by subtractive hybridization. One of these was expressed exclusively by a bilaterally symmetric structure within the posterior hypothalamus. They subsequently cloned cDNAs covering the entire coding region, which encodes a putative secretory protein of 130 amino acids. According to its primary sequence, they predicted that this protein gives rise to two peptide products that are structurally related to each other. Because these predicted peptides were expressed in the *hypo*thalamus and had similarity to se*cretin*, they named them hypocretin-1 and -2 *(1)*.

It was later learned that hypocretins (orexins) are, in fact, not relatives of the incretin family; receptors for these peptides have considerably different structures from those of the incretin recepor superfamily, which usually couple to the Gs family of G proteins. *Hypocretin* mRNA was detected only in the brain, and antibodies raised against prepro-hypocretin identified hypocretin-positive cell bodies exclusively in the perifornical area of the hypothalamus and hypocretin-positive nerve fibers in many brain areas. It was further demonstrated by electron microscopy and electrophysiology studies that hypocretin is present in synaptic vesicles, and also that hypocretin-2 has excitatory effects on hypothalamic neurons but not on hippocampal dentate granule neurons. Although their prediction of mature peptides processed from prepro-hypocretin later turned out to be inexact, their study strongly suggested that hypocretin peptides are novel neurotransmitters exclusively expressed in a population of neurons in the perifornical area.

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Around the same time as the report by de Lecea et al., Sakurai et al. *(2)* reported identification of two peptides (orexin-A and orexin-B) that were endogenous ligands of two orphan G-protein-coupled receptors (GPCRs) whose cognate ligands had not been identified, which they named orexin receptor 1 (OX_1R) and orexin receptor 2 (OX_2R). These peptides are cleaved from a single-precursor polypeptide prepro-orexin, which is expressed by a select population of neurons clustered around the perifornical lateral hypothalamus. It was later learned that prepro-orexin and prepro-hypocretin were identifical and that orexin-A and -B corresponded to hypocretin-1 and -2, respectively. However, the original structures of predicted hypocretin-1 and -2 were not identical to purified orexin-A and -B because of an incorrect prediction of proteolytic sites, as well as the loss of two intrachain disulfate bonds and N-terminal pyroglutamylation in hypocretin-1, which were found in orexin-A. Since these peptides were exclusively expressed in neurons of the lateral hypothalamus, which has been implicated as a "feeding center," intracerebroventricular (icv) administration of orexin-A or -B in the lateral ventricle was examined and found to increase food intake in rats dose dependently; this was the reason these peptides were named "orexin" after the Greek word *orexis*, meaning appetite. Furthermore, expression of *prepro-orexin* mRNA was upregulated more than twofold upon fasting, just like *neuropeptide Y* (*NPY*) mRNA, whose product is a well-known feeding peptide.

3. LOSS OF OREXIN/HYPOCRETIN SIGNALING CAUSES NARCOLEPSY–CATAPLEXY

Soon after the discovery of orexin/hypocretin, two independent studies utilizing dog forward genetics and mouse reverse genetics dramatically increased our understanding of orexin/hypocretin biology. Human narcolepsy–cataplexy is a debilitating neurological disease characterized by excessive daytime sleepiness, premature transitions to REM sleep (so-called sleep-onset REM periods), and cataplexy (sudden bilateral skeletal muscle weakness without impairment of consciousness) *(3,4)*. Scientists at Stanford University had established and been maintaining canine breeds with autosomal recessive inheritance of a narcolepsy syndrome for decades. In 1999, Lin et al. *(5)* at Stanford succeeded in identifying mutations in the *OX_2R/hypocretin* receptor 2 *(hcrtr2)* gene responsible for canine narcolepsy–cataplexy by positional cloning.

In the same month, Chemelli et al. *(6)* reported *prepro-orexin* knockout mice that exhibited a phenotype strikingly similar to human narcolepsy–cataplexy, characterized by cataplexy-like abrupt behavioral arrests, sleep/wake state fragmentation, non-REM sleep, and direct transitions from wakefulness to REM sleep. Orexin/hypocretin-immunoreactive nerve terminals were found on neurons implicated in arousal regulation, thus demonstrating the important role of orexin/hypocretin in sleep/wake regulation.

Subsequently, disruptions of the orexin/hypocretin system in human narcolepsy–cataplexy were confirmed. Nishino et al. *(7,8)* found that orexin-A/hypocretin-1 was undetectable in the cerebrospinal fluid (CSF) of up to 95% of narcolepsy–cataplexy patients but was readily detected in normal control individuals. Drastic reductions of *orexin/hypocretin* mRNA and immunoreactivity in postmortem brains of narcolepsy–cataplexy patients were also shown by Peyron et al. *(9)* and Thannickal et al. *(10)*. Furthermore, an unusually severe, early-onset case of human narcolepsy–cataplexy was associated with a mutation in the *orexin/hypocretin* gene that impairs peptide trafficking and processing *(9)*. These studies of human and animals alike established that the failure of signaling mediated by orexin/hypocretin neuropeptides causes narcolepsy–cataplexy.

4. MORE THAN JUST SLEEP/WAKE REGULATION

Even before the discovery of their linkage to narcolepsy–cataplexy, a detailed description of projection patterns of orexin/hypocretin–positive neurons suggested their involvement in a wide range of physiological functions, such as feeding, autonomic regulation, neuroendocrine regulation, and sleep/wake regulation *(11,12)*. Distribution of orexin/hypocretin–responsive neurons, marked with Fos expression induced by icv administration of orexin/hypocretin peptides, supported this prediction *(12)*.

Orexin/hypocretin administration experiments have demonstrated their multiple pharmacological effects. Since the initial report of orexin/hypocretin as feeding peptides *(2)*, increased food intake by laboratory animals following icv administration of orexin/hypocretin peptides has been reproduced. In addition to their effect on feeding, central administration of orexin/hypocretin peptides has been reported to cause a wide variety of physiological responses, such as an increase in motor activity, wakefulness (and suppression of sleep), energy expenditure, sympathetic outflow, gastric acid secretion and gastric motility, and hypothalamus–pituitary–adrenal (HPA) axis activity, as well as suppression of secretion of some hormones such as growth hormone, prolactin, and thyroid-stimulating hormone *(13–18)*.

Subsequently, many microinjection experiments into local brain areas identified putative target neurons that mediate effects of central orexin/hypocretin administrations. A confounding factor of microinjection experiments has been the dual innervation of orexin/hypocretin neurons to both excitatory and inhibitory areas as regards a certain physiological function. For example, REM sleep-related muscle atonia can be facilitated or inhibited by local orexin/hypocretin administration depending on the site of injection in brainstem areas *(19–21)*. Similarly, orexin/hypocretin neurons make excitatory input connections to both autonomic excitatory and inhibitory areas of the heart rate: the rostral ventrolateral medulla and the rostral ventromedial medulla, which mediates sympathetic tachycardia; and the nuclei of the solitary tract and ambiguus, which mediate sympathoinhibition and vagal bradycardia *(22–25)*. The balance between inputs into excitatory and inhibitory areas seems important, and therefore the dynamics of the action of the orexin/hypocretin system, as well as its interactions with other neuronal systems, should be considered *(26)*.

Orexin/hypocretin peptides have always been characterized as neurotransmitters *(1,27)*, and examination of neuronal responses to orexin/hypocretin application by in vitro electrophysiological recordings has also been carried out to identify downstream neurons of the orexin/hypocretin system and their signaling mechanisms. Site of action (pre or post), receptor subtype responsible, and downstream intracellular signaling cascade depend on the types of neuron examined, but the actions reported so far are mostly excitatory on postsynaptic cells and facilitatory on presynaptic release.

5. INTEGRATIVE PHYSIOLOGY OF THE OREXIN/HYPOCRETIN SYSTEM

As described above, central administration of orexin/hypocretin peptides causes a wide variety of effects. The critical question is whether these pharmacological effects are relevant to functions of endogenous orexin/hypocretin neurons in physiological conditions. The essential role of orexin/hypocretin on regulation of the sleep/wake cycle has been supported by several different approaches and seems valid. As described above, loss of orexin/hypocretin signaling causes narcolepsy–cataplexy in the human, dog, and mouse. Icv administration of orexin/hypocretins increases wakefulness and suppresses both non-REM and REM sleep. Orexin/hypocretin–positive nerve terminals and orexin/hypocretin receptors are found in

nuclei previously implicated in sleep/wake regulation, such as the tuberomammillary nucleus, locus ceruleus, dorsal raphe nucleus, pedunculopontine tegmental nucleus/laterodorsal tegmental nucleus, and basal forebrain. Microinjection of orexin/hypocretin into these areas in vivo increases wakefulness and suppresses sleep, and application of orexin/hypocretins excites neurons of these areas in vitro *(28)*. Nevertheless, several researchers are now claiming that orexin/hypocretin peptides regulate motor activity rather than wakefulness *per se*, and maintenance of wakefulness by orexin/hypocretins may be secondary to increased activity *(26,29,30)*. Whether orexin/hypocretins regulate wakefulness and/or motor activity or even higher functions, such as alertness and attention, requires further study.

So what about other functions of orexin/hypocretins proposed by pharmacological studies? One pharmacological effect of orexin/hypocretin initially reported was stimulation of food intake *(2)*. Although the efficacy of orexin/hypocretin was lower than that of NPY, a well-known feeding peptide, it was as potent as other appetite-stimulating peptides such as melanin-concentrating hormone (MCH), and this effect of orexin/hypocretin was reproduced in several laboratories *(31,32)*. In support of this idea, administration of anti-orexin/hypocretin antibody or an OX_1R-selective antagonist reduced food intake *(33,34)*, and *prepro-orexin* knockout mice and transgenic mice lacking orexin/hypocretin neurons ate less than control wild-type mice *(35,36)*. However, some researchers claim that the increase in food intake caused by orexin/hypocretin administration is secondary to an increase in wakefulness and activity *(37)*. However, other arousal-promoting substances do not always increase food intake. For example, corticotropin-releasing factor has a strong arousal effect yet suppresses appetite.

The "feeding peptides" such as NPY usually decrease energy expenditure while increasing food intake, which is more reasonable under the situations animal need to save energy *(38)*. In contrast, orexin/hypocretin increase energy expenditure. Therefore, orexin does not simply act as a system that maintains long-term body weight homeostasis. The function of orexins might be necessary for food seeking and feeding behaviors, especially when animals are faced with scarcity. Food seeking and food intake require more vigilant states and more energy expenditure. Recent evidence has suggested that orexin/hypocretin neurons are capable of sensing indicators of energy balance such as glucose and leptin; negative energy balance activates orexin/hypocretin neurons *(39–41)*. These findings raised a hypothesis that orexin/hypocretin neurons link energy homeostasis and sleep/wake regulation so that they allow animals to increase arousal under negative energy balance *(41)*. Similarly, other proposed roles of orexin/hypocretins, such as regulation of autonomic and endocrine systems, need to be studied further. Detailed characterization of disturbances in these systems in animals lacking orexin/hypocretin signaling pathways (including human narcolepsy–cataplexy patients) is needed.

Overall, it is likely that the orexin/hypocretin system not only promotes arousal but also regulates autonomic and endocrine systems so that physiological conditions of the whole body are well coordinated on demands of external and internal circumstances to awake and execute behaviors. We know that the orexin/hypocretin system regulates many downstream targets, but we have little knowledge about when orexin/hypocretin neurons are activated or about the upstream signals that regulate activities of orexin/hypocretin neurons. These questions have been addressed by counting Fos expression in orexin/hypocretin neurons, monitoring release of orexin/hypocretin by measuring orexin/hypocretin content in the CSF or microdialysis perfusates, and in vitro recording of activities of orexin/hypocretin neurons. These studies have suggested several factors as regulators of orexin/hypocretin neurons, such as energy balance, stress, and the circadian pacemaker *(39–44)*. It is very helpful to

systematically describe afferent pathways to orexin/hypocretin neurons. In addition, monitoring activities of orexin/hypocretin neurons in vivo with high temporal resolution, such as single-unit recording in unanethetized animals, would be essential to understand regulation of orexin/hypocretin neurons, as well as to understand their physiological roles.

The orexin/hypocretin system seems to have multiple upstream activators and downstream targets. Thus, it is important to ask whether physiological responses induced by activation of orexin/hypocretin neurons are stereotyped or variable depending on the natural conditions that cause activation of orexin/hypocretin neurons. It might be the combination of the orexin/hypocretin system and the other neuronal systems that determines the specific pattern of responses upon a certain circumstance, or the orexin/hypocretin system might have some heterogeneity within itself so that each subpopulation of orexin/hypocretin neurons can elicit physiological responses different from each other (for example, *see* ref. *45*).

6. CLINICAL POTENTIALS OF OREXIN/HYPOCRETIN PEPTIDES

In addition to providing a better understanding of the regulation of the sleep/wake cycle, discovery that the cause of human narcolepsy–cataplexy correlates with the loss of orexin/hypocretin production has had a tremendous impact on our clinical understanding of human narcolepsy–cataplexy. Narcolepsy–cataplexy is thought to be an autoimmune disease because of its strong association with certain human leukocyte antigen alleles *(46)*. Narcolepsy–cataplexy may result from selective autoimmune degeneration of orexin/hypocretin neurons. Indeed, residual gliosis was observed in the perifornical area of postmortem brains of narcolepsy–cataplexy patients *(10)*. Thus, we can now concentrate on orexin/hypocretin neurons as a target of research to understand the molecular and genetic mechanisms surrounding the development of narcolepsy–cataplexy, which might lead to accurate estimation of disease risk and eventually to prevention of disease onset.

This research on orexin/hypocretin peptides has led to the development of a novel, definitive diagnostic test for human narcolepsy–cataplexy. Mignot and his colleagues at Stanford *(7)* found that low orexin-A/hypocretin-1 levels in CSF is specifically correlated to patients with narcolepsy–cataplexy. The current nosology of narcolepsy–cataplexy is controversial. Measurements of CSF orexin-A/hypocretin-1 levels are expected to complement current diagnosis of narcolepsy–cataplexy.

Finally, the discovery of orexin/hypocretin has brought about the possibility of novel therapies for narcolepsy–cataplexy. Currently, excessive sleepiness is treated using psychostimulants, and cataplexy is treated with tricyclic antidepressants.γ-Hydroxybutyrate (sodium oxybate) is also used to consolidate nocturnal sleep and reduce cataplexy *(47)*. This therapeutic regimen is problematic owing to limited effectiveness, undesirable side effects such as insomnia or symptom rebounds, and the potential for abuse. We recently showed that ectopic expression of a *prepro-orexin/hypocretin* transgene or administration of orexin-A/hypocretin-1 in the brain of orexin/hypocretin neuron-ablated mice suppressed the narcoleptic phenotype of these mice *(48)*. These results indicate that orexin/hypocretin neuron-ablated mice retain the ability to respond to orexin/hypocretin neuropeptides and that a temporally regulated and spatially targeted secretion of orexin/hypocretins is not necessary to prevent narcoleptic symptoms. A similar result was also obtained by Fujiki et al. *(49)*, demonstrating that orexin-A/hypocretin-1 administered intravenously at an extremely high dose induces a very short-lasting anticataplectic effect in an orexin/hypocretin-deficient narcoleptic dog. Thus, an orexin/hypocretin receptor agonist would be of potential value for treating human narcolepsy–cataplexy. The development of stable, blood-brain barrier-permeable agonists for orexin/hypocretin receptors is still

awaited. Such agonists might also be useful in the treatment of other conditions involving excessive daytime sleepiness in humans. In addition, orexin/hypocretin receptor antagonists may also prove useful as safe hypnotics and antiobesity drugs *(50).*

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II

Overview of the Orexin/Hypocretin Neuronal System

2
Orexin and Orexin Receptors

Takeshi Sakurai, MD, PhD

1. INTRODUCTION

"Reverse pharmacological" approaches, i.e., ligand identification using cell lines expressing orphan receptors, combined with genetic engineering techniques, have increased our understanding of novel signaling systems in the body *(1)*. Orexin/hypocretin is the first example of the factors that were successfully applied using such approaches *(2)*. Our group initially identified orexin-A and orexin-B as endogenous peptide ligands for two orphan G-protein–coupled receptors found as human expressed sequence tags (ESTs) *(2)*. This chapter discusses structures and functions of orexin neuropeptides and their receptors.

2. IDENTIFICATION OF OREXIN

Most neuropeptides work through G-protein–coupled receptors (GPCRs). There are numerous (approx 100–150) "orphan" GPCR genes in the human genome; the cognate ligands for these receptor molecules have not been identified yet. We performed an approach, so-called reverse pharmacology, that aims to identify ligands for orphan GPCRs. We expressed orphan GPCR genes in transfected cells and used them as a reporter system to detect endogenous ligands in tissue extracts that can activate signal transduction pathways in GPCR-expressing cell lines. We identified orexin-A and orexin-B as endogenous ligands for two orphan GPCRs found as human ESTs *(2)*.

Structures of orexins were chemically determined by biochemical purification and sequence analysis by Edman sequencing and mass spectrometry *(2)*. Orexins constitute a novel peptide family, with no significant homology with any previously described peptides. Orexin-A is a 33-amino-acid peptide of 3562 Dalton, with an N-terminal pyroglutamyl residue and C-terminal amidation (Fig. 1). The molecular mass of the purified peptide as well as its sequencing analysis indicated that the four Cys residues of orexin-A formed two sets of intrachain disulfide bonds. The topology of the disulfide bonds was chemically determined to be [Cys6-Cys12, Cys7-Cys14]. This structure is completely conserved among several mammalian species (human, rat, mouse, cow, sheep, dog, and pig). On the other hand, rat orexin-B is a 28-amino-acid, C-terminally amidated linear peptide of 2937 Dalton, which was 46% (13/28) identical in sequence to orexin-A (Fig. 1A). The C-terminal half of orexin-B is very similar to that of orexin-A, whereas the N-terminal half is more variable. Mouse orexin-B was predicted to be identical to rat orexin-B. Human orexin-B has two amino acid substitutions from the rodent sequence within the 28-residue stretch. Pig and dog orexin-B have one amino acid substitution from the human or rodent sequence.

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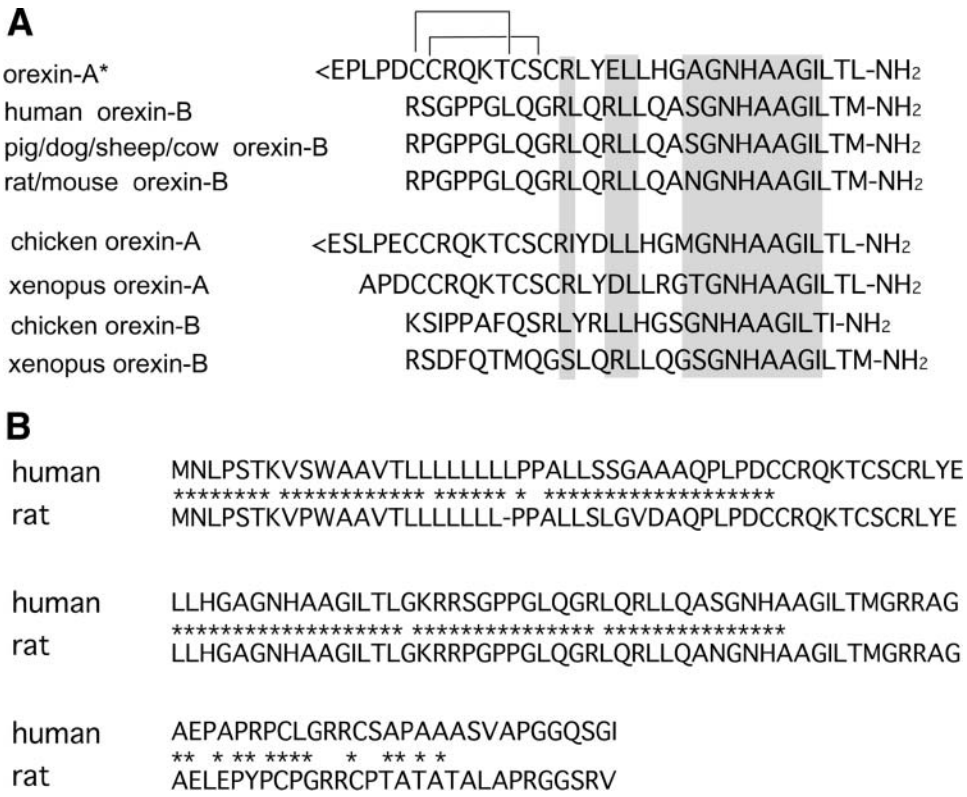

Fig. 1. **(A)** Structures of mature orexin-A and -B peptides. The topology of the two intrachain disulfide bonds in orexin-A is indicated above the sequence. Amino acid identities are indicated by shaded boxes. **(B)** Amino acid sequences of human and rat prepro-orexin. Asterisks indicate the identical amino acids between human and rat sequences.

Other than mammalian species, structures of *Xenopus* and chicken orexin-A and orexin-B, which also have conserved structures compared with mammalian sequences, have been elucidated (Fig. 1A).

The prepro-orexin cDNA sequences revealed that both orexins are produced from the same 130-residue (rodent) or 131-residue (human) polypeptide, prepro-orexin, by proteolytic processing. The human and mouse prepro-orexin sequences are 83 and 95% identical to the rat counterpart, respectively (Fig. 1B) *(2)*. The majority of amino acid substitutions were found in the C-terminal part of the precursor, which appears unlikely to encode another bioactive peptide (Fig. 1B).

An mRNA encoding the same precursor peptide was independently isolated by de Lecea et al. *(3)* as a hypothalamus-specific transcript. They predicted that this transcript encodes two neuropeptides, named hypocretin-1 and -2. The terms “hypocretin” and “orexin” are currently used as synonyms.

We reported orexins initially as orexigenic peptides *(2)*. Subsequently, they have been reported to have a variety of pharmacological actions (*see* other chapters). In particular, recent observations implicate orexins/hypocretins in sleep disorder narcolepsy and in the regulation of the normal sleep process. The biological activities of orexins are discussed in other chapters of this book.

3. THE PREPRO-OREXIN GENE: STRUCTURE AND REGULATION OF EXPRESSION

The human prepro-orexin gene, which is located on chromosome 17q21, consists of two exons and one intron distributed over 1432 bp *(4)*. The 143-bp exon 1 includes the 5´-untranslated region and the coding region that encodes the first seven residues of the secretory signal sequence. Intron 1, which is the only intron found in the human prepro-orexin gene, is 818-bp long. Exon 2 contains the remaining portion of the open reading frame and the 3´-untranslated region.

The human prepro-orexin gene fragment, which contains the 3149-bp 5´-flanking region and the 122-bp 5´-noncoding region of exon 1, was reported to have an ability to express *lacZ* in orexin neurons without ectopic expression in transgenic mice, suggesting that this genomic fragment contains most of the necessary elements for appropriate expression of the gene *(4)*. This promoter is useful to examine the consequences of expression of exogenous molecules in orexin neurons of transgenic mice, thereby manipulating the cellular environment in vivo *(4–6)*. For example, this promoter was used to establish several transgenic lines, including orexin neuron-ablated mice and rats and mice in which orexin neurons specifically express green fluorescent protein (GFP) *(5,6)*.

The regulation of expression of the prepro-orexin gene still remains unclear. Prepro-orexin mRNA was shown to be upregulated under fasting conditions, indicating that these neurons somehow sense the animal's energy balance *(2)*. Several reports have shown that orexin neurons express leptin receptor- and STAT-3-like immunoreactivity, suggesting that orexin neurons are regulated by leptin *(7)*. We consistently found that continuous infusion of leptin into the third ventricle of mice for 2 wk resulted in marked downregulation of prepro-orexin mRNA level *(5)*. Therefore, reduced leptin signaling may be a possible factor that upregulates expression of prepro-orexin mRNA during starvation. Prepro-orexin levels were also increased in hypoglycemic conditions, suggesting that expression of the prepro-orexin gene is also regulated by plasma glucose levels *(8)*. These observations are consistent with our electrophysiological study of GFP-expressing orexin neurons in transgenic mice, which showed that orexin neurons are regulated by extracellular glucose concentration and leptin *(5)*.

4. STRUCTURES AND PHARMACOLOGY OF OREXIN RECEPTORS

The actions of orexins are mediated by two G-protein–coupled receptors termed orexin-1 receptor (OX_1R) and orexin-2 receptor (OX_2R) (Fig. 2) *(2)*. Among various classes of G-protein–coupled receptors, OX_1R is structurally more similar to certain neuropeptide receptors, most notably to the Y2 neuropeptide Y (NPY) receptor (26% similarity), followed by the thyrotropin-releasing hormone (TRH) receptor, cholesystokinin type-A receptor, and NK2 neurokinin receptor (25,23, and 20% similarity, respectively).

The amino acid identity between the deduced full-length human OX_1R and OX_2R sequences is 64%. Thus, these receptors are much more similar to each other than they are to other GPCRs. Amino acid identities between the human and rat homologs of each of these receptors are 94% for OX_1R and 95% for OX_2R, indicating that both receptor genes are highly conserved between the species. Competitive radioligand binding assays using Chinese hamster ovary (CHO) cells expressing OX_1R suggested that orexin-A is a high-affinity agonist for OX_1R. The concentration of cold orexin-A required to displace 50% of specific radioligand binding (IC_{50}) was 20 n*M*. Human orexin-B also acted as a specific agonist on CHO cells expressing OX_1R. However, human orexin-B has significantly lower affinity compared with

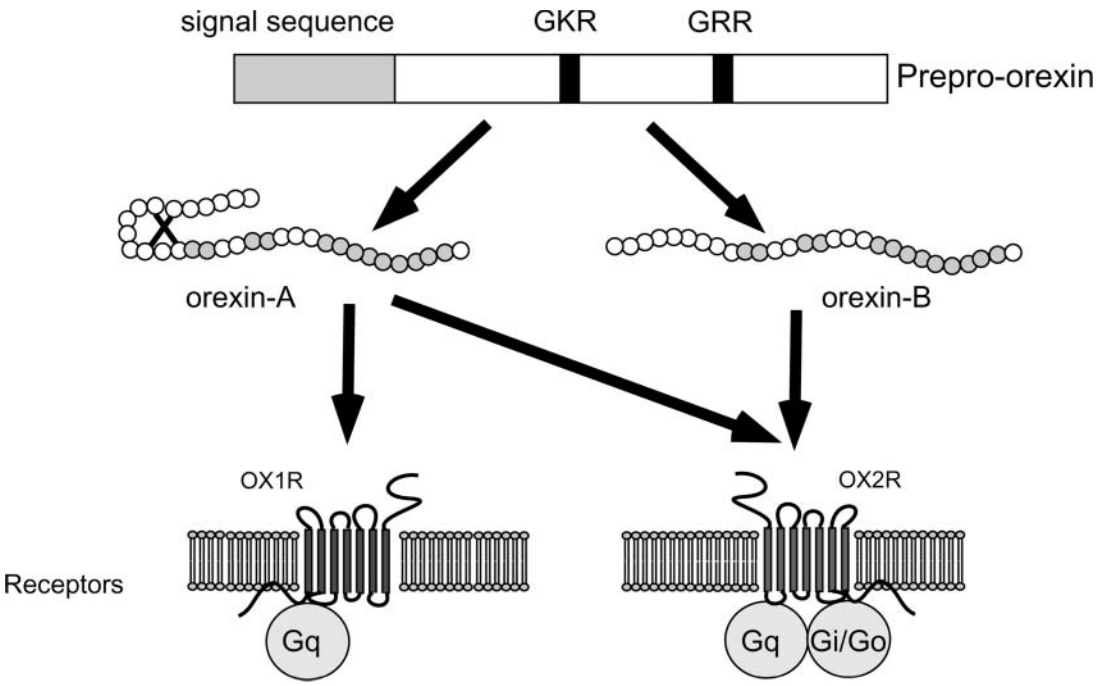

Fig. 2. Schematic representation of the orexin system. Orexin-A and -B are derived from a common precursor peptide, prepro-orexin. The actions of orexins are mediated via two G-protein–coupled receptors named orexin-1 (OX_1R) and orexin-2 (OX_2R) receptors. OX_1R is selective for orexin-A, whereas OX_2R is a nonselective receptor for both orexin-A and orexin-B.

human OX_1R: the calculated IC_{50} in a competitive binding assay was 250 n*M* for human orexin-B, indicating two orders of magnitude lower affinity compared with orexin-A (Fig. 2).

On the other hand, binding experiments using CHO cells expressing the human OX_2R cDNA demonstrated that OX_2R is a high-affinity receptor for human orexin-B with an IC_{50} of 20 n*M*. Orexin-A also had high affinity for this receptor with an IC_{50} of 20 n*M*, which is similar to the value for orexin-B, suggesting that OX_2R is a nonselective receptor for both orexin-A and orexin-B (Fig. 2).

5. MOLECULAR GENETIC STUDIES OF OREXIN RECEPTORS

Earlier genetic studies revealed that dogs with a mutation of the OX_2R gene or OX_2R-knockout mice displayed a narcolepsy-like phenotype *(9,10)*, whereas OX_1R knockout mice did not reveal any obvious abnormality in the sleep/wake states *(10)*. These studies provide strong evidence for the roles of OX_2R in regulating the vigilance state in human and animals. However, double receptor knockout (OX_1R- and OX_2R-null) mice appear to be a phenocopy of prepro-orexin knockout mice, suggesting that OX_1R also has additional effects on sleep/wakefulness. Consistent with this, the behavioral and electroencephalographic phenotype of OX_2R knockout mice is less severe than that found in prepro-orexin knockout mice *(9)*. These findings suggest that loss of signaling through both receptor pathways is necessary for severe narcoleptic characteristics. Indeed, OX_2R knockouts are only mildly affected with cataplexy-like attacks of REM sleep, whereas orexin knockout mice are severely affected *(9)*.

The phenotypes of orexin receptor knockout mice are discussed more precisely in another chapter.

6. HOW MANY OREXIN RECEPTOR GENES?

Two genes for orexin receptors have been identified in mammalian species thus far. The phenotypes of OX_1R and OX_2R double-deficient mice were analyzed and shown to have sleep state abnormality, which was indistinguishable from that of prepro-orexin gene-deficient mice. This observation suggests that only two receptors for orexins might exist in mammals, at least in vigilance state control. However, it is possible that there are other subtypes of receptors produced from OX_1R or OX_2R genes by alternative splicing. In fact, two alternative C-terminus splice variants of the murine OX_2R, termed m $OX_2\alpha R$ (443 amino acids) and m $OX_2\beta R$ (460 amino acids) have been identified *(11)*. However, orexin-A and orexin-B showed no difference in binding characteristics between the splice variants.

7. DISTRIBUTION OF OREXIN RECEPTORS

Although orexin receptors are expressed in a pattern consistent with orexin projections, mRNA for OX_1R and OX_2R were shown to be differentially distributed throughout the brain. For instance, within the hypothalamus, a low level of OX_1R mRNA expression is observed in the dorsomedial hypothalamus (DMH), whereas a higher level of OX_2R mRNA expression is observed in this region. Other areas of OX_2R expression in the hypothalamus are the arcuate nucleus, paraventricular nucleus (PVN), lateral hypothalamic area (LHA), and, most significantly, the tuberomammillary nucleus (TMN) *(12)*. In these regions, there is little or no OX_1R signal. In the hypothalamus, OX_1R mRNA is abundant in the anterior hypothalamic area and ventromedial hypothalamus (VMH). Outside the hypothalamus, high levels of OX_1R mRNA expression are detected in the tenia tecta, hippocampal formation, dorsal raphe nucleus, and, most prominently, the locus ceruleus (LC). OX_2R mRNA is abundantly expressed in the cerebral cortex, nucleus accumbens, subthalamic nucleus, paraventricular thalamic nuclei, anterior pretectal nucleus, and the raphe nuclei.

Within the brain, OX_1R is most abundantly expressed in the LC, whereas OX_2R is most abundantly expressed in the TMN, regions highly important for maintenance of arousal. The raphe nuclei contain both receptor mRNAs. These observations suggest strong interaction between orexin neurons and the monoaminergic systems. More precise distribution of orexin recepters is discussed in Chapter 3.

8. STRUCTURE-ACTIVITY RELATIONSHIPS OF OREXINS

Activities of synthetic orexin-B analogs in cells transfected with either OX_1R or OX_2R were examined to define the structural requirements for activity of orexins on their receptors *(13)*. The ability of N- or C-terminally truncated analogs of orexin-B to increase cytoplasmic Ca^{2+} levels in the cells showed that the absence of N-terminal residues had little or no effect on the biological activity and selectivity of both receptors. Truncation from the N-terminus to the middle part of orexin-B resulted in moderate loss of activity, in the order of peptide length. In particular, deletion of the conserved sequence between orexin-A and orexin-B caused a profound loss of biological activity, and the C-terminally truncated peptides were also inactive for both receptors. These results suggest that the consensus region between orexin-A and orexin-B is important for the activity of both receptors.

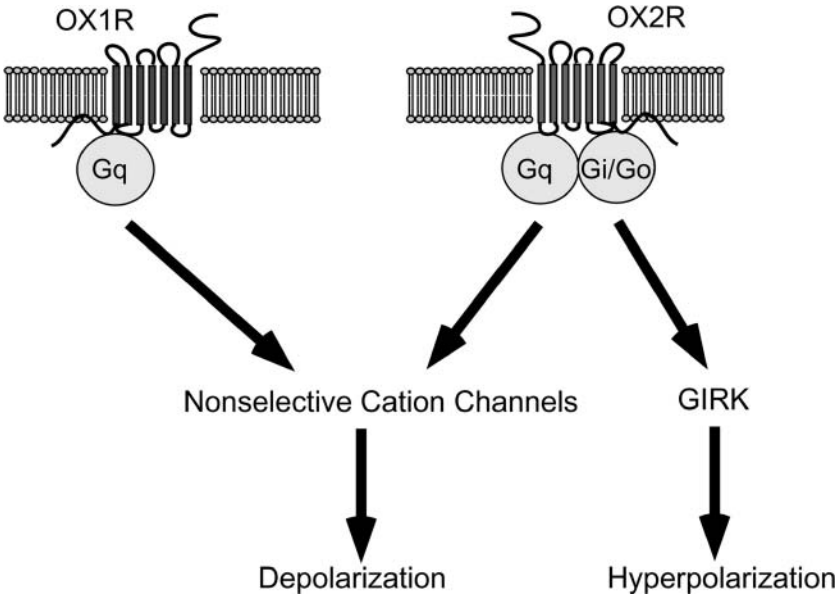

Fig. 3. Schematic representation of the intracellular signal transduction systems of orexin receptors. OX_1R is coupled exclusively to the G_q subclass of heterotrimeric G proteins, whereas OX_2R may couple to $G_{i/o}$, and/or G_q. GIRK, G-protein-gated inwardly rectifying potassium channel.

Substitution of each amino acid of the natural sequence of orexin-B by L-alanine revealed that the residues in the N-terminal region could be substituted by L-alanine without loss of activity of both receptors. However, substitution in the C-terminal region (especially at positions 24–28) decreased the activity, just as C-terminal truncation did. Substitution of each amino acid of orexin-B by the corresponding D-amino acid also showed that the C-terminal region is highly important for the activity of orexin-B.

Orexin-A (positions 15–33), the C-terminal half of orexin-A, and orexin-B (positions 10–28) have similar sequences, however, their selectivity to OX_1R and OX_2R is different. This finding indicates that not only the activity but also the ligand/receptor selectivity is closely related to the C-terminal half of the orexin sequence.

9. SIGNAL TRANSDUCTION SYSTEM

Both OX_1R and OX_2R are G-protein–coupled receptors, which transmit information into cells by activating heterotrimeric G proteins. Activation of the signaling pathways associated with distinct G proteins may contribute to the diverse physiological roles of orexin in particular neurons. Although many G-protein–coupled neurotransmitter receptors are potentially capable of modulating both voltage-dependent calcium channels and G-protein–gated inwardly rectifying potassium channels (GIRKs), there might be a substantial degree of selectivity in the coupling to one or other of these channels in neurons (Fig. 3). The signal transduction pathways of orexin receptors were examined in cells transfected with OX_1R or OX_2R. In OX_1R-expressing cells, forskolin-stimulated cAMP was not affected by orexin administration. In addition, PTX treatment did not show any effects on orexin-induced increases in $[Ca^{2+}]_i$.

These results suggest that OX_1R does not couple to PTX-sensitive $G_{i/o}$ proteins *(14)*. In contrast, orexin inhibited forskolin-stimulated cAMP production in a dose-dependent manner

in OX_2R-expressing cells. The effect was abolished by pretreatment with PTX. However, orexin-induced increases in $[Ca^{2+}]_i$ were not affected by PTX treatment in OX_2R-expressing cells. These results indicate that the OX_2R couples to PTX-sensitive G proteins that were involved in the inhibition of adenylyl cyclase by orexin. They also suggest that OX_1R couples exclusively to PTX-insensitive G proteins, and OX_2R couples to both PTX-sensitive and -insensitive proteins. The relative contribution of these G proteins in the regulation of neuronal activity remains unknown.

Orexins have been shown to have an excitatory activity in many types of neurons in vivo. For instance, noradrenergic cells of the LC *(15)*, dopaminergic cells of the ventral tegmental area *(16)*, and histaminergic cells from the TMN *(17)* have been shown to be activated by orexins. Because LC neurons exclusively express OX_1R, whereas TMN neurons exclusively express OX_2R, these observations suggest that both OX_1R and OX_2R signaling are excitatory on neurons. However, these studies only examined the effect of orexins on receptor-expressing cell bodies. There is a possibility that orexin receptors locate on presynaptic terminals, because Li et al. *(18)* reported that orexin increases local glutamate signaling by facilitation of glutamate release from presynaptic terminals. Therefore, it is possible that activation of PTX-sensitive G proteins downstream of OX_2R might be involved in functions other than activation of neurons, such as in the tips of developing neurites and on presynaptic nerve terminals, leading to growth cone collapse and enhanced synaptic release of the transmitter. Alternatively, OX_2R-mediated activation of Gi might result in inhibition of some populations of neurons. In fact, orexin was recently reported to inhibit preopio-melanocortin neurons in the arcuate nucleus in vitro *(19)*.

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3

Orexin Projections and Localization of Orexin Receptors

Jacob N. Marcus, BS and Joel K. Elmquist, DVM, PhD

1. INTRODUCTION

The past several years have provided important insights into the physiological significance of the central orexin system. An important component of these is an increased understanding of the unique neuroanatomy of the orexin/hypocretin system. The orexin peptides, orexin-A and orexin-B, are produced in a restricted region of the central nervous system, the neurons of the lateral hypothalamic area. From this small neuronal source, on the order of a few thousand neurons, orexin-expressing neurons project over virtually the entire brain and spinal cord. At the terminals of these projections, orexin interacts with two distinct receptors, the orexin-1 receptor and the orexin-2 receptor. Given the diffuse projection pattern of orexin-containing axons, it is not surprising that orexin receptor expression has been described in a large number of brain areas. This chapter briefly outlines the brain regions innervated by hypothalamic orexin neurons and provides an overview of the distribution of each receptor subtype.

The location of orexin neurons combined with the orexin fiber innervation and receptor distribution pattern have been used to formulate anatomic hypotheses as to the likely physiological roles of the orexin system. These roles include the control of wake and sleep states, feeding and drinking behavior, neuroendocrine and autonomic regulation, locomotor activity, and many others. Notably, several of these models have been supported by physiological, pharmacological, and genetic evidence. The physiological importance of orexins in these different systems is addressed in detail by others in this text; in this chapter we consider the anatomical evidence for the role of orexins in these multiple systems.

2. LOCATION AND NEUROCHEMICAL PROPERTIES OF OREXIN NEURONS

The discovery of the orexin (hypocretin) peptides was met with enthusiasm, in part because of the unique expression pattern of the peptides *(1,2)*. It was immediately evident that neurons expressing orexin peptides are found in a very small region of the central nervous system. These neurons are located predominantly in the perifornical region and the lateral hypothalamus, at the tuberal level of the hypothalamus where the median eminence is evident. This region of the lateral hypothalamus has classically been implicated in a wide variety of behavioral and homeostatic regulatory systems, and thus the simple location of orexin-expressing neurons generated hypotheses as to their physiological relevance *(3,4)*.

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Within the rat brain, in the rostral to caudal plane, the orexin neuronal field extends from just caudal to the paraventricular hypothalamic nucleus to just rostral to the tuberomammillary nucleus. In the medial to lateral plane, whereas orexin neurons encroach medially through the dorsomedial nucleus as far as the third ventricle and laterally as far as the optic tracts, most orexin neurons reside within the perifornical area. Similarly, the vast majority of orexin neurons are found dorsal to the fornix, and only scattered cells exist in the ventral portion of the hypothalamus. Although some orexin neurons clearly exist outside of the perifornical lateral hypothalamus including portions of the dorsal medial hypothalamus, for the remainder of the chapter we will refer to the location of orexin-containing neurons as the lateral hypothalamic area (LHA).

The orexin peptide family consists of two known peptides, orexin-A (33 amino acids) and orexin-B (27 amino acids), which are proteolytically cleaved from one gene product, the prepro-orexin peptide *(1,2)*. Whereas the pharmacological and physiological effects of each singular orexin peptide may differ and have yet to be fully characterized, it appears that neurons expressing prepro-orexin probably contain both orexin-A and orexin-B peptides. Orexin peptides localize within secretory vesicles, implying that both orexin-A and orexin-B are coreleased at orexinergic synapses *(5)*. There is general agreement in immunohistochemical staining for prepro-orexin, orexin-A, orexin-B, further implying that both orexin peptides are produced in orexin-containing neurons.

Several other neurochemical features distinguish the population of orexin-expressing neurons. Orexin neurons contain the peptide dynorphin *(6,7)* and express the secretory marker secretogranin II *(8)*. Galanin *(9)* and neuropeptide (NPY)-Y4 receptors *(10)* have also been reported to be coexpressed by orexin neurons. Many orexin neurons express vesicular glutamate transporters, suggesting that many if not all orexin neurons are glutamatergic *(11,12)*. In contrast, orexin neurons do not express GAD-67 mRNA, suggesting that orexin neurons are not GABAergic *(11)*. Given their location in the LHA, it is likely that orexin neurons receive multiple inputs. For example, norepinephrine and serotonin have direct inhibitory actions on orexin neurons, implying the presence of adrenergic and serotinergic receptors on orexin neurons *(13)*. In contrast, histamine and acetylcholine have little direct effect *(13)*.

Given the purported role of the central orexin system in regulating metabolic homeostasis, it is also important to note that orexin neurons reside in the lateral hypothalamus intermingled among, but completely distinct from, another group of peptidergic neurons, those expressing melanin-concentrating hormone (MCH) *(14,15)*. Thus, the orexin- and MCH-expressing cells comprise two neurochemically unique neuronal populations in the lateral hypothalamus, both of which have been suggested to play a role in regulating body weight homeostasis *(3,16)*. Notably, the orexin population is distinct from yet a third neuronal population within the lateral hypothalamus, those neurons that express neuronal nitric oxide synthase (nNOS) *(17)*.

Studies on identified orexin neurons have shown that several circulating metabolic cues can influence their electrical activity. For example, orexin neurons are excited by application of ghrelin *(18)* and glucagon-like peptide-1 (GLP-1) *(19)*. In contrast, orexin neurons are inhibited in response to application of leptin *(18)*. Orexin neurons can also be activated by decreases and inhibited by increases in the extracellular glucose concentration *(18,20)*. Whereas it is possible that these factors may influence orexin neuons directly in vivo, it is apparent that orexin neurons may also be regulated indirectly through afferents to the LHA from regions that are directly affected by circulating cues, such as the arcuate nucleus *(14,21–23)* and the dorsal vagal complex *(19)*.

Orexin peptides interact with two known G-protein–coupled receptor subtypes, the orexin-1 receptor (OX_1R) and the orexin-2 receptor (OX_2R), which each have slightly different affinities for the orexin peptides. OX_1R is approx 100 times more selective for orexin-A compared with orexin-B, whereas OX_2R is relatively nonselective between the two orexin peptides. These two receptor subtypes are, as yet, the only known receptors that respond to application of orexin peptides. Whereas orexin peptides are produced in the restricted location of the hypothalamus, orexin receptors are expressed in many diverse regions of the brain and spinal cord.

3. TECHNICAL CONSIDERATIONS

Before discussing the distributions of orexin fibers and receptors throughout the central nervous system, a brief technical comment is warranted. There is a general scientific consensus on the areas of the brain thought to be innervated by orexin neurons. Orexin-expressing neurons may be visualized using routine *in situ* hybridization (mRNA detection) and immunohistochemical (protein detection) techniques. Messenger RNA expression, as visualized using *in situ* hybridization and immunohistochemical staining for orexin peptides, is robust, which is consistent with the presumption that orexin peptides are produced in large amounts and secreted at axon terminals. Antisera to orexin peptides and to prepro-orexin have been generated and directly reconciled with prepro-orexin mRNA expression using *in situ* hybridization in both normal and orexin-deficient rodent and human tissue. The use of validated antisera has allowed the detection of orexin fibers and terminals throughout the brain and spinal cord. Furthermore, classic retrograde and anterograde tracing studies have clearly demonstrated the projections emanating from the LHA *(24,25)*, although the topography of the orexin inputs to the respective central nervous system (CNS) targets remains to be elucidated. As orexin fibers may only originate from this location, any fiber staining present in a region not already known to receive innervation from the LHA may be an indication of nonspecific binding.

The detection of orexin receptor expression lacks this degree of certainty. First, receptor mRNA expression levels as assessed by *in situ* hybridization histochemistry are inherently less robust than that of the mRNA for the peptide ligand precursor. For this reason, sensitive histological and histochemical procedures must be used to reliably detect receptor mRNA expression. Even using sensitive procedures, the resultant mRNA detection is dependent on the specificity and validity of the riboprobe used. Furthermore, these procedures invariably result in some loss of mRNA, and therefore one must assume that most *in situ* hybridization mapping studies may be underrepresenting the expression profiles of receptor mRNA. In addition, receptor mapping studies often lack corroborating parallel data, such as genetic knockouts or antibody verification. Unfortunately, at the time of this writing, the reliability and specificity of antisera generated against orexin receptors remain to be established (*see* ref. *26* for discussion). Moreover, the visualization of orexin receptor protein expression may not overlap with that of mRNA expression in the same way that the ligand mRNA and protein expression do. For example, receptor protein expression might be restricted to the soma, as is mRNA expression. However, receptor protein may be transported to axon terminals or even distal dendrites in regions far removed from the soma to exert its effect at a region that is innervated by orexin fibers. In this case, detection of mRNA in the soma does not yield specific information about receptor location. Indeed, orexin has been shown to act presynaptically to affect the release of neurotransmitter from axon terminals *(13),* suggesting that orexin receptor proteins can function at axon terminals.

Because of these concerns, a large amount of inherent uncertainty exists in the descriptions of orexin receptor distributions. For these reasons we will comment at length on orexin receptor distributions from the point of view of mRNA expression only, bearing in mind the limitations of this view. It should also be noted that the vast majority of anatomic data has been collected on rat brain tissue, and it is these data that will be discussed in this chapter. To a much more limited extent, the anatomy of the orexin system has also been explored in the primate, mouse, sheep, and other species *(27–29)*.

4. OREXIN NEURONS PROJECT DIFFUSELY

From a hypothalamic point of origin, orexin fibers project across the entire neuraxis from the cortex to the spinal cord (Fig. 1). The diffuse nature of orexin efferents has implicated the orexin neurons in a wide variety of physiological systems *(5)*. The lateral hypothalamic area, the origin of orexin projections, has long been considered a master integration and command center for numerous homeostatic, autonomic, endocrine, and behavioral systems *(25)*. For example, retrograde and anterograde tracing studies have shown that the LHA is a primary recipient of projections, presumably containing visceral sensory information, from the brainstem *(30)* and provides a reciprocal descending projection to these brainstem regions *(30)*. Similarly, the LHA both innervates and is innervated by the cortex *(31,32)*. The LHA also innervates neuroendocrine control regions in the hypothalamus such as the arcuate nucleus (ARC) and the paraventricular hypothalamic nucleus (PVH) *(33)*.

Not surprisingly, the orexin fibers that exit the LHA innervate many if not all of the regions shown previously to be innervated by the LHA. Numerous studies have reported the locations of orexin fibers *(5,8,34–39)* using immunohistochemical techniques. Several studies detailing orexin receptor mRNA expression using *in situ* hybridization *(40–42)* and immunohistochemistry *(43–46)* have also been performed. In general, these studies are consistent with each other. For example, areas receiving dense innervation of orexin fibers also express high levels of receptor mRNA. In addition, all central areas innervated by fibers express mRNA for either of the two orexin receptors or both of them. The results of these distribution studies are summarized in Figs. 1–7 and Table 1, and within the text below in a survey of the central nervous system.

4.1. Hypothalamic Innervation and Receptor Distribution

4.1.1. Orexin Fibers

Orexin fiber innervation of the hypothalamus is extremely robust. Fiber innervation is present at all levels of the hypothalamus, from the preoptic level to the level of the mammillary bodies. Not surprisingly, the densest concentrations of fibers are seen in the lateral and posterior hypothalamus. Many if not most of these fibers are exiting the orexin neuronal field, but some of these fibers probably terminate on neurons within the LHA itself, including on orexin neurons *(13,29)* and MCH neurons *(47)*. Large numbers of fibers are present in the parvocellular portions of the paraventricular nucleus, whereas few fibers enter the magnocellular portion (Fig. 2B). Innervation of the dorsomedial nucleus is moderate, as is innervation of the ventromedial nucleus (VMH), especially the region anterior to the VMH. More rostrally, the preoptic nuclei are innervated by a moderate number of fibers. Orexin fibers are seen around the supraoptic nucleus and suprachiamatic nucleus, but few fibers enter these areas *(8)*. The ARC is densely innervated throughout its extent, and fibers extend into the median eminence *(29,36)*. Fibers are also seen in the periventricular nucleus, with some fibers appearing to extend into the third ventricle *(38)*. In the caudal hypothalamus, the tuberomammillary and suprammamillary nuclei are densely innervated (Fig. 2C).

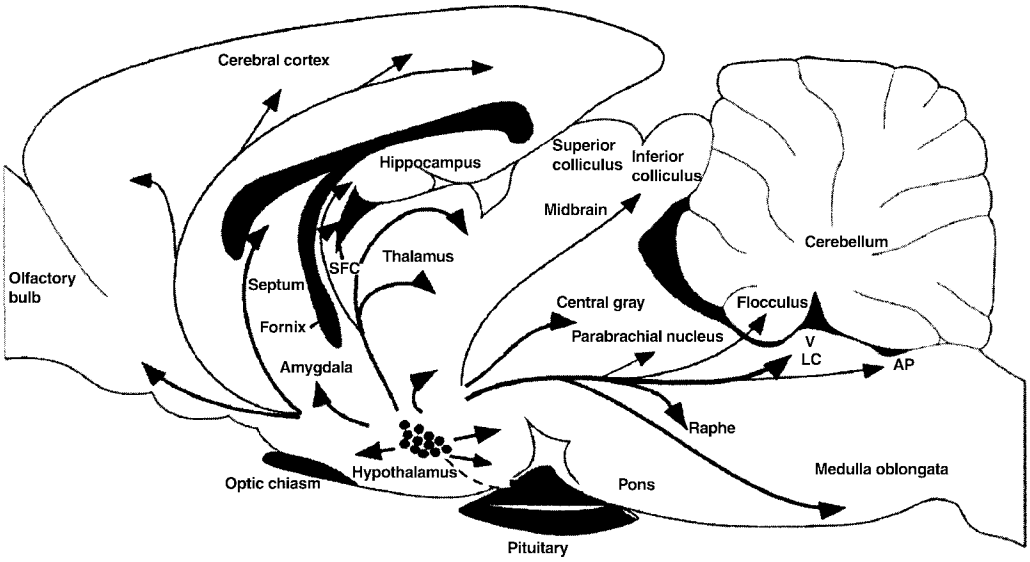


Fig. 1. Schematic depiction of the rat brain showing widespread distribution of orexin-containing fibers (arrows) originating from the lateral hypothalamus. AP, area postrema; LC, locus ceruleus; SFC, subfornical organ; V, fourth ventricle; (Modified from ref. *34*.)

4.1.2. Hypothalamic OX_1R Distribution

OX_1R mRNA is expressed in a subset of the hypothalamic nuclei described above. For example, low levels of expression are present in the preoptic nuclei, with more moderate levels in the medial preoptic nucleus. Robust expression is seen in the anterior hypothalamic nucleus (Fig. 3C) and the dorsomedial portion of the VMH (Fig. 3E and F). Moderate levels of OX_1R mRNA are seen in the dorsal hypothalamus, ventral premammillary nucleus (Fig. 3G), posterior hypothalamus, and supramammillary nucleus. Diffuse expression is present in the LHA (Fig. 3F). Messenger RNA expression is absent in the paraventricular, arcuate, suprachiasmatic, and tuberomammillary nuclei (Fig. 5).

4.1.3. Hypothalamic OX_2R Distribution

OX_2R mRNA expression is present in many hypothalamic nuclei. For example, the tuberomammillary nucleus contains the most robust OX_2R expression in the hypothalamus (Fig. 4G). These tuberomammillary neurons are histaminergic *(48,49)*. As with OX_1R, the preoptic nuclei (Fig. 4C), supramammillary nucleus, and posterior hypothalamus express OX_2R mRNA. OX_2R mRNA is less robust than OX_1R mRNA in the ventromedial nucleus and dorsal hypothalamus (Fig. 4F). However, many hypothalamic nuclei show preferential expression of OX_2R. These include the arcuate (Fig. 4E), dorsomedial (Fig. 4F), parvocellular paraventricular (Fig. 4D), medial mammillary (Fig. 4G), lateral mammillary (Fig. 4G), and tubermammillary nuclei (Fig. 5). Diffuse OX_2R mRNA is also present in the lateral hypothalamic area (Fig. 4F).

4.2. Ascending Projections From the Hypothalamus

4.2.1. Ascending Orexin Fibers

The ascending projections of orexin neurons are extremely widespread and extend to cortical and subcortical regions. In the cortex, fibers are widespread and present in low but diffuse concentrations across all cortical levels. Moderate amounts of cortical innervation are

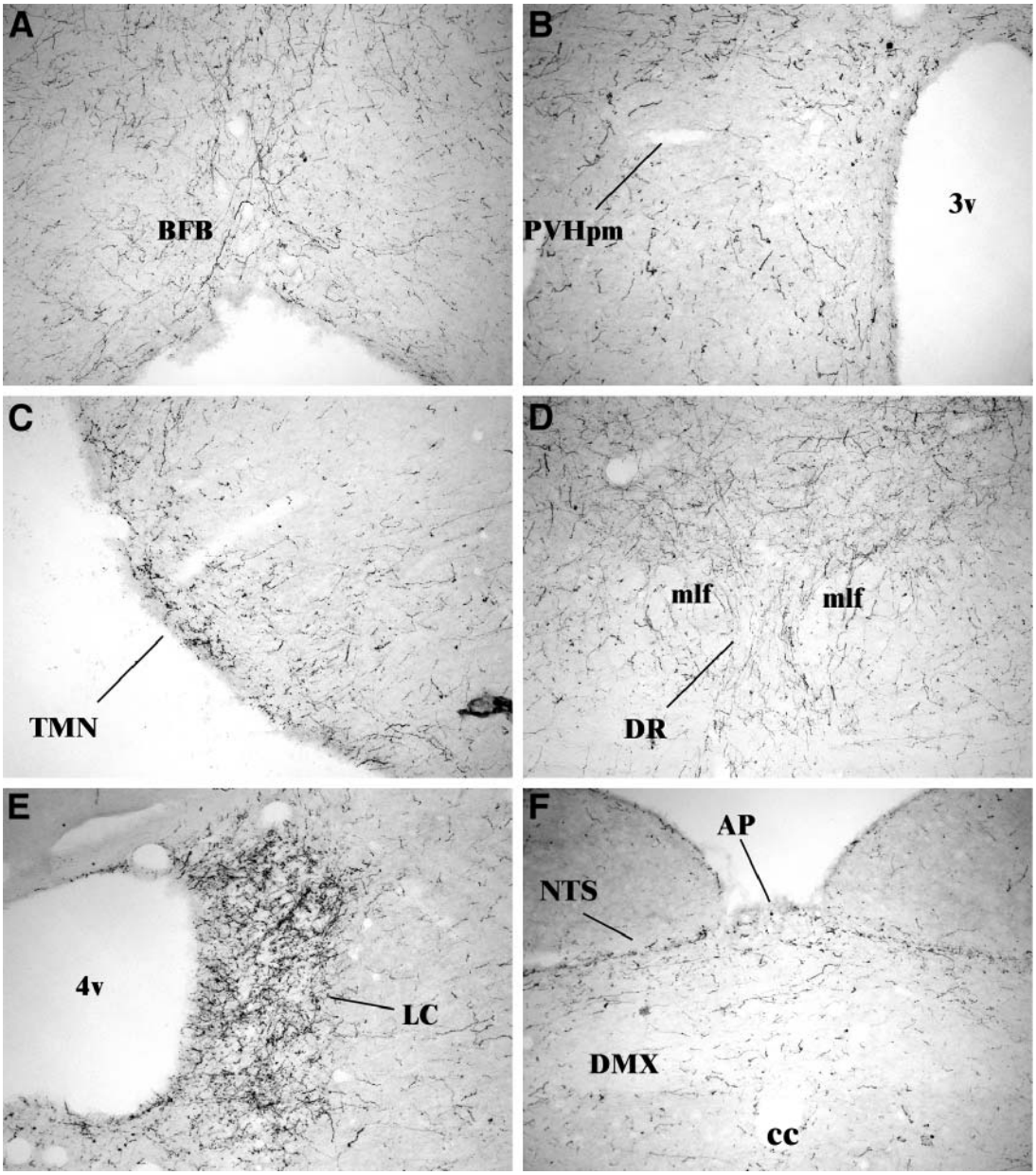
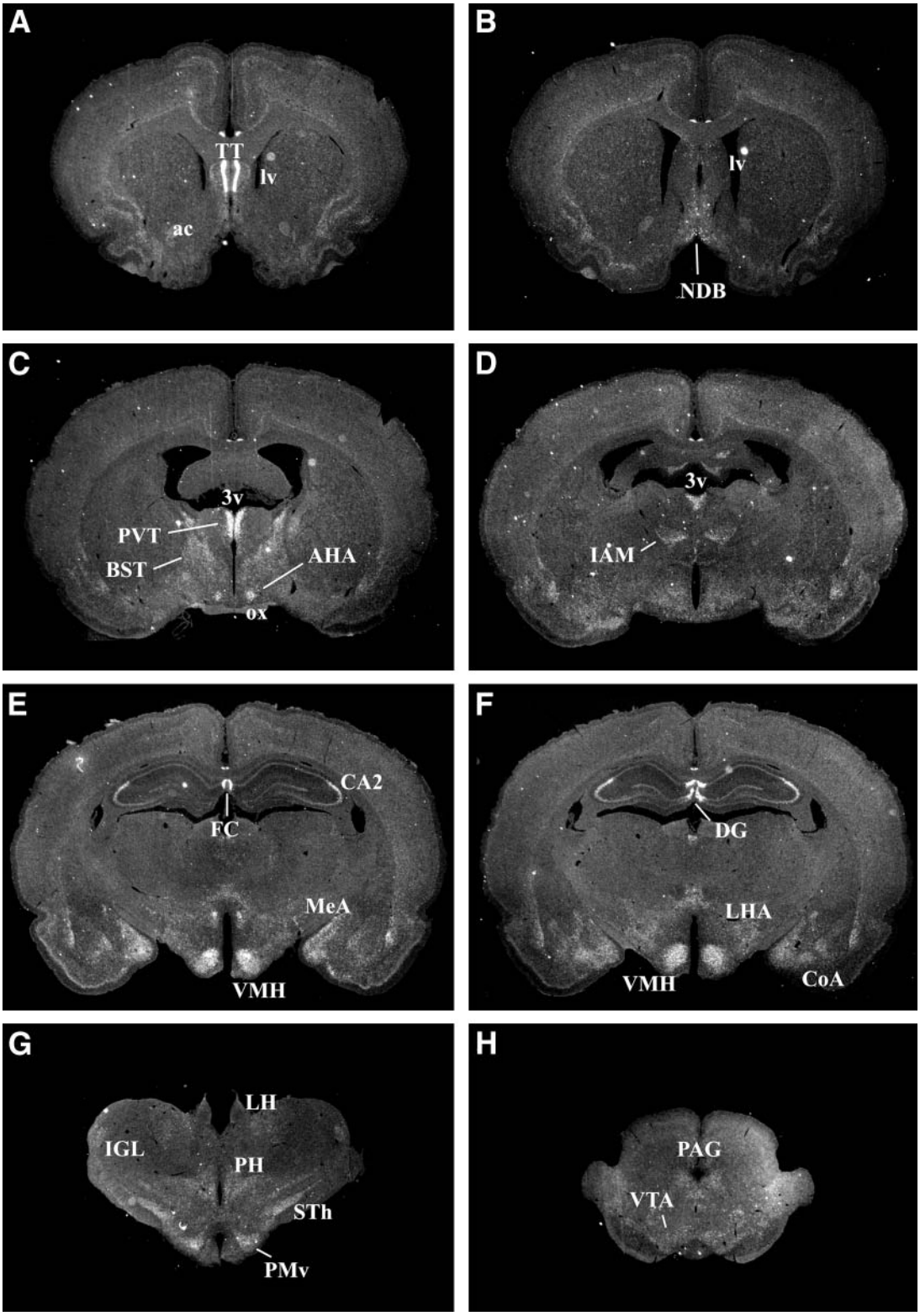


Fig. 2. Photomicrographs depicting the orexin-fiber innervation of the **(A)** basal forebrain, **(B)** paraventricular hypothalamic nucleus, **(C)** tuberomammillary hypothalamic nucleus, **(D)** dorsal raphe nucleus, **(E)** locus coeruleus, and **(F)** dorsal vagal complex. Orexin fibers are visualized as the dark DAB precipitate. Abbreviations: BFB, basal forebrain, PVHpm, paraventricular hypothalamic nucleus, posterior magnocellular, 3v, third ventricle, TMN, tubermammillary hypothalamic nucleus, DR, dorsal raphe nucleus, mlf, medial longitudinal fasiculus, LC, locus coeruleus, 4v, fourth ventricle, AP, area postrema, NTS, nucleus of the solitary tract, DMX, dorsal motor nucleus of the vagus nerve, cc, central canal.

seen in the insular, prelimbic, piriform, and infralimbic cortices, with fewer fibers in the sensory and motor cortices *(5)*. In the olfactory system, fibers are seen in the anterior olfactory bulb. Orexin fibers avoid the caudate putamen and globus pallidus, but a moderate number of fibers exist in the medial nucleus accumbens. Within the basal forebrain, orexin fibers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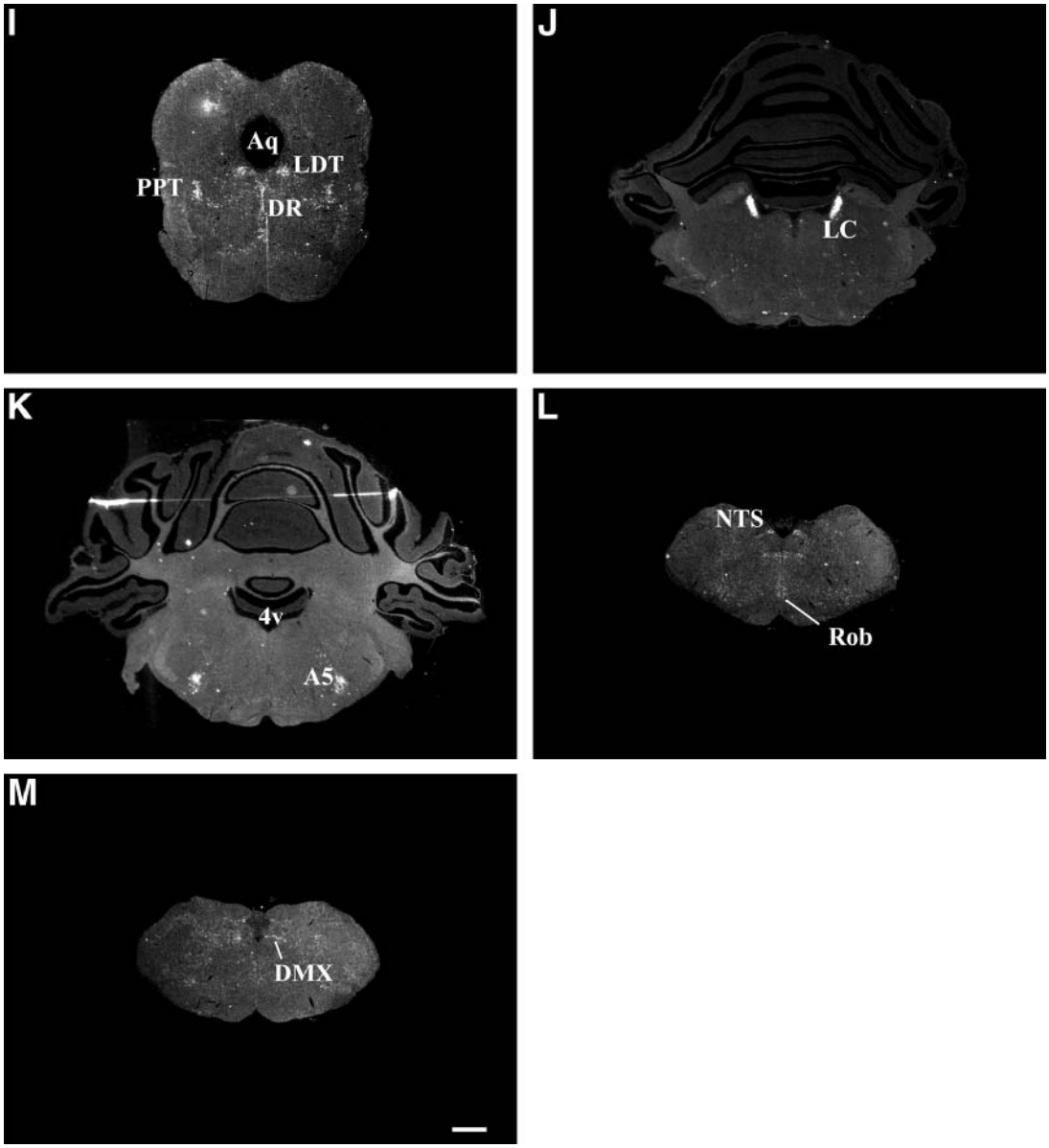
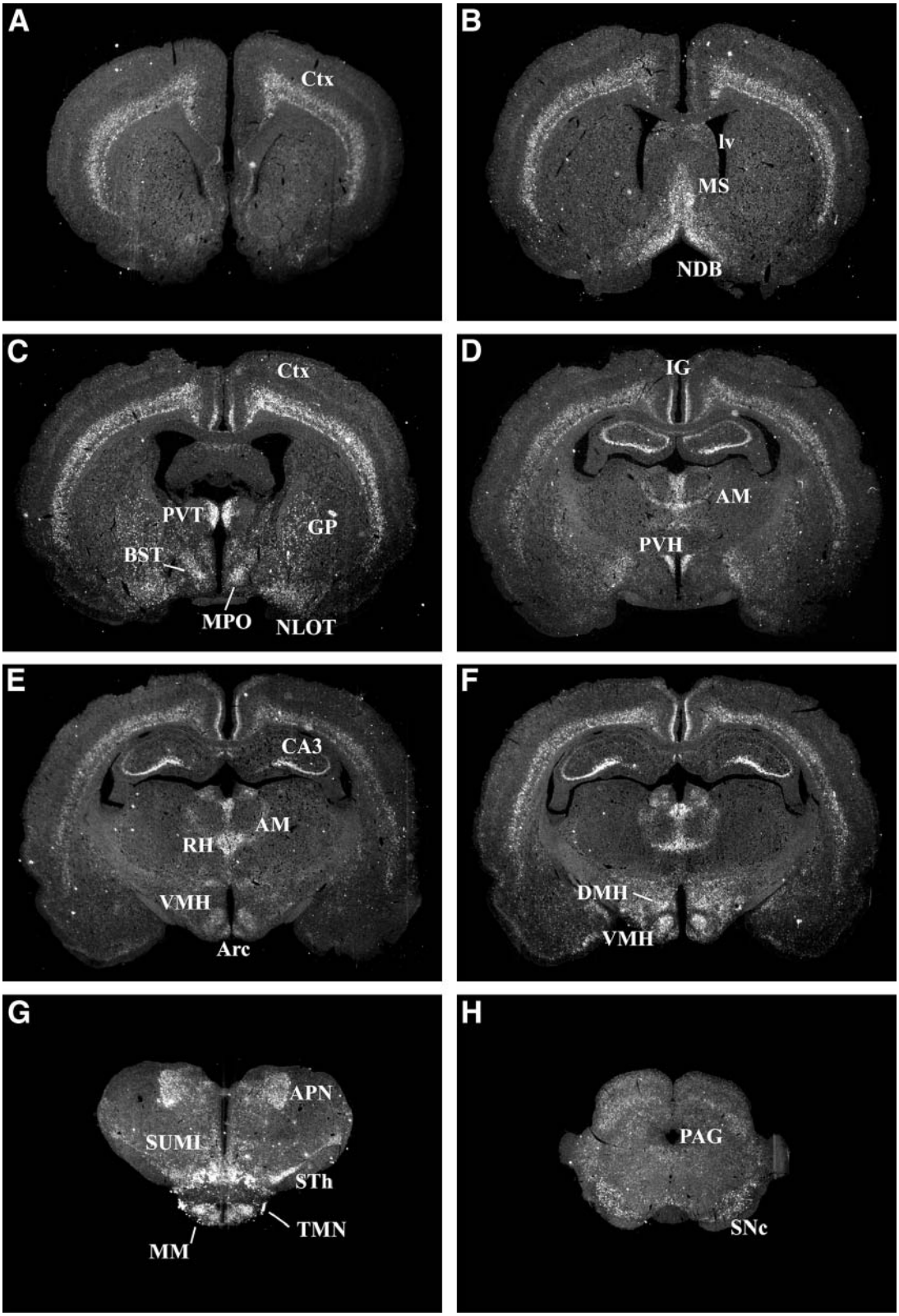


Fig. 3. A series of low-power photomicrographs summarizing OX_1R mRNA expression across the rat brain. Note mRNA expression in the **(A)** tenia tecta (TT), **(B)** basal forebrain, **(C)** paraventricular thalamic nucleus (PVT), bed nucleus of the stria terminalis (BST), and anterior hypothalamic area (AHA), **(D)** ventromedial hypothalamic nucleus (VMH), anteromedial thalamic nucleus (IAM), and medial amygdala, **(E)** hippocampal formation, especially the CA2 field and the fasciola cinerea (FC), rhomboid nucleus, dorsal hypothalamic area, ventromedial hypothalamic nucleus (VMH), and medial amygdala (MeA), **(F)** dentate gyrus (DG), cortical nucleus amygdala (CoA), and lateral hypothalamic area (LHA), **(G)** lateral habenula (LH), intergeniculate leaflet (IGL), posterior hypothalamic area (PH), subthalamic nucleus (STn), and ventral premammillary nucleus (PMv), **(H)** periaqueductal gray (PAG), ventral tegemental area (VTA), and central tegmental fields, **(I)** laterodorsal tegmental nucleus (LDT), pedunculopontine tegmental nucleus (PPT), and dorsal raphe nucleus (DR), **(J);** locus coeruleus (LC) **(K)** A5 cell group, **(L)** nucleus raphe obscurus (Rob) and nucleus of the solitary tract (NTS), **(M)** dorsal motor vagal nucleus (DMX), Scale bar = 1 mm in M (applies to all). Abbreviations: 3v, third ventricle, 4v, fourth ventricle, ac, anterior commissure, Aq, cerebral aqeduct, lv, lateral ventricle, NDB, nucleus of the diagonal band, ox, optic chiasm. (Reprinted from ref. *41*.)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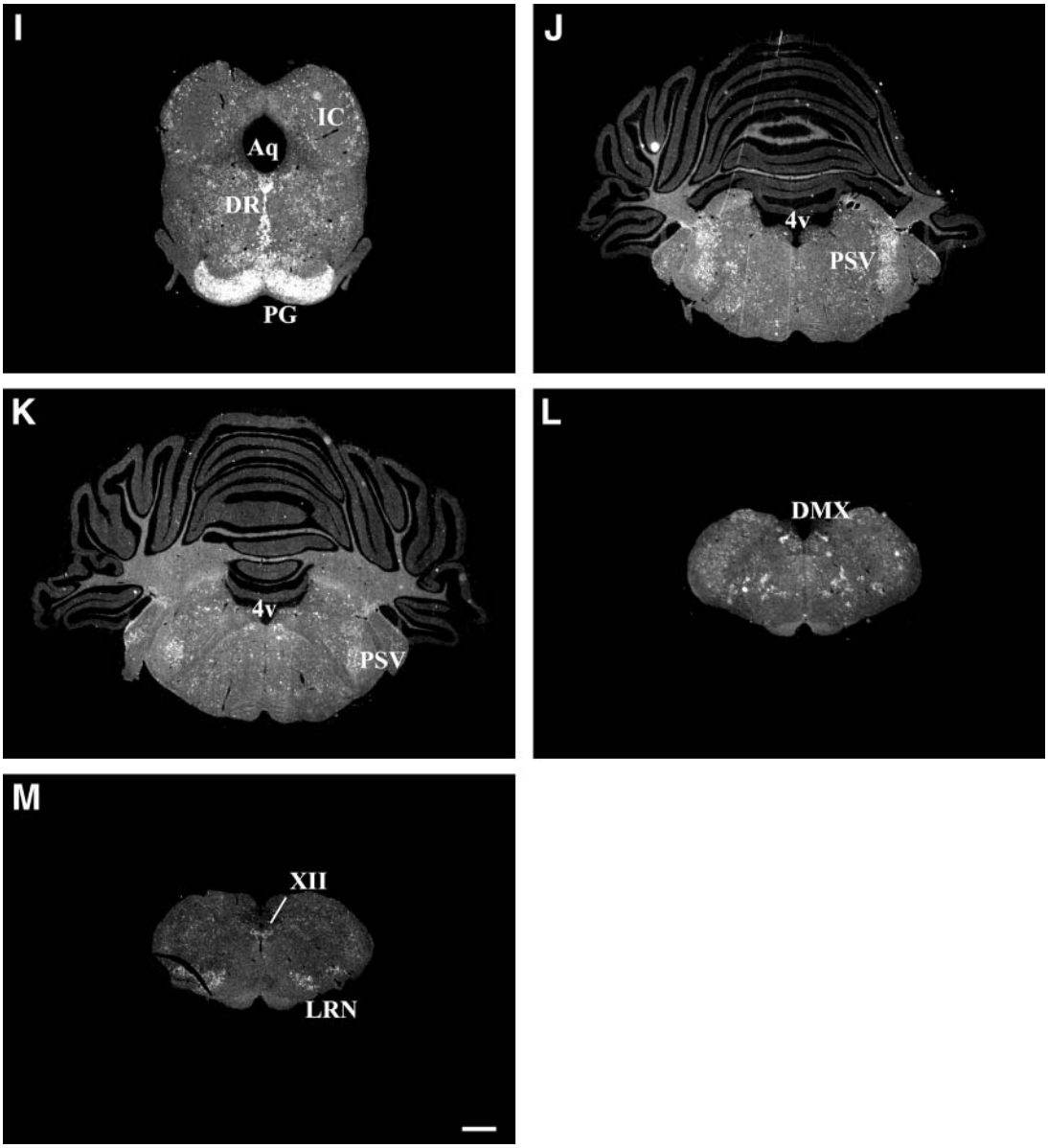

Fig. 4. A series of low-power photomicrographs summarizing OX_2R mRNA expression across the rat brain. Note mRNA expression in the cerebral cortex (Ctx) **(A)** throughout figure, **(B)** basal forebrain, including the medial septal nucleus (MS) and nucleus of the diagonal band (NDB), **(C)** paraventricular thalamic nucleus (PVT), globus pallidus (GP), bed nucleus of the stria terminalis (BST), medial preoptic nucleus (MPO), and nucleus of the lateral olfactory tract (NLOT), **(D)** paraventricular thalamic nucleus, anteromedial thalamic nucleus (AM), and paraventricular hypothalamic nucleus (PVH), **(E)** hippocampal formation, especially the CA3 field and induseum griseum (IG), rhomboid nucleus (RH), ventromedial hypothalamic nucleus (VMH), and arcuate hypothalamic nucleus (Arc), **(F)** hippocampal formation and midline thalamic nuclei as in, (E), dorsomedial hypothalamic nucleus (DMH), and ventromedial hypothalamic nucleus (VMH), **(G)** anterior pretectal nucleus (APN), supramammillary nucleus (SUMI), subthalamic nucleus (STh), lateral hypothalamic area, medial mammilary nucleus (MM), and tuberomammillary nucleus (TMN) **(H)** periaqueductal gray (PAG), and substantia nigra par compacta (SNc), **(I)** inferior colliculus (IC), dorsal raphe nucleus (DR), pontine gray (PG), **(J,K)** primary sensory nucleus of the trigeminal

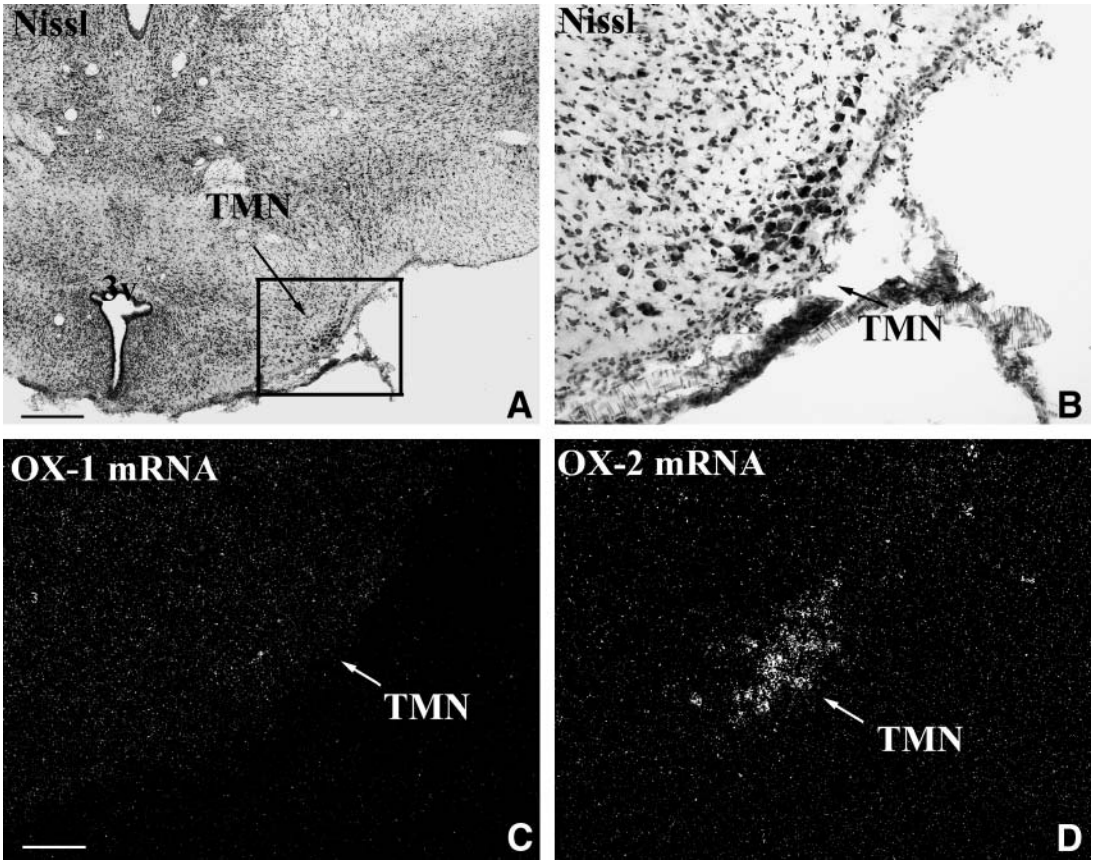

Fig. 5. A series of photomicrographs contrasting OX_1R and OX_2R mRNA expression in the tuberomammillary nucleus of the hypothalamus (TMN). Note the lack of OX_1R mRNA in the TMN. **(A)** Low-power magnification of a Nissl-stained brain section, showing the TMN and the third ventricle (3v). **(B)** A higher power view of the boxed section in (A). **(C)** Adjacent section of the brain shown in (B), showing OX_1R receptor mRNA. White clusters represent specific hybridization of riboprobe to orexin receptor mRNA. **(D)** Adjacent section of the brain shown in (B), showing OX_2R receptor mRNA. Scale bars = 400 μm in (A); 100 μm in (C) (applies to [B–D]) (Reprinted from ref. *41*.)

densely innervate the medial and lateral septal nuclei as well as the vertical and horizontal bands of the diagonal band of Broca (Fig. 2A). There is a diffuse, low-level innervation of all regions of the hippocampus. Within the amygdala, orexin fibers extend to the central and medial nuclei.

In the thalamus, orexin fibers primarily innervate various midline nuclei. The paraventricular thalamic nucleus is most densely innervated. Thalamic regions with more moderate numbers of fibers include the reuniens, rhomboid, and centromedial nuclei. The traditional primary sensory relay nuclei are mainly devoid of fibers, as is the reticular formation. Other forebrain structures receiving orexin innervation include the zona incerta, lateral habenula,

Fig. 4. (*Continued*) nerve (PSV), **(L)** dorsal motor nucleus of the vagus (DMX), **(M)** hypoglossal nucleus (XII), and lateral reticular nucleus (LRN). Scale bar = 1 mm in M (applies to all). Abbreviations: 4v, fourth ventricle, Aq, cerebral aqueduct, lv, lateral ventricle. (Reprinted from ref. *41*.)

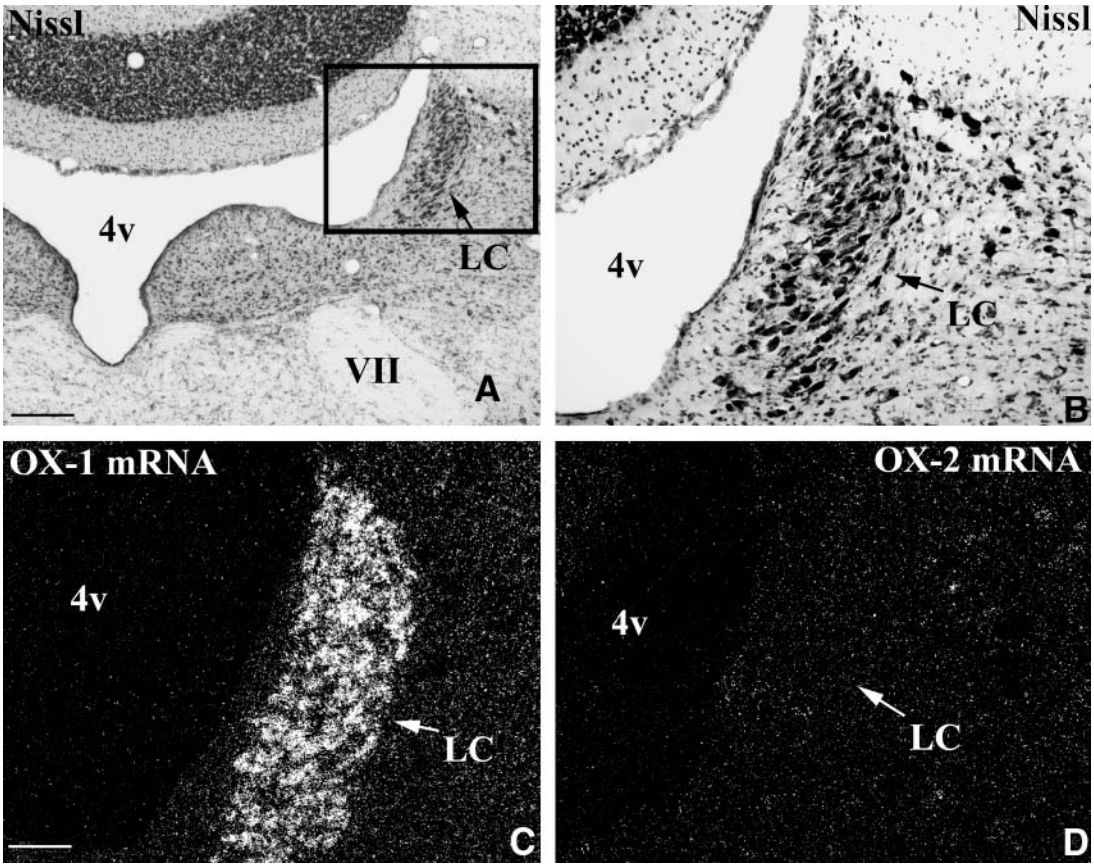

Fig. 6. A series of photomicrographs contrasting OX_1R and OX_2R mRNA expression in the locus coerulus (LC). Note the lack of OX_2R mRNA. **(A)** Low-power magnification of a Nissl stained brain section. **(B)** A higher power view of the boxed section in (A). **(C)** Adjacent section of the brain shown in (B), showing OX_1R receptor mRNA. White clusters represent specific hybridization of riboprobe to orexin receptor mRNA. **(D)** Adjacent section of the brain shown in (B), showing OX_2R receptor mRNA. Scale bars = 250 µm in (A); 100 µm in (C) (applies to [B–D]). Abbreviations: 4v, fourth ventricle; VII, genu of the seventh cranial nerve. (Reprinted from ref. *41.)*

substantia innominata, tenia tecta, induseum griseum, subthalamic nucleus, and bed nucleus of the stria terminalis.

4.2.2. Forebrain OX_1R mRNA

As is the case in the hypothalamus, the distribution of orexin receptor mRNA mirrors that of the fiber distribution. In cortical regions, OX_1R mRNA is prominent in layers 5 and 6 of the cingulate, infralimbic, and prefrontal cortices, with less present in layer 2. OX_1R mRNA is expressed across the amygdala at low levels. High levels of expression are seen in the bed nucleus of the stria terminalis (Fig. 3C). Within the basal forebrain, OX_1R mRNA can be seen in the nucleus of the diagonal band (Fig. 3B). In the hippocampus, OX_1R mRNA is primarily located in the dentate gyrus, and in the CA1 and CA2 regions of Ammon's horn (Fig. 3E, and F). In the thalamus, the paraventricular nucleus contains robust expression of OX_1R mRNA, whereas low levels of expression are seen in other midline thalamic nuclei (Fig. 3C).

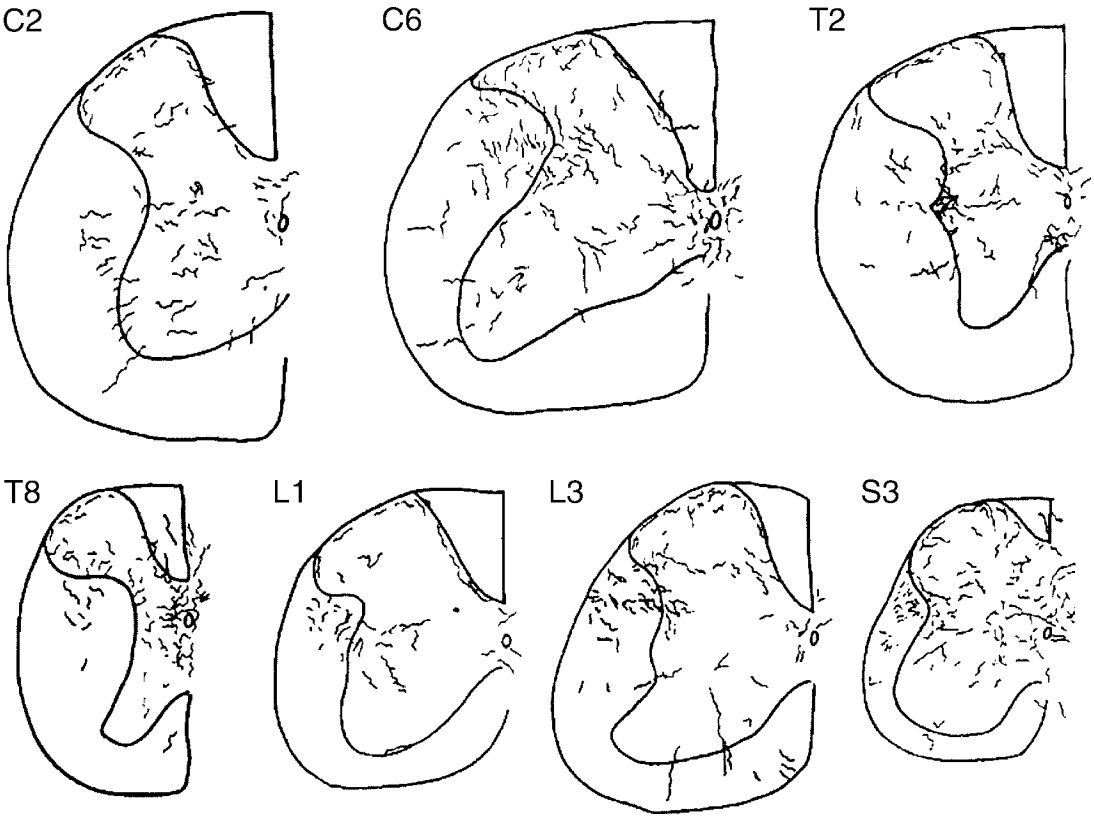

Fig. 7. Camera lucida drawings of orexin fiber distribution within selected levels of the rat spinal cord. Fibers are shown as thin lines (not to scale) in their location in the gray and white matter at cervical (C2 and C6 levels), thoracic (T2 and T8 levels), lumbar (L1 and L3 levels), and sacral (S3 level). (Modified from ref. *39*.)

4.2.3. Forebrain OX_2R mRNA

In the cortex, OX_2R mRNA is most noticeable in layers 2 and 6 of the neocortex (Fig. 4B). OX_2R mRNA is also expressed at moderate levels in the bed nucleus of the stria terminalis (Fig. 4C) and within the cortical nucleus of the amygdala. The basal forebrain contains robust OX_2R mRNA expression in the medial septal nucleus and within both the vertical and horizontal limbs of the nucleus of the diagonal band (Fig. 4B). Within the hippocampus, OX_2R mRNA is seen in the CA3 region of Ammon's horn (Fig. 4E, and F). In addition to several other midline thalamic nuclei listed in Table 1, the rhomboid and paraventricular thalamic nuclei contain noteworthy OX_2R mRNA expression in the thalamus (Fig. 4E).

4.3. Descending Projections From the Hypothalamus

4.3.1. Descending Orexin Fibers

Midbrain, medullary, and forebrain regions receive a similarly dense innervation. Travelling caudally from the hypothalamus, orexin fibers extend through the ventral tegmental area and central gray. Some fibers are seen in the substantia nigra pars compacta. Orexin fibers are present in the raphe nuclei, especially the dorsal raphe (Fig. 2D), median raphe, and raphe magnus nuclei. Fibers extend throughout the periaqueductal gray and continue into the superior and inferior colliculi. The pedunculopontine and laterodorsal tegmental nuclei receive a

Table 1
Relative Densities of Orexin Receptor mRNA Expression in the Rat Brain[a]

Brain area	Orexin-1	Orexin-2
Cerebral cortex and cerebellum		
Neocortex, layer 6	+	++
Neocortex, layer 5	+	−
Neocortex, layer 2	−	++
Cingulate cortex, layer 3	++	−
Anterior olfactory nucleus	++	−
Insular area, agranular	+	−
Endopiriform area	+	−
Piriform cortex	−	++
Infralimbic cortex	++	+
Prefrontal cortex	++	−
Cerebellum	−	−
Claustrum	+	−
Globus pallidus	−	+
Nucleus accumbens, rostral	−	+
Amygdala and bed nucleus of the stria terminalis		
Bed nucleus of the stria terminalis, principal	+++	++
Nucleus of the lateral olfactory tract	++	+
Medial nucleus amygdala, anterodorsal part	++	+
Cortical nucleus amygdala	+	++
Amygdala–hippocampal transition zone	++	−
Central nucleus amygdala	+	−
Basomedial nucleus	+	−
Substantia innominata	+	+
Hippocampus and septum		
Taenia tecta	+++	+
Septohippocampal nucleus	−	−
Faciola cinerea	++	++
Induseum griseum	+++	++
Septofimbrial nucleus	−	+
Doral septal nucleus	+	−
Lateral septal nucleus	−	+
Medial septal nucleus	−	+++
Nucleus of the diagonal band, vertical limb	+	++
Nucleus of the diagonal band, hoizontal limb	++	+++
Dentate gyrus	++	+
CA1 field	+	−
CA2 field	++	−
CA3 field	−	+++
Thalamus		
Anteromedial nucleus thalamus, dorsal	++	+
Centrolateral thalamus	+	++
Centromedial nucleus thalamus	+	++
Interanterodorsal nucleus thalamus	+	−
Interanteromedial nucleus thalamus	+	++
Medial dorsal nucleus thalamus	−	−

Table 1 *(Continued)*

Brain area	Orexin-1	Orexin-2
Nucleus reuniens, medial	+	+
Paraventricular nucleus thalamus	+++	++
Rhomboid nucleus	+	+++
Intergeniculate leaflet	++	+
Intralaminar nucleus	−	+
Pericentral nucleus	−	+
Hypothalamic preoptic nuclei		
Anteroventral preoptic area	+	+
Magnocellular preoptic area	+	++
Medial preoptic area	−	++
Medial preoptic nucleus	++	++
Median preoptic nucleus	+	+
Supraoptic nucleus	+	+
Ventrolateral preoptic area	−	+
Ventromedial preoptic area	+	+
Hypothalamic nuclei		
Anterior hypothalamic area, anterior	++	+
Anterior hypothalamic nucleus	+++	+
Anteroventral periventricular nucleus hypothalamus	+	++
Arcuate nucleus hypothalamus	−	+++
Dorsal hypothalamic area	++	+
Dorsomedial nucleus hypothalamus, anterior	+	++
Dorsomedial nucleus hypothalamus, posterior	−	++
Lateral hypothalamic area	+	+++
Lateral mammillary nucleus	−	++
Medial mammillary nucleus	−	++
Paraventricular nucleus hypothalamus, dorsal parvocellular	−	+
Paraventricular nucleus hypothalamus, lateral parvocellular	−	+
Paraventricular nucleus hypothalamus, medial parvocellular	−	+++
Paraventricular nucleus hypothalamus, posterior magnocellular	−	−
Paraventricular nucleus hypothalamus, ventral parvocellular	−	+
Posterior hypothalamus	++	++
Premammillary nucleus, dorsal	+	++
Premammillary nucleus, ventral	++	+++
Ventromedial nucleus hypothalamus, central	++	+
Ventromedial nucleus hypothalamus, dorsomedial	+++	++
Subparafasicular nucleus	−	++
Supramammillary nucleus	++	++
Tuberomammillary nucleus, medial	−	+++
Tuberomammillary nucleus, lateral	−	+++
Vascular organ of the lamina terminalis	+	−
Ventromedial nucleus hypothalamus, ventrolateral	+	−
Ventromedial nucleus hypothalamus, anterior	+	−
Midbrain, pons, and medulla oblongata		
A4 cell group	+++	−
A5 cell group	+++	−

(*Continued*)

Table 1 *(Continued)*

Brain area	Orexin-1	Orexin-2
A7 cell group	+++	–
Anterior pretectal nucleus	–	++
Barrington's nucleus	–	++
Dorsal motor nucleus of vagus	++	++
Dorsal raphe nucleus	++	++
External cuneate nucleus	–	+
Facial motor nucleus	–	++
Fields of Forrel	++	–
Hypoglossal nucleus	–	++
Inferior colliculus, external nucleus	+	–
Inferior colliculus, superficial and central layers	–	+
Interpeduncular nucleus, dorsal cap	+	++
Lateral habenula	–	++
Lateral reticular nucleus	–	++
Lateral vestibular nucleus	–	+
Laterodorsal tegemental nucleus	++	+
Locus coerulus(A6 cell group)	+++	+
Medial raphe nucleus	++	++
Medial vestibular nucleus	+	+
Midbrain reticular formation	–	++
Motor trigeminal	+	–
Nucleus ambiguous	–	+
Nucleus of the brachium of the inferior colliculus	–	+
Nucleus of the posterior commissure	–	++
Nucleus of the solitary tract	++	+
Nucleus prepositus	–	+
Nucleus raphe magnus	+	+
Nucleus raphe obscurus	++	–
Nucleus trapezoid body	–	++
Parabigeminal nucleus	–	++
Parabrachial nucleus	–	+
Peduncular pontine nucleus	++	+
Periaqueductal gray matter	++	++
Pontine gray	++	++
Principal sensory nucleus trigeminal nerve	–	++
Spinal trigeminal nucleus	–	++
Substantia nigra pars reticulata	–	–
Substantia nigra, pars compacta	++	++
Subthalamic nucleus	+	++
Superior colliculus	+	+
Superior olivary complex, inferior part	–	++
Ventral cochlear nucleus	–	+
Ventral tegmental area	++	++
Zona incerta	++	++

[a]Qualitative estimates of orexin receptor mRNA expression as described in Marcus et al. *(41)*. The following scale was used: +++, highest density; ++, moderate density; +, low density slightly above background; –, background density.

moderate orexin fiber innervation. The locus coeruleus is the site of the highest concentration of orexin fibers (Fig. 2E). Fibers are also present in the subceruleus area and in the region of the A5 noradrenergic cell group, opposing noradrenergic neurons *(50)*. More caudally, orexin fibers exist in the parabrachial nucleus and both the pontine and medullary reticular formations. In the brainstem sensory nuclei, orexin fibers are seen in the dorsal cochlear nucleus and the nucleus of the solitary tract (Fig. 2F). In brainstem motor nuclei, the dorsal motor nucleus of the vagus nerve is prominently innervated. Additionally, collateral projections arising from single orexin neurons have been shown to project to both the dorsal vagal complex and the nucleus ambiguous *(51)*. Few fibers are seen in the facial motor and trigeminal motor nuclei. Within the cerebellum, few orexin fibers have been reported, with the exception of the flocculus *(34)*.

Orexin fibers also extend throughout the spinal cord (Fig. 7). Fibers have been reported at all levels of the spinal cord from cervical to sacral, although the cervical and rostral thoracic levels contain the highest number of fibers *(37,39,52)*. Within the white matter of the cord, orexin fibers primarily descend in the dorsal portion of the lateral white matter *(39)*. In the gray matter, orexin fibers terminate in laminae known to receive innervation from the lateral hypothalamus. These regions include the superficial dorsal horn (laminae I and II), the interomediolateral cell column (IML), lamina VII, and the area around the central canal including the central autonomic area (CAA, lamina X). Whereas orexin fibers appose and form synapses with cholinergic sympathetic preganglionic neurons in the IML at the T1 and T2 levels, appositions at more caudal levels are more rare. Throughout the cord, orexin fibers appose cholinergic neurons in lamina X *(52)*.

4.3.2. Midbrain, Pons, and Medulla OX_1R mRNA

OX_1R mRNA is expressed in a wide variety of brain regions caudal to the hypothalamus. The brain areas that express OX_1R mRNA most robustly are the locus coeruleus and A5 cell group (Figs. 3J, and K, 6). The A4 and A7 noradreneric groups also contain high levels of OX_1R mRNA expression. More moderate levels of mRNA expression are seen in the ventral tegmental area, the substantia nigra, periaqueductal gray, and zona incerta (Fig. 3H). The pedunculopontine and laterodorsal tegmental nuclei both express OX_1R mRNA (Fig. 3I). The raphe nuclei, including the dorsal raphe (Fig. 3I), medial raphe, and raphe obscurus (Fig. 3L) have moderate levels of OX_1R mRNA expression. In the hindbrain, there is moderate expression of OX_1R mRNA in the dorsal motor nucleus of the vagus, the nucleus of the solitary tract, and the reticular formation, extending to possible A1/C1 neurons in the ventrolateral medulla (Fig. 3M).

4.3.3. Midbrain, Pons, and Medulla OX_2R mRNA

Caudal to the hypothalamus, OX_2R mRNA is also expressed in a wide variety of brain regions. Many of these sites, listed in Table 1, have low levels of diffuse mRNA expression, including the nucleus of the solitary tract, nucleus ambiguous, and parabrachial nucleus. Several sites have more robust mRNA expression. The sites include the pontine gray (Fig. 4I), the dorsal and medial raphe nuclei (Fig. 4I), the dorsal motor nucleus of the vagus (Fig. 4L), the spinal trigeminal nucleus, the hypoglossal nucleus (Fig. 4M), and the facial motor nucleus. OX_2R mRNA is present in sensory nuclei such as the principal trigeminal sensory nucleus (Fig. 4J). The midbrain reticular formation and (in the hindbrain) the lateral reticular formation also have moderate levels of OX_2R mRNA expression (Fig. 4M).

5. FUNCTIONAL IMPLICATIONS

The anatomy of the orexin system has given rise to numerous models that predict a number of physiological roles of the orexins and orexin receptors (*see* other chapters in this book). Whereas orexin neurons themselves are located in a restricted location within the lateral hypothalamus, orexin fibers and orexin receptors are found across the CNS. It is thus not surprising that orexins have been implicated in numerous physiological systems, including sleep/wake states, arousal, feeding, neuroendocrine regulation, autonomic control, and locomotor activity. Indeed, from an anatomic point of view, the lateral hypothalamus is ideally positioned to regulate these responses, and furthermore, orexin receptor mRNA is found in many central regions known to be involved each of these responses. The relative contribution of each orexin peptide and each orexin receptor in any given brain region remains unclear and is particularly hard to tease apart.

The functional relevance of the orexin system has been most strongly linked to central systems controlling sleep/wake states and arousal. Orexins are thought to constitute a wake-promoting neuronal group *(53)*. The loss of orexin neurons in rodents leads to a narcolepsy-like phenotype *(54–56)*, and in human narcoleptics orexin neurons are absent *(57)*. Conversely, both central orexin administration by pharmacological means and overexpression by transgenesis cause wakefulness and can reverse the narcolepsy/cataplexy phenotype of mice without orexin neurons *(58)*. In addition to a general innervation of the cerebral cortex, by which orexin peptides might help maintain arousal, orexin neurons project to several central regions thought to be involved in the control of arousal and sleep. Orexin fibers densely innervate the basal forebrain, including the medial septum and the nucleus of the diagonal band. Both OX_1R and OX_2R mRNA are found in these sites. Orexin peptides have been shown to excite basal forebrain cholinergic neurons that may be important for maintaining arousal *(59,60)*. The dorsal raphe nucleus is another arousal-related region receiving innervation from orexin neurons and expressing mRNA for both receptor subtypes. Orexin application onto neurons of the dorsal raphe also has been shown to cause direct excitation *(61–63)*. These serotinergic neurons, in turn, may provide general cortical activation and ensuing wakefulness.

Interestingly, some of these brain regions contain only one orexin receptor subtype. For example, the locus coeruleus, laterodorsal tegmental nucleus (LDT), and pedunculopontine tegmental nucleus (PPT) are examples of regions, densely innervated by orexin fibers, that contain solely the OX_1R mRNA. These regions have been implicated in general cortical arousal, in the case of the locus coeruleus, and in REM sleep maintanance, in the case of the PPT/LDT, and will be discussed elsewhere in this text. Orexin application in these regions causes neuronal excitation *(50,64–67)*. Ultrastructural examination of noradrenergic neurons in the locus coeruleus innervated by orexin fibers has confirmed the presence of synaptic contacts *(50)*.

On the other hand, the tuberomammillary nucleus of the hypothalamus, containing histaminergic neurons also important for maintaining arousal, solely expresses OX_2R mRNA. Orexin application onto tubermammillary neurons is excitatory *(49,68)*, and orexin fibers have been shown to form synapses on tuberomammillary neurons *(12)*. Furthermore, the wakefulness induced by orexin administration requires an intact histaminergic system *(69)*. Future genetic, pharmacological, and electrophysiological studies will probably tease apart receptor subtype-specific components of arousal and sleep/wake state control.

In addition to sleep/wake and arousal, orexins have been implicated in feeding behavior, neuroendocrine and metabolic regulation, and autonomic control. In support of this notion,

orexin fibers innervate the paraventricular and arcuate hypothalamic nuclei, as well as the LHA itself. These hypothalamic nuclei have all been classically implicated in neuroendocrine, autonomic, and metabolic regulation. Orexin receptor mRNA, particularly OX_2R mRNA, is expressed in these regions. Arcuate neurons in particular, some of which express NPY and are thought to be involved in the control of feeding and body weight homeostasis, are excited by orexin administration *(70,71)*. Similarly, MCH neurons in the lateral hypothalamus are activated in response to application of orexins *(47,72)*. Several hindbrain regions involved in central autonomic control and metabolic regulation are innervated by orexin neurons and include the dorsal motor nucleus of the vagus, the nucleus of the solitary tract, the nucleus ambiguous, and the rostral ventral lateral medulla. These regions express orexin receptor mRNA, and several studies have shown direct excitatory and in some cases inhibitory effects of orexin administration in these regions *(73–75)*. Furthermore, sympathetic preganglionic neurons in the spinal cord, a portion of which express orexin receptor mRNA (Marcus and Elmquist, unpublished observations), also receive direct synaptic contacts from orexin neurons *(52)*, and these neurons respond electrically to orexin peptide *(76,77)*.

From all the anatomic foundations described above, recent physiological experiments can be placed in context. As mentioned earlier, orexin neurons themselves can detect and respond to metabolic cues such as glucose, ghrelin, and leptin *(18,78)*. Finally, it is interesting to speculate that orexin neurons may utilize a complex combination of both hypothalamic and subhypothalamic effectors through both receptor subtypes to direct integrated responses and maintain metabolic and autonomic homeostasis, in addition to their role in regulating sleep/wake states.

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4
Neuronal Responses to Hypocretin/Orexin

Anthony N. van den Pol, PhD and Claudio Acuna-Goycolea, PhD

1. INTRODUCTION

The peptides hypocretin-1 and -2 (also called orexin-A and-B) were first described in 1998 in two independent papers by de Lecea et al. *(1)* and Sakurai et al. *(2)*. These two peptides are synthesized from the same prepropeptide, and therefore both peptides are found in the same cells and are probably released simultaneously from axon terminals. The neurons that synthesize the hypocretins are found selectively in the lateral hypothalamus perifornical area, and axons from these cells project widely throughout the brain *(3)* and spinal cord *(4)*. Two receptors have been identified *(2)*. These appear to be Gq protein coupled. The hypocretin receptor 1 (orexin receptor 1) has a greater affinity for hypocretin-1, whereas the hypocretin receptor 2 (orexin receptor 2) is activated similarly by both hypocretin-1 and -2 *(2)*.

Many brain regions are innervated by hypocretin-immunoreactive axons including the locus ceruleus, dorsal tegmentum, tuberomammillary nucleus, dorsal raphe, substantia gelatinosa of the cord, midline thalamus, and a diffuse projection to the cerebral cortex *(3,4)*. A high density of hypocretin axons is also found within the hypothalamus, in both the lateral and medial parts. Just as hypocretin axons have a wide distribution throughout the brain, with selective areas being more highly innervated, hypocretin receptors are similarly distributed throughout the central nervous system (CNS), with some regions showing high levels of expression *(5)*. Some of these brain regions express one receptor subtype predominantly, for instance, the locus ceruleus expresses primarily hypocretin receptor 1, whereas the tuberomammillary nucleus may predominantly express the hypocretin receptor 2 *(5)*. Other parts of the brain express both types of receptor.

Hypocretin plays an important role in arousal, and the absence of hypocretin or its receptors leads to narcolepsy in mice, dogs, and humans *(6–10)*. Hypocretin might also be involved in the regulation of feeding; CNS injections of hypocretin are reported to increase food intake *(2,11)*, fasting increases hypocretin mRNA levels *(12)*, and hypocretin knockout animals may show reduced food intake *(13)*.

From the first studies of its effects on hypothalamic neurons *(1,14)*, hypocretin has been shown to exert excitatory actions (Fig. 1). These excitatory actions are mediated by a wide range of cellular mechanisms, with different mechanisms accounting for hypocretin actions in different types of cells, as discussed in more detail below.

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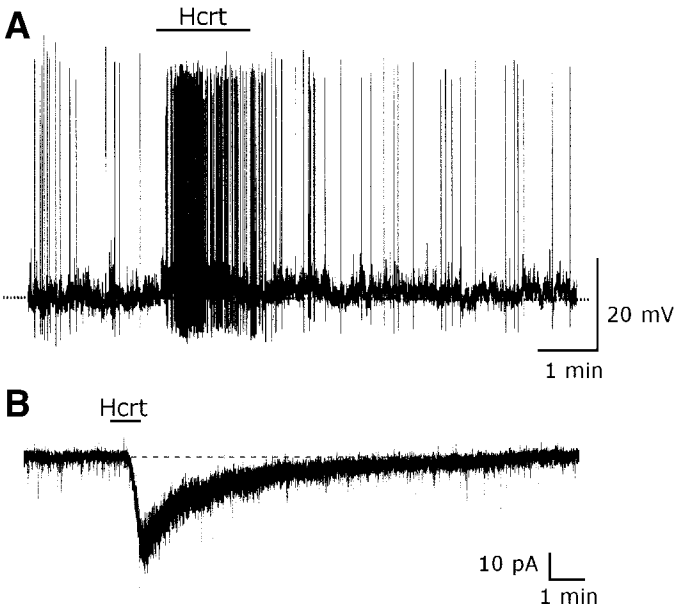

Fig. 1. (A) Hypocretin (Hcrt) increased spike frequency in an LC neuron. **(B)** In voltage clamp (−60 mV holding potential), hypocretin evoked an inward current in a spinal cord dorsal horn neuron. (Modified from ref. *26* [A] and *20* [B].)

2. MEMBRANE MECHANISMS OF HYPOCRETIN EXCITATION

2.1. Hypocretin Enhances Na-Dependent Currents

Hypocretin exerts depolarizing effects on the membrane potential and increases spike frequency in several brain regions, including the hypothalamus *(15,16)*, thalamus *(17)*, brainstem *(18),* and spinal cord *(19,20)* (Fig. 1A). These excitatory actions of hypocretin appear to be dependent on external sodium; the replacement of extracellular NaCl with NMDG-Cl abolished the depolarization by hypocretin in hypothalamic neurons *(21)*. In parallel to its effects on the membrane potential, hypocretin can induce a robust inward current at holding potentials near −60 mV (Fig. 1B). This hypocretin-induced current is dependent on Na^+, as a decrease in extracellular Na^+ (by replacing with choline, Tris, or NMDG to preserve the osmolarity) consistently suppressed the inward current induced by hypocretin (Fig. 2) *(22–26)*.

2.2. Nonselective Cation Current

The experiments described above show that Na^+ can be a critical component of hypocretin's excitatory actions. Depending on the recording conditions, the reversal potential for a Na^+ current would be positive to +40 mV according to the Nernst equation. Hypocretin-mediated current reversed at –19 mV in the dorsal raphe *(24)* and at –44 mV in the nucleus of the solitary tract *(27)*. As these values are substantially negative to the reversal potential predicted for sodium, it is unlikely that a pure sodium conductance accounts for these results. The difference between the two values is owing to a combination of recording conditions and cell characteristics.

One mechanism that would account for the reversal potentials reported for hypocretin-mediated excitation is based on activation of a nonselective cation channel *(24,27)*. Liu et al. *(24)* recorded from serotonin cells from the dorsal raphe identified electrophysiologically by a long spike duration (1 ms, at half amplitude), an input resistance >200 MΩ, and a serotonin-mediated hyperpolarization *(28)*. In current clamp hypocretin depolarized these serotonin

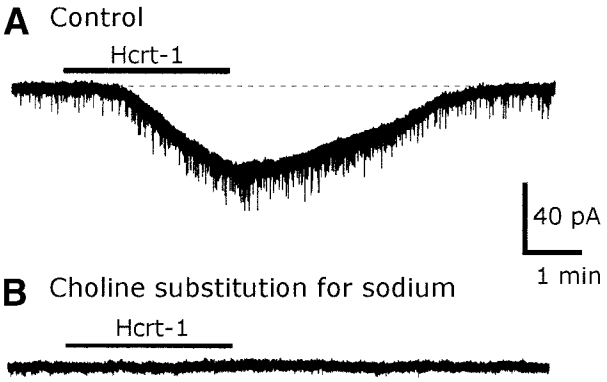

Fig. 2. The hypocretin (Hcrt-1)-induced inward current **(A)** was abolished by choline substitution for sodium **(B)** in the bath. (Modified from ref. *26.)*

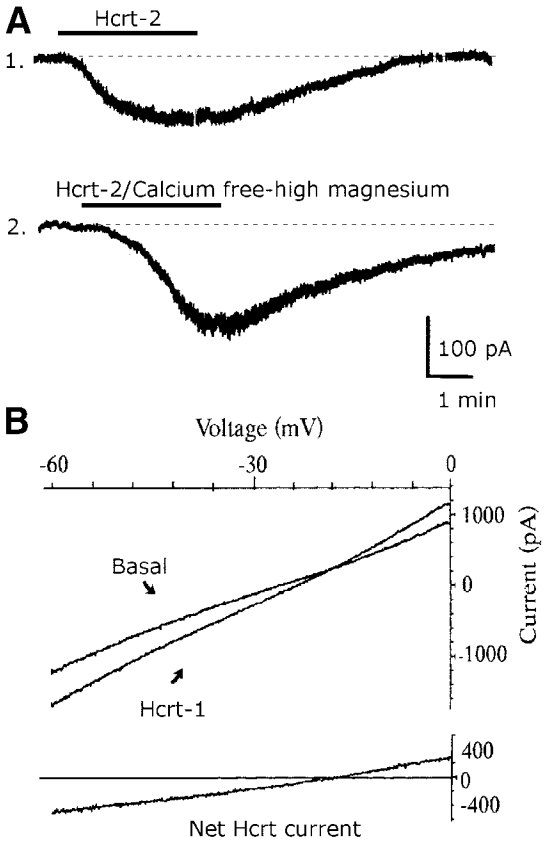

Fig. 3. Hypocretin (Hcrt) activates a nonselective cationic current in a serotonin neuron of the dorsal raphe. **(A)** Upper trace. Hypocretin induced a robust inward current (−60 mV holding potential). Lower trace. In nominal calcium-free/high-magnesium external solution, the hypocretin-evoked current was not depressed. **(B)** The reversal potential for the hypocretin-sensitive current was near −19 mV. CsCl was used in the recording pipets in these experiments. (Modified from ref. *24.*)

cells, and in voltage clamp, hypocretin generated an inward current (Fig. 3A, upper trace), with an associated increase in current noise suggestive of increased ion channel opening. These hypocretin actions were dependent on extracellular sodium; when sodium was

exchanged for choline or Tris, hypocretin evoked little inward current. To determine whether the current was dependent on calcium or on calcium-dependent transmitter release, a calcium-free high magnesium extracellular buffer was used. This calcium-free buffer did not block the inward current; in fact it enhanced it (Fig. 3A, lower trace). Inclusion of the calcium chelator BAPTA in the recording pipet had little effect on the hypocretin-mediated current. With CsCl in the pipet, the inward current reversed near −19 mV (Fig. 3B).

Together, the increase in inward current in the absence of extracellular calcium and the lack of dependence on intracellular calcium, the importance of extracellular sodium, and the reversal potential suggest activation of a nonselective cation current (NSCC), at a channel with both sodium and potassium conductance *(24)*. This was further corroborated by the absence of an effect of the Na^+/Ca^{2+} exchange inhibitor KB-R7943 on hypocretin-mediated currents. Similar conclusions were arrived at independently in another study of dorsal raphe serotonin neurons *(29)*; Brown et al. found that these serotonin neurons were stimulated not only by hypocretin, but also by histamine and norepinephrine. In cholinergic laterodorsal tegmental neurons, another important group of neurons involved in arousal/sleep regulation, hypocretin induced an inward current that persisted under low extracellular calcium conditions, consistent with the activation of an NSCC *(30)*. Additional data consistent with a hypocretin-activated NSCC were found in the nucleus of the solitary tract *(27)* and dorsal motor nucleus of the vagus nerve *(22)*, supporting the idea that NSCCs represent one mechanism of hypocretin excitation found in several regions of the brain.

2.3. Na^+/Ca^{2+} Exchanger

Another mechanism that may account for some Na-dependent excitatory actions of hypocretin is the activation of an Na^+/Ca^{2+} exchanger. The Na^+/Ca^{2+} exchanger can alter the activity of excitable cells due to its electrogenic activity. In the forebrain, the application of a high concentration of nickel, which blocks the Na^+/Ca^{2+} exchanger, decreased *(21)* or eliminated *(23)* the hypocretin-induced inward current, suggesting a role for the Na^+/Ca^{2+} exchanger in these actions (Fig. 4). Consistent with this view, when the specific Na^+/Ca^{2+} exchange blocker KB-R7943 was added to the bath, the hypocretin-sensitive current was depressed *(21,23,31)*. Finally, these hypocretin actions appear to depend on the availability of intracellular calcium. The inclusion of the calcium chelator BAPTA (10 m*M*) in the recording pipete completely abolished the direct actions of hypocretin *(31)*. All these results support the idea that some of the hypocretin excitatory effects may involve the activation of the Na^+/Ca^{2+} exchanger, leading to depolarization and increase in spike frequency in target cells. Although this mechanism may account for the excitatory actions of hypocretin in some neurons, other neurons that are excited by hypocretin show little evidence for this particular mechanism *(24,27)*.

2.4. Hypocretin Depresses K^+ Currents

Changes in potassium conductance can substantially alter neuronal excitability. In some regions of the brain, hypocretin excitation and depolarization is accompanied by an increase in membrane input resistance, suggesting that activation of the hypocretin receptors may lead to the closure of some ion channels, most likely those with K^+ conductance. In the locus coeruleus (LC), hypocretin depolarizes the membrane potential, with a parallel increase in the apparent input resistance of the recorded cells. When the membrane potential of these neurons was shifted to −90 mV, a value close to the reversal potential for K^+, by constant injection of negative current through the recording pipet, the hypocretin-induced depolarization was substantially reduced, supporting the idea that hypocretin excitation of LC neurons may

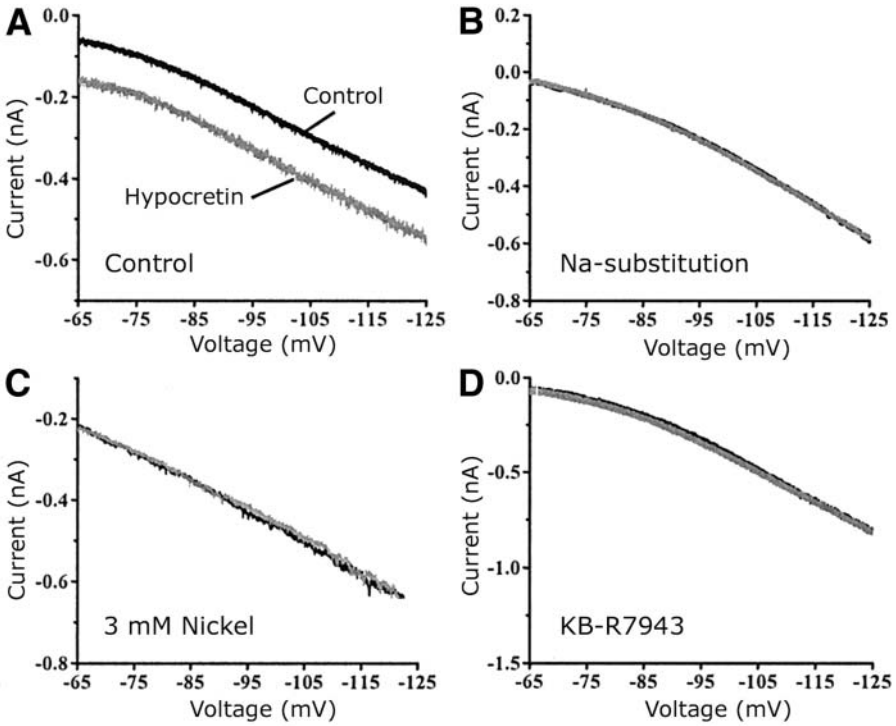

Fig. 4. In the medial septum/diagonal band, hypocretin activates a Na^+/Ca^{2+} exchanger. **(A)** Voltage ramp protocols from –125 to –65 mV reveal an inward current with hypocretin application. Substitution of extracellular sodium with choline **(B)** and application of nickel **(C)** or the Na^+/Ca^{2+} exchanger blocker KB-R7943 **(D)** to the bath blocked the hypocretin actions. (Modified from ref. *23*.)

in part be attributable to the suppression of K^+ currents *(32)*. Similar hypocretin actions have been observed in the intralaminar and midline thalamic nuclei *(17)*. Here neurons are sensitive to very low concentrations of hypocretin, and hypocretin-mediated depolarization is accompanied by an increase in membrane input resistance. Similar to LC neurons, when thalamic cells were held near the K^+ reversal potential, hypocretin evoked little change in the membrane potential *(17)*. Hypocretin has also been reported to excite cortical neurons by mechanisms based on closure of K^+ channels via hypocretin receptor-2 activation *(54)*. Interestingly, these excitatory cortical actions appear to mainly target sublayer 6B neurons with little direct effect on other cortical cell populations *(54)*.

In the nucleus of the solitary tract, hypocretin also appears to affect a K^+ conductance. Application of hypocretin induced a broadening in the spikes, and when depolarizing voltage steps were applied, a consistent reduction in the current response was detected consistent with a suppression of K^+ channels *(27)*. Additionally, hypocretin was initially postulated to act by reducing a K^+ current in dorsal raphe neurons *(33)*, but subsequent analysis by the same group showed that the substantive mechanism of hypocretin action was more dependent on activation of a mixed cation conductance (*see* ref. *29* for further discussion).

Finally, hypocretin depressed the after-hyperpolarization (AHP) at the end of the action potential in LC neurons (Fig. 5) *(34)*. The AHP is dependent on the activation of a subset of K^+ currents, including Ca^{2+}-activated K^+ channels, and can be modulated by a number of transmitters, including neuropeptides *(35)*. The depression of the AHP by hypocretin might be explained by depression of these K^+ channels, or by modulation of intracellular Ca^{2+} which

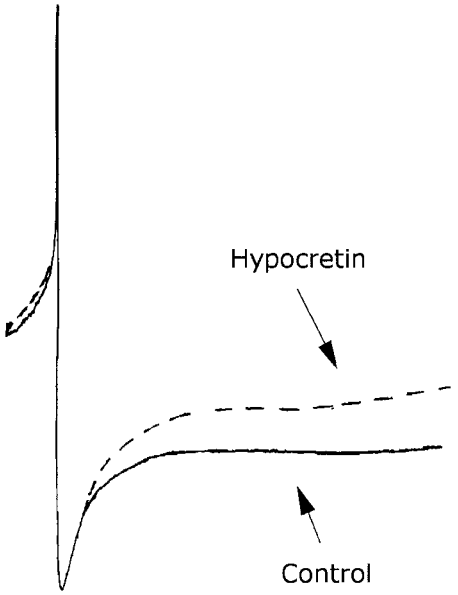

Fig. 5. In locus coeruleus neurons, hypocretin depressed the afterhyperpolarization at the end of an action potential. Peak of spike is truncated. (Modified from ref. *34.*)

may be important for activation of some K^+ channels. Reducing the AHP can increase spike frequency.

When K^+ channels are blocked, an increase in excitation may occur. However, as noted above, activation of an NSCC is excitatory and leads not only to an increase in sodium conductance, but also to an increase in K^+ conductance through the same channel. As shown in the nucleus of the solitary tract *(27)*, hypocretin may act by multiple mechanisms that would lead to an increase in K^+ conductance at the NSCC and a depression of K^+ conductance at selective K^+ channels.

2.5. Hypocretin Increases Intracellular Calcium

In the first physiological study on hypocretin actions on neurons, digital imaging with the calcium-sensitive ratiometric dye fura-2 was used. A proportion of neurons cultured from the rat hypothalamus showed an increase in calcium in the presence of hypocretin, and this returned to baseline levels in the absence of hypocretin (Fig. 6A). Repeated peptide application generated repeated calcium rises. When tested in the presence of tetrodotoxin (TTX) to block spike-mediated synaptic activity, hypocretin still generated calcium increases (Fig. 6B), indicating the activation of a postsynaptic receptor on the cell body and dendrites independent of synaptic modulation *(14)*. To determine whether hypocretin might induce calcium release from intracellular organelles, neurons were pretreated with thapsigargin to deplete intracellular stores, but this had no effect on the hypocretin response (Fig. 6C). When extracellular calcium was removed and replaced with EGTA, hypocretin no longer increased calcium levels; similarly, adding cadmium to the extracellular buffer eliminated intracellular calcium rises (Fig. 6D and E).

Together these data indicate that hypocretin increased cytoplasmic calcium by enhancing extracellular calcium entry by calcium-permeable ion channels. To test the second messenger system involved, cells were treated with the selective protein kinase C (PKC) inhibitor bisindolylmaleimide, thereby blocking hypocretin-mediated calcium rises. In contrast, little effect

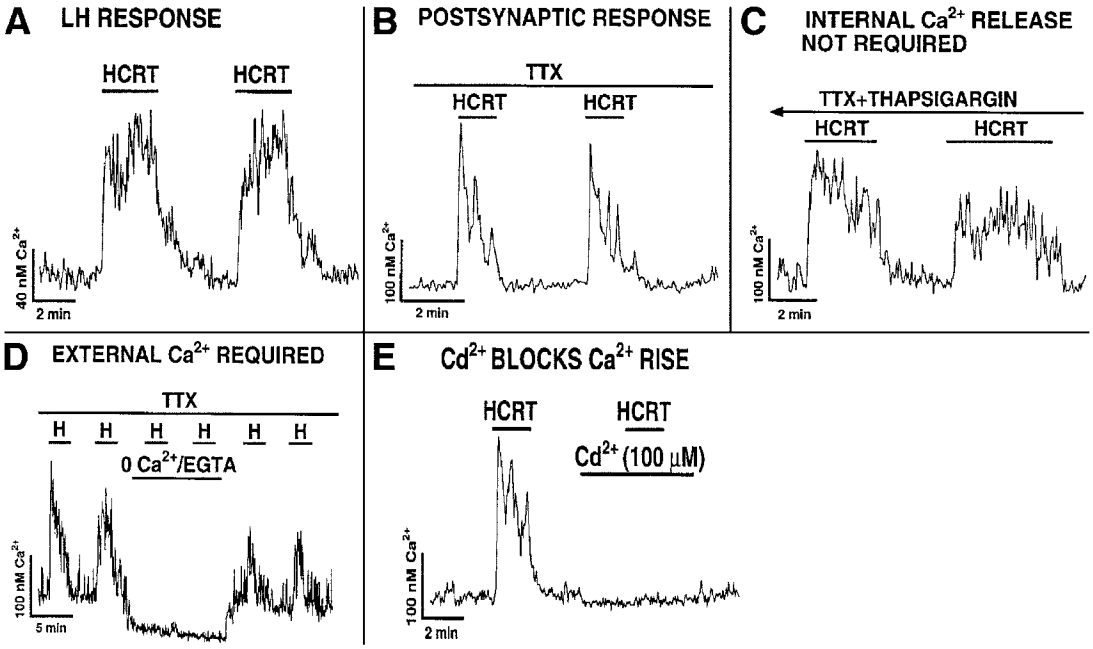

Fig. 6. Hypocretin (HCRT or H) raises intracellular calcium. **(A,B)** Hypocretin increased cytoplasmic calcium in normal buffer (**A**) and in the presence of tetrodotoxin (TTX) (**B**). **(C)** The calcium rise was not dependent on internal calcium stores, as thapsigargin-mediated depletion of intracellular calcium did not alter the hypocretin response. **(D,E)** The hypocretin effects were dependent on extracellular calcium (**D**) and were abolished by cadmium in the bath (**E**). (Modified from ref. *14*.)

of hypocretin on cAMP levels was found. Recent experiments have confirmed the role of PKC in the mediation of hypocretin excitatory actions in the nucleus of the solitary tract *(18)*. Together, these data suggested that hypocretin activated a PKC pathway leading to a phosphorylation of calcium channels and an increased calcium influx *(14)*.

In studies of laterodorsal tegmentum and dorsal raphe brain slices by Kohlmeier et al. *(36)*, hypocretin increased cytoplasmic calcium by mechanisms independent of the intracellular stores, not based on a sodium calcium exchanger, but dependent on extracellular calcium and in part on activation of L-type calcium channels. Depletion of intracellular calcium stores with cyclopiazonic acid, thapsigargin, or ryanodine did not attenuate hypocretin responses, but lowering extracellular calcium and blocking L-type calcium channels with nifedipine did reduce hypocretin-mediated calcium increases. A similar mechanism appears to account for hypocretin actions in neurons dissociated from the ventral tegmental area *(37)*.

The mechanisms underlying the hypocretin-dependent increase in calcium levels have also been studied in Chinese hamster ovary (CHO) cells transfected with DNA coding for hypocretin receptors *(38,39)*. In CHO cells, hypocretin raised intracellular calcium and inositol triphosphate (IP3), consistent with a Gq-coupled mechanism. Similar to neurons, in CHO cells the hypocretin actions have been shown to depend on external calcium. Removal of extracellular calcium abolished both the increase in intracellular calcium and the IP3 response to hypocretin. If these hypocretin-mediated calcium actions were dependent on the external calcium influx, a depression in the response after a reduction in driving force for calcium ions would be expected. Using whole cell voltage clamp, Lund et al. *(39)* studied the hypocretin effects on intracellular calcium when the holding potential was shifted from

normal −50 mV to +60 mV, a value near the calcium reversal potential. With the cells held at +60 mV, thereby suppressing the driving force for calcium, the hypocretin-mediated increase in intracellular calcium was abolished. When the membrane potential was returned to −50 mV under continuous application of hypocretin, a rapid increase in intracellular calcium was detected, consistent with the view that hypocretin-mediated elevation of intracellular calcium is dependent on the influx of extracellular calcium *(39)*.

3. HYPOCRETIN MODULATION OF SYNAPTIC ACTIVITY

3.1. Hypocretin Increases Transmitter Release by Activating Receptors on Axon Terminals

Synaptic activity in both cultures and brain slices is enhanced by hypocretin (Figs. 7 and 8). This could be due to activation of receptors on the cell body, or to receptors on axonal boutons, or both. To address the question of whether hypocretin could act directly on axon terminals, action potential-mediated synaptic activity was blocked with TTX. Even when spikes are blocked, many axons continue to release neurotransmitters, although at a lower level, detected as miniature postsynaptic currents (mPSCs) or potentials. When hypocretin was added to cultures of hypothalamic neurons, increases in spontaneous excitatory (Fig. 7) and inhibitory (Fig. 8A) synaptic activity were found; an increase in the frequency of both glutamate-mediated m excitatory (E)PSCs and GABA-dependent m inhibitory (I)PSCs was also detected. In both cases, the frequency of miniature release events was increased, with no rise in the amplitude cumulative probability (Fig. 7B and C). This suggests that the increased synaptic frequency was not owing to an enhanced receptor response in the postsynaptic neuron, but rather was due to an increased release probability from both glutamate and GABA axon terminals *(14)*. In parallel studies in the nucleus of the solitary tract, hypocretin increased the frequency of mEPSCs but not IPSCs *(40)*; in this part of the brain, hypocretin appeared to selectively activate release from excitatory axons, but not from inhibitory axons. This suggests that hypocretin is not indiscriminantly excitatory, but rather can selectively activate subsets of cells.

In studies of responses in the prefrontal cerebral cortex, hypocretin increased release of glutamate from presumptive thalamocortical axons by a mechanism dependent on TTX-sensitive spikes *(41)*. These data suggest that spikes may be initiated in the presynaptic boutons and that hypocretin may depolarize the boutons sufficiently to generate a spike leading to glutamate release and a postsynaptic response, even in the absence of the bouton's parent cell body.

Recently, a novel mechanism has been identified by which hypocretin may modulate glutamatergic transmission, based on the activation of retrograde endocannabinoid signaling. Haj-Dahmane and Shen *(55)* found that under some conditions, hypocretin reduced the release of glutamate from presynaptic axons onto serotonin neurons in the dorsal raphe. Unexpectedly, these hypocretin actions were abolished when postsynaptic serotonin cells were loaded with GDPβS (an inhibitor of G proteins) via the patch pipet, suggesting that hypocretin acted on its postsynaptic receptors and indirectly evoked a decrease in the release of glutamate from presynaptic terminals. The selective cannabinoid receptor 1 (CB1) agonist WIN 55, 212-2 mimicked these actions of hypocretin, whereas the application of AM 251, a selective cannabinoid receptor 1 antagonist, depressed the hypocretin-mediated reduction in glutamate transmission. Together, these results are consistent with the idea that postsynaptic activation of hypocretin receptors in serotoninergic dorsal raphe neurons can induce the release of endocannabinoids that activate CB1 receptors in presynaptic glutamate terminals, leading to a decrease in transmitter release onto postsynaptic targets *(55)*.

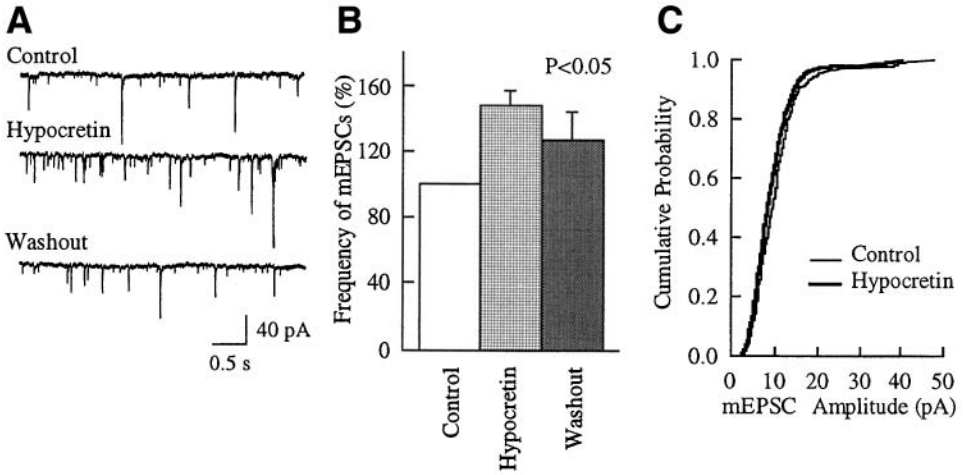

Fig. 7. Hypocretin increases excitatory activity by presynaptic mechanisms. **(A)** Bath application of hypocretin increased the frequency of miniature excitatory postsynaptic currents (mEPSCs). In the presence of TTX, hypocretin increased the frequency **(B)**, but not the amplitude **(C)**, of mEPSCs recorded in cultured hypothalamic neurons. (Modified from ref. *14*.)

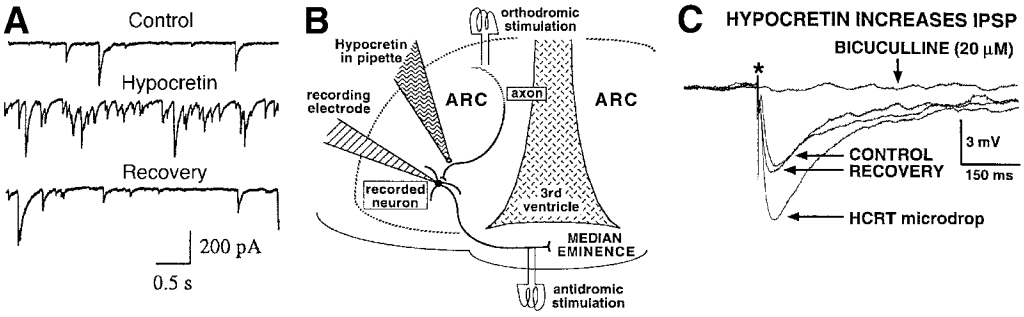

Fig. 8. Hypocretin enhances GABA release onto neuroendocrine neurons. **(A)** Hypocretin increased the frequency of inhibitory synaptic currents in hypothalamic neurons. **(B)** Experimental paradigm for **(C)**, showing recording pipet, hypocretin-containing pipet, orthodromic electrode to stimulate axons projecting to the recorded cell, and antidromic electrode to stimulate neuroendocrine neurons from their axons in the median eminence ARC, arcuate nucleus. **(C)** Hypocretin (HCRT) enhanced the GABA-mediated inhibitory-evoked potential (IPSP) in arcuate neurons projecting to the median eminence, suggesting a role for hypocretin in neuroendocrine regulation. (Modified from ref. *14*.)

3.2. Network Actions of Hypocretin

In addition to its effects on pre- and postsynaptic sites, hypocretin can indirectly modulate the neuronal excitability of a given neuron by acting on the soma of local glutamate or GABA interneurons that make synaptic contacts onto other cells. Voltage clamp recording of serotonin neurons in the dorsal raphe revealed a TTX-sensitive increase in the IPSC frequency with the application of hypocretin, and immunostaining showed hypocretin-containing boutons near the cell body of GABA-expressing cells, consistent with the idea that hypocretin may excite GABA neurons that innervate serotonin neurons *(24)*. Similar spike-dependent indirect actions of hypocretin on GABA interneurons have been observed in the medial septum/diagonal band *(23)*. These hypocretin actions on inhibitory interneurons can serve to prevent overexcitation of neuronal networks due to high levels of hypocretin release.

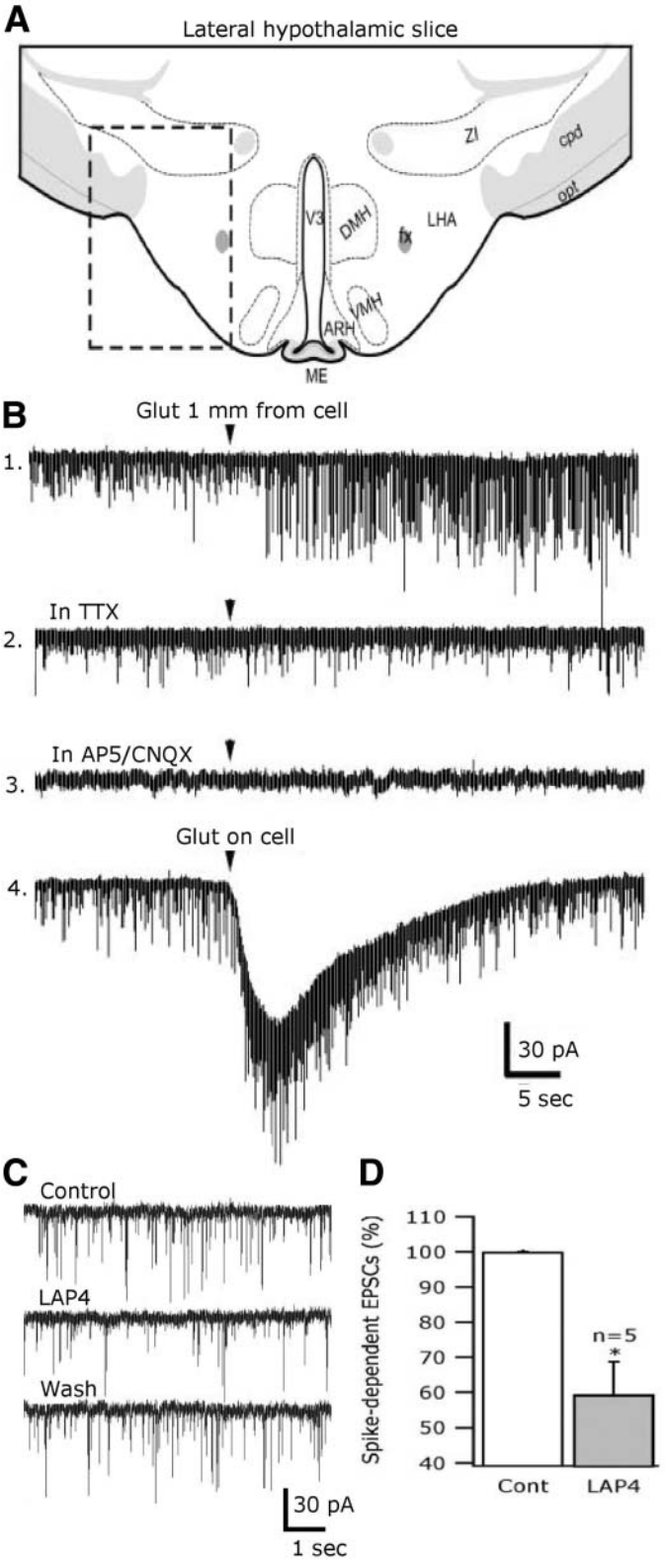
A
Lateral hypothalamic slice
ZI
cpd
opt
V3
DMH
LHA
fx
VMH
ARH
ME
B
Glut 1 mm from cell
1.
In TTX
2.
In AP5/CNQX
3.
Glut on cell
4.
30 pA
5 sec
C
Control
LAP4
Wash
30 pA
1 sec
D
Spike-dependent EPSCs (%)
110
100
90
80
70
60
50
40
n=5
*
Cont
LAP4

Fig. 9.

Hypocretin cells not only send long-distance projections throughout the CNS, but also densely innervate the lateral hypothalamus *(14)*, where they can make contact with the dendrites and cell bodies of local interneurons or with other hypocretin neurons. To evaluate the actions of hypocretin on the cells that produce it, hypocretin-1 or -2 was applied to hypocretin cells in the presence of TTX, but it exerted relatively little effect on their membrane potential. In contrast, in the absence of TTX, hypocretin increased spike frequency, in large part by increasing glutamate release onto hypocretin cells *(16)*. The hypocretin-induced increase in the release of glutamate onto hypocretin neurons may have been due to the direct activation of receptors located in the presynaptic glutamate terminals or attributable to the activation of hypocretin receptors in the somatodendritic region of glutamate neurons innervating hypocretin cells. To address this question, the action of hypocretin on the mEPSCs in the presence of TTX was studied. Under these conditions, hypocretin still increased glutamate release, suggesting that these hypocretin actions were in part caused by activation of its receptors located on presynaptic axons.

The glutamate axons synapsing onto hypocretin neurons could arise from local glutamate interneurons in the LH or from glutamatergic cells elsewhere in the brain. To evaluate this, glutamate microdrop experiments were performed in minislices containing only the lateral hypothalamus (Fig. 9A) *(42)*. Glutamate microdrops, which do not stimulate axons of passage, were applied 1 mm away from the recorded hypocretin cells, and an increase in EPSC frequency was detected in hypocretin neurons (Fig. 9B1). These microdrop actions were completely blocked by TTX (Fig. 9B2), suggesting that they were spike-dependent and not owing to a direct effect of glutamate on the recorded cell (Fig. 9B4). They were also blocked by AP5 and CNQX (Fig. 9B3), confirming that the cells stimulated by the microdrop released glutamate. These results support the idea that glutamate interneurons in the LH innervate hypocretin cells. Some of these LH glutamate interneurons might be activated by hypocretin and could play a role in recruiting the output of a network of dispersed hypocretin neurons. Although most ionotropic glutamate receptor responses to glutamate are excitatory, the group 3 metabotropic glutamate receptor agonist L-AP4 reduced excitatory synaptic activity in hypocretin neurons (Fig. 9C). L-AP4 reduced the frequency of large spike-dependent EPSCs (Fig. 9D), suggesting an action on local glutamate neurons. The mechanism was based on activation of presynaptic metabotropic glutamate receptors on local circuits neurons *(42)*.

Another mechanism by which hypocretin can affect network activity is by modulating timing of discharge of specific cell populations. In the LC, individual neurons may be coupled to others by gap junctions *(43)*, a characteristic that may facilitate the occurrence of coupled oscillations and synchronous firing during directed attention *(44)*. Hypocretin enhances membrane oscillations and synchronous firing in identified noradrenergic LC cells *(26)*. The application of hypocretin-1 to these neurons induced regular oscillations in their membrane potential that were absent in control conditions. In some spontaneously oscillating LC neurons, hypocretin-1 increased the frequency of oscillations by up to 300%. Subthreshold

Fig. 9. (*opposite page*) Local glutamate interneurons innervate hypocretin neurons. **(A)** Schematic representation of a lateral hypothalamic (LH) minislice used to study local circuits. **(B)** Trace 1. Glutamate (Glut) microdrop applied 1 mm away from the recorded cell induced a robust increase in the frequency of excitatory postsynaptic currents (EPSCs) in hypocretin cells. The glutamate microdrop effect was completely abolished by bath application of TTX (trace 2) or by the presence of glutamate receptors antagonists AP5 and CNQX in the bath (trace 3). Direct application of the glutamate microdrop to the recorded hypocretin cell evoked a direct inward current (trace 4). **(C,D)** The group III metabotropic glutamate receptor agonist LAP-4 reduced synaptic glutamate release onto hypocretin neurons. (Modified from ref. *42*.)

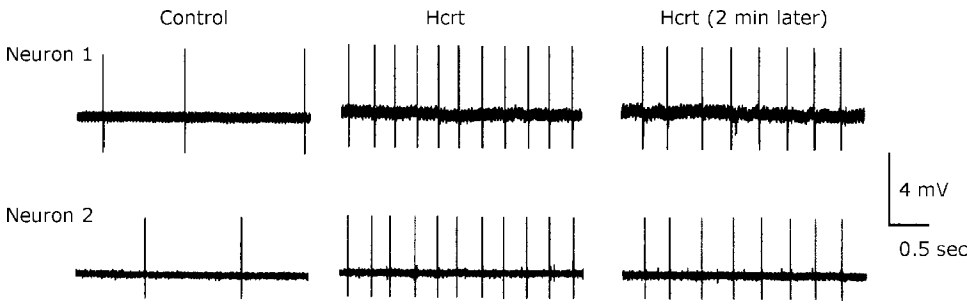

Fig. 10. Hypocretin (Hcrt) enhanced the synchronous firing of two locus coeruleus neurons recorded with two electrodes in an LC slice. (Modified from ref. *26.)*

membrane potential oscillations might underlie the occurrence of synchronic firing in neurons connected by electrical junctions *(45)*. Using dual cell recording, an increase in the synchrony of spikes was detected with the application of hypocretin to the LC cells (Fig. 10) *(26)*. A similar hypocretin effect has been detected in the spinal cord *(19)*. Here hypocretin increased the amplitude and frequency of membrane potential oscillation and induced synchronous discharge in electrically coupled pairs of spinal neurons previously silent. As hypocretin does not modify the number of coupling junctions in these cells, these results suggest that this peptide directly modulates synchronous firing in neural networks previously silent.

3.3. Many Neurotransmitter Systems Are Excited by Hypocretin

Both hypocretin axons and receptors are widespread within the brain. As hypocretin generally exerts excitatory actions, a number of other transmitter systems would be activated by hypocretin. For instance, the noradrenergic neurons of the LC receive a substantial direct synaptic hypocretin innervation as confirmed with electron microscopy *(34)*, and a number of papers have described different aspects of the excitatory responses in the LC *(26,32,46)*. Thus hypocretin activation of the LC would increase the release probability of norepinephrine in a wide variety of LC targets. In the lateral hypothalamus, norepinephrine and other catecholamines inhibit the neurons that produce hypocretin *(16,56)*. Hypocretin also activates the serotonin neurons of the dorsal raphe *(24,29,33)* leading to an increased serotonin release. Similarly, hypocretin activates the serotonin neurons of the dorsal raphe *(24,29,33)*, leading to an increased serotonin release. Similar to the LC neurons, the dorsal raphe has a widespread pattern of axonal innervation. In addition to activation of serotonin neurons, hypocretin also activates local GABAergic neurons in the raphe that terminate on nearby serotonin cells *(24)*.

Hypocretin-1 and -2 excited neurons of the tuberomammillary region identified as histaminergic by their electrophysiological properties *(21,47)*. Histamine neurons also send axons throughout many regions of the brain *(48)*.

The neurons of the lateral hypothalamus that synthesize melanin-concentrating hormone (MCH) receive a synaptic innervation from hypocretin neurons *(49,50)*. Hypocretin excites MCH neurons by a direct effect on the cell body and by increasing release of glutamate onto MCH neurons *(50)*. Since MCH-containing axons and MCH receptors are found throughout the CNS, activation of the MCH system by hypocretin would increase the probability of MCH release in many different brain regions.

In the spinal cord and brainstem, a number of different neuron types are excited by hypocretin. Hypocretin increases release of the inhibitory amino acid transmitter glycine, and the excitatory transmitter ATP in the dorsal horn of the spinal cord *(20)*. Hypocretin also

excites a subset of cells in the nucleus of the solitary tract in the dorsal medulla *(40)*. A subpopulation of neurons in the dorsal medulla synthesize glucagon-like peptide-1, a peptide that exerts multiple excitatory actions on hypocretin-containing cells *(57)*.

Neurons of the medial septum/diagonal band (MSDB) project to a number of sites, with an important projection to the hippocampus. Whereas the MSDB receives a strong hypocretin innervation that makes synaptic contact with both GABAergic and cholinergic cells *(23,25)*, the hippocampus itself receives relatively little direct hypocretin innervation *(3)*. Interestingly, hypocretin excites the GABAergic neurons of the MSDB that project to the hippocampus, identified by retrograde transport of tracer injected into the hippocampus back to the recorded neurons *(23)*. These MSDB GABA neurons terminate on inhibitory neurons of the hippocampus. Activation of the MSDB inhibitory cells would lead to an increase in hippocampal activity by reducing the inhibitory tone from local hippocampal neurons. Furthermore, hypocretin also excites the cholinergic cells of the MSDB *(25)*, and these cholinergic neurons project directly to the principal neurons of the hippocampus, also leading to excitation. Thus the combination of activating both the GABA and cholinergic cells in the MSDB would lead to a potentially powerful means of activating hippocampal circuits. Hypocretin-1 and -2 excited cholinergic neurons of the basal forebrain but had little effect on the sleep-promoting GABA neurons of the preoptic area *(51)*.

Within the hypothalamus, hypocretin increased the actions of GABAergic neurons that make synaptic contact with neuroendocrine neurons that maintain axons terminating in the median eminence *(14)*. Thus hypocretin would activate an inhibitory circuit that in this case would probably inhibit release of a pituitary tropin into the blood stream of the median eminence. A typical experiment of this sort is shown in Fig. 8B and C, in which a neuroendocrine neuron is identified by antidromic stimulation from the median eminence, and inhibitory projections to the recorded neurons are electrically stimulated. Some neurons in the arcuate nucleus, for instance those that synthesize NPY, receive direct synaptic contact from hypocretin-immunoreactive axons *(52)* and are excited by hypocretin *(53)*. NPY-containing neurons in the arcuate nucleus and other brain regions send their axons to the lateral hypothalamus where the hypocretin neurons are located; NPY depresses the activity of hypocretin neurons by activation of potassium currents, depression of calcium channels, and reduction in the release of glutamate from presynaptic axons *(58)*.

Hypocretin neurons have a strong projection to the midline thalamic nuclei. Both hypocretin-1 and -2 increased the activity of the centromedial and rhomboid nucleus by a direct action but had no effect on the sensory relay nuclei of the ventral posterolateral or dorsal lateral geniculate *(17)*. The midline thalamic nuclei maintain a nonspecific cortical projection, which may enhance cortical activation.

In summary, neurons in many regions of the brain show excitatory responses to hypocretin. Multiple pre- and postsynaptic mechanisms underlie hypocretin's actions. These mechanisms are owing to complex signal transduction pathways that couple hypocretin receptor activation, primarily through Gq protein, with changes in Na^+, K^+, and Ca^{2+} ion channel conductance.

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5
Afferent System of Orexin Neurons

Akihiro Yamanaka, PhD

1. INTRODUCTION

A small number of orexin/hypocretin neurons are specifically located in the lateral hypothalamic area (LHA), although these neurons project to almost all parts of the brain except the cerebellum *(1,2)*. Like other neurons in the brain, orexin neurons receive many excitatory or inhibitory inputs, and they output to projection sites by integrating these input signals. Because orexin neurons play a crucial role in the regulation of feeding and sleep/wakefulness *(3–6)*, it is important to explore the neural network connecting orexin neurons and other neurons for further understanding of the feeding and sleep/wakefulness regulation system. Little is known about the afferent pathway to orexin neurons, that is, which neurons in the brain project to orexin neurons, what kinds of neurotransmitter are released from these neurons, and how orexin neurons respond to these neurotransmitters. Because orexin neurons of the LHA are scarce, diffusely distributed, and lack distinct morphological features, the classical electrophysiological method is not sufficient for studying the afferent system of this type of neuron. To facilitate the analysis of orexin neurons, we created transgenic mice as a powerful analytical tool.

2. ELECTROPHYSIOLOGICAL APPROACH

Electrophysiological analysis is widely used for the study of neureons, membrane characteristics. However, it is difficult to determine by electrophysiological study alone what kind of neurotransmitter the recording neuron contains. To identify the characteristics of neurons, histochemical study is often combined with electrophysiology. However, this combination is not sufficient to identify and directly examine the electrophysiological properties of orexin neurons because orexin neurons in the LHA are scarce, and diffusely distributed. To facilitate the search for orexin neurons in living tissue, such as brain slice preparations, we created transgenic mice in which orexin neurons specifically express enhanced green fluorescent protein (EGFP). The orexin promoter, 3.2 kb upstream of the orexin gene *(6–8)*, was used for specific expression of the gene (Fig. 1A). In this transgenic mouse brain, approx 80% of orexin neurons express EGFP (Fig. 1B). No ectopic expression of EGFP was observed in these transgenic mice. We prepared brain slices from these mice and subjected them to whole cell patch-clamp recordings. Fluorescence imaging of EGFP was strong enough to identify neurons in freshly prepared slice preparations (Fig. 2A).

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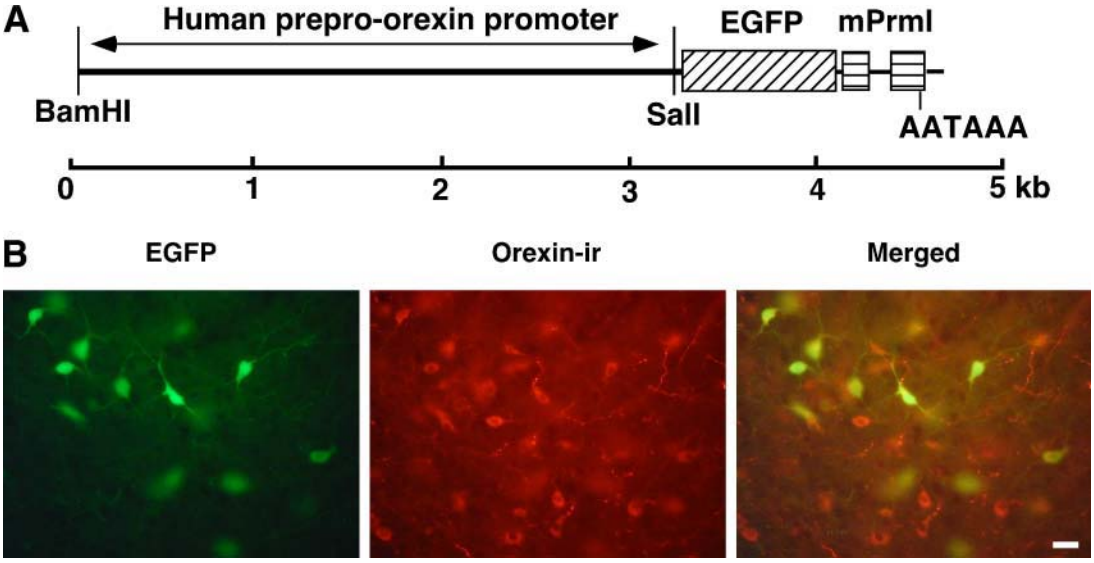

Fig. 1. Generation of transgenic mice expressing enhanced green fluorescent protein (EGFP) in orexin neurons. (**A**) Structure of the *orexin/EGFP* transgene. (**B**) Specific expression of EGFP by orexin-containing neurons in the LHA region of *orexin/EGFP* transgenic mice. Left panel, EGFP (green). Middle panel, orexin-like immunoreactivity visualized by Cy-3 (red). Right panel, merged image. Scale bar = 50 μ*M*.

2.1. Glutamatergic and GABAergic Input to Orexin Neurons

To study the effects of the classical neurotransmitters glutamate and γ-aminobutyric acid (GABA) on orexin neurons, *N*-methyl-D-aspartate (NMDA) and α-amino-3-hydroxy-5-methyl-4-isoxazole-propionate (AMPA), (glutamate receptor agonists) and muscimol and baclofen (GABA receptor agonists) were used. In whole cell current clamp, orexin neurons were depolarized by AMPA (50 μ*M*) and NMDA (200 μ*M*), whereas muscimol (200 μ*M*) and baclofen (100 μ*M*) hyperpolarized orexin neurons in the presence of tetrodotoxin (TTX) (1 μ*M*). (Fig. 2B). To determine whether orexin neurons actually received glutamatergic or GABAergic input in the brain, excitatory postsynaptic currents (EPSCs) and inhibitory (I) PSCs were recorded at a holding potential of –30 mV. Under this condition, EPSCs and IPSCs were observed as inward and outward currents, respectively (Fig. 2C, top). Simultaneous application of AP-5 (50 μ*M*), NBQX (50 μ*M*), glutamate receptor antagonists, and bicuculline (50 μ*M*), a $GABA_A$ receptor antagonist, almost blocked EPSCs and IPSCs, suggesting that transmission via glutamate and GABA forms the primary synaptic input to orexin neurons in the slice preparation (Fig. 2C, middle). EPSCs and IPSCs were again observed after washout of these antagonists (Fig. 2C, bottom).

2.2. Other Neurotransmitters

2.2.1. Input From Serotonergic Neurons

It has been reported that serotonin (5-HT) neurons in the raphe nuclei play an important role in the regulation of sleep/wakefulness because the destruction of 5-HT neurons of the raphe nuclei or the inhibition of 5-HT synthesis with *p*-chlorophenylalanine (pCPA) induces severe insomnia *(9,10)*. Orexin neurons densely innervate the raphe nuclei, which express

orexin receptors 1 and 2 (OX_1 R and OX_2 R) *(11)*. Additionally, orexins directly or indirectly activate serotonergic neurons in the dorsal raphe nucleus *(12,13)*. On the other hand, serotonergic neurons reciprocally innervate orexin neurons *(14)*. Serotonin transporter-ir (red), which is located on serotonergic presynaptic membranes that function to recycle 5-HT, are in apposition to somata or dendrites of orexin-ir neurons (green) (Fig. 3A). To study the effect of 5-HT on orexin neurons, whole cell current clamp recordings were made on slice preparations of *orexin/EGFP* transgenic mice. All EGFP-positive neurons (orexin neurons) tested were hyperpolarized by 5-HT, whereas EGFP-negative neurons in the same area showed a variety of responses to 5-HT: 33% of the neurons exhibited hyperpolarization, 20% exhibited depolarization, and 47% exhibited no effect. 5-HT hyperpolarized orexin neurons in a concentration-dependent manner; the E_{max} was –32.0 mV at 100 μ*M*, and half maximal response (EC_{50}) was 0.87 μ*M*. Hyperpolarization was accompanied by a decrease in membrane resistance. In the presence of TTX, 5-HT (10 μ*M*) application significantly decreased membrane resistance to 60%. The reversal potential estimated from the *I–V* relationship was –111 mV. This value is similar to the theoretical K^+ equilibrium potential (–116 mV) calculated from the Nernst equation at the given K^+ concentration of the external and pipet solutions. These results suggest that 5-HT decreases input resistance through an increase in potassium conductance. WAY100635, a selective antagonist for the $5\text{-}HT_{1A}$ receptor, inhibited 5-HT-induced hyperpolarization in a concentration-dependent manner (Fig. 3D), suggesting the involvement of $5\text{-}HT_{1A}$ in this response. 5-HT-induced hyperpolarization was also inhibited by a low concentration of Ba^{2+}, the G-protein–coupled inward rectifier potassium (GIRK) channel inhibitor, showing involvement of GIRK channel activation (Fig. 3E). Also, single-channel recordings revealed that the conductance of 5-HT-induced single-channel activity (33.8 pS) was consistent with previously reported GIRK conductance (32–39 pS) *(15,16)*. The $5\text{-}HT_{1A}$ receptor is known to couple with the Gi-type α subunit of G protein, and βγ subunits directly activate GIRK without the involvement of any second messengers *(17,18)*. These results suggest that 5-HT-induced hyperpolarization of orexin neurons is mediated by the $5\text{-}HT_{1A}$ receptor and by subsequent activation of the GIRK channel. If the serotonergic neurons in the raphe nuclei innervate orexin neurons, this serotonergic inhibitory input might form a negative feedback loop, because it has been reported that orexin directly or indirectly activates 5-HT neurons in the raphe nuclei *(12,13)*.

2.2.2. Input From Noradrenergic Neurons

Noradrenaline (NA) is a potent modulator of the forebrain and behavioral activity state and is also known to be an important regulator of sleep/wakefulness. The locus ceruleus (LC) is the main source of noradrenergic neurons and receives the densest innervations from orexin neurons. The LC dominantly expresses OX_1R, and orexins activate LC neurons *(19,20)*. Noradrenergic neurons spread their axons widely through the brain (i.e., the cortex, thalamus, and hypothalamus). Many tyrosine hydroxylase-ir nerve endings are observed near the orexin neurons in the LHA. Tyrosine hydroxylase-ir axons (black) are in apposition to somata or dendrites of orexin-ir neurons (brown) (Fig. 4A). To study the effect of NA on orexin neurons, whole cell current clamp recordings were made on slice preparations of *orexin/EGFP* transgenic mice. All orexin neurons tested were hyperpolarized by NA. NA hyperpolarized orexin neurons in a concentration-dependent manner; the E_{max} was 17.3 mV at 100 μ*M*, and the EC_{50} was 6.7 μ*M*. Like the 5-HT response, hyperpolarization was accompanied by a decrease in membrane resistance. The reversal potential estimated from the *I–V* relationship was –106 mV. This value is similar to the theoretical K^+ equilibrium potential (–116 mV), suggesting

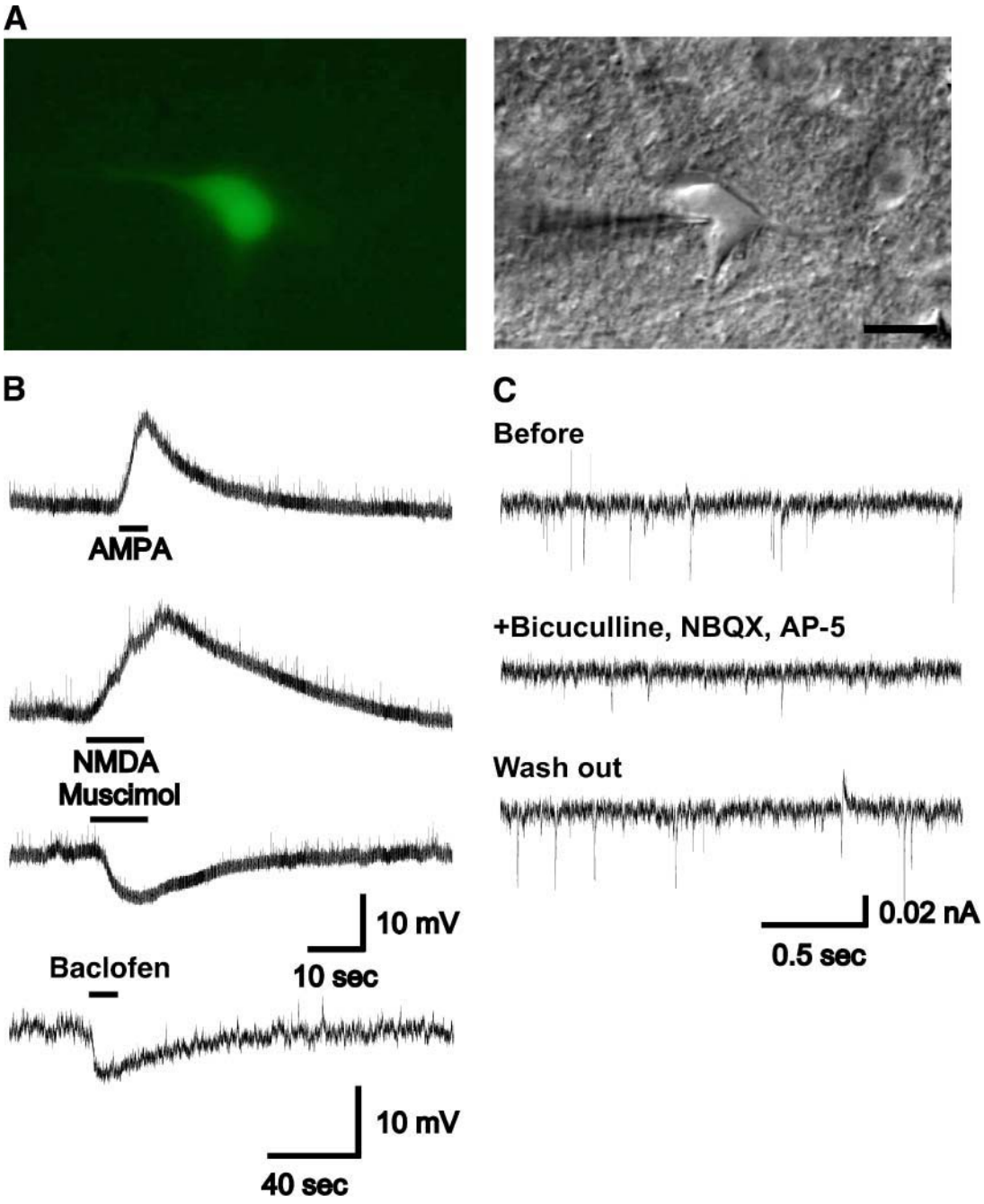

Fig. 2. Identification of orexin neurons using EGFP fluorescence in a hypothalamic slice preparation from *orexin/EGFP* transgenic mice. **(A)** Fluorescence (left) and IR-DIC (right) images of an EGFP-expressing orexin neuron. Scale bar = 20 μ*M*. **(B)** Effect of AMPA (50 μ*M*) NMDA (200 μ*M*), muscimol (200 μ*M*) and baclofen (100 μ*M*) on orexin neurons. In current clamp mode, NMDA and AMPA depolarized, while muscimol and baclofen hyperpolarized the membrane potential of orexin neurons. The Membrane potential was set at –60 mV and –45 mV before NMDA, AMPA, baclofen, and muscimol application, respectively, by current injection. All recordings were performed in the presence of TTX (1 μ*M*). NMDA, AMPA, muscimol, and baclofen were applied locally through a fine

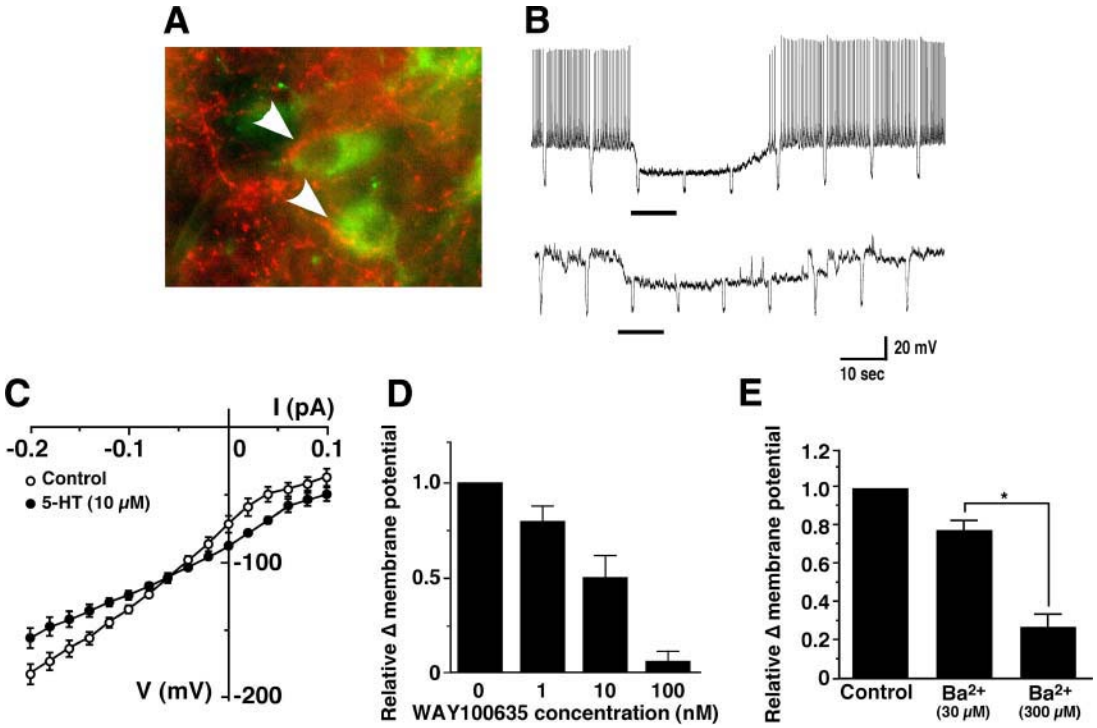

Fig. 3. The effect of 5-HT on orexin neurons. (**A**) 5-HT transporter-ir nerve endings are in close apposition to orexin-ir neurons. 5-HT transporter-ir (red, Cy3) varicosities are closely apposed to an orexin-ir (green, FITC) cell. 5-HT hyperpolarizes orexin neurons in the presence or absence of TTX. (**B**) In current clamp mode, 5-HT (10 μ*M*) was applied to orexin neurons in the absence (upper trace) or presence (lower trace) of TTX (1 μ*M*). TTX was administered by bath application, and 5-HT was applied locally through a fine polyethylene tube during the period indicated by the bars. Input resistance was monitored by the amplitude of electrotonic potentials generated by injection of a rectangular wave current pulse (–20 pA, 500 ms, 0.1 Hz). (**C**) Current-voltage (*I–V*) relationship derived from records of membrane potential in response to a series of 100-ms current steps (in 20-pA increments, –200 pA to 180 pA) from resting potential (–60 mV) in the absence (control, open circles) or presence (5-HT, filled circles) of 5-HT (10 μ*M*). The estimated reversal potential was –111 mV. (**D**) WAY100635, a 5-HT_{1A} receptor-selective antagonist, concentration-dependently inhibited 10 μ*M* 5-HT-induced hyperpolarization (1–100 n*M*). (**E**) The GIRK inhibitor Ba^{2+} concentration-dependently inhibited 5-HT-induced hyperpolarization. Values are mean ± SEM.

that NA also decreases input resistance through an increase in potassium conductance. Idazoxan, a selective α2 receptor antagonist, inhibited NA-induced hyperpolarization in a concentration-dependent manner. However, NA induced weak depolarization in the presence of a high concentration of idazoxan. This weak depolarization was inhibited by prazosin, an α1 receptor selective antagonist, suggesting that both α2 and α1 receptors are involved in the

Fig. 2. (*Continued*) polyethylene tube during the period indicated by the bars. (**C**) Voltage clamp recording held at –30 mV shows EPSCs and IPSCs (top). AP-5 (50 μ*M*) and NBQX (50 μ*M*), glutamate receptor antagonists, and $GABA_A$ receptor antagonist were administered by bath application. EPSCs and IPSCs were blocked by simultaneous treatment of these antagonists (middle), and recovered by removal of these antagonists (bottom).

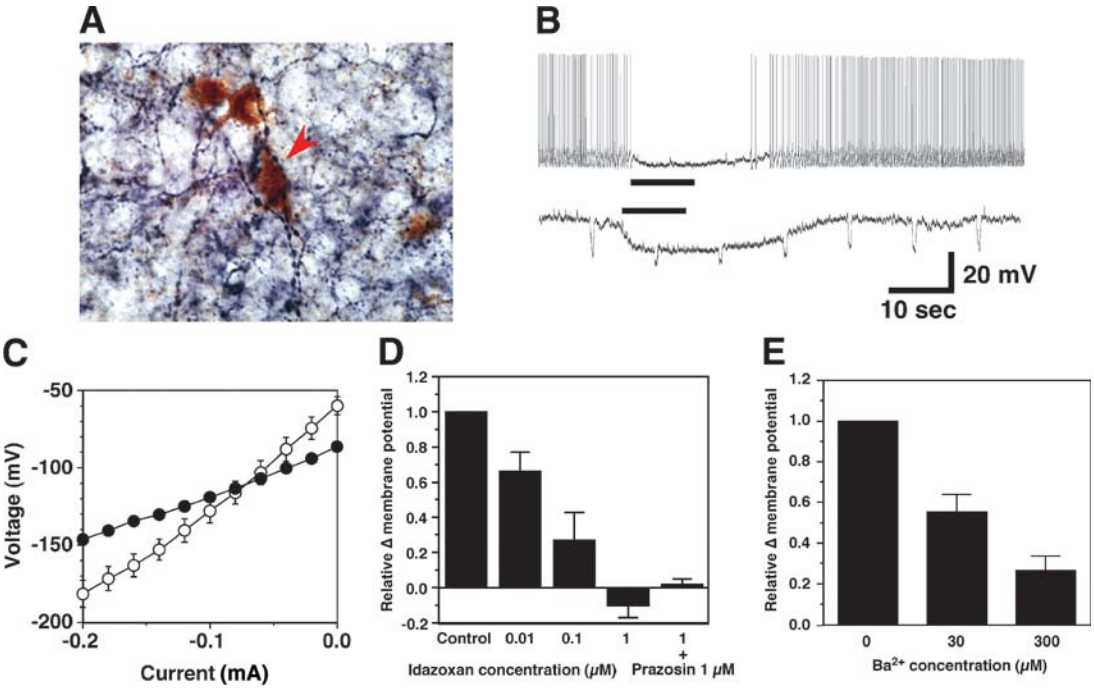

Fig. 4. The effect of NA on orexin neurons. (**A**) Tyrosine hydroxylase-ir nerve endings are in close apposition to orexin-ir neurons. Tyrosine hydroxylase-ir (black) varicosities are closely apposed to an orexin-ir (brown) cell. (**B**) In current clamp mode, NA (30 μ*M*) was applied to orexin neurons in the absence (uppertrace) or presence (lowertrace) of TTX (1 μ*M*). TTX was administered by bath application, and NA was applied locally through a fine polyethylene tube during the period indicated by the bars. Input resistance was monitored by the amplitude of electrotonic potentials generated by injection of a rectangular wave current pulse (–20 pA, 500 ms, 0.1 Hz). (**C**) Current-voltage (*I–V*) relationship derived from records of membrane potential in response to a series of 100-ms current steps (in 20-pA increments, –200 pA to 180 pA) from resting potential (–60 mV) in the absence (open circles) or presence (filled circles) of NA (30 μ*M*). The estimated reversal potential was –106 mV. (**D**) Idazoxan, an α2 receptor antagonist, concentration-dependently inhibited NA-induced hyperpolarization. (**E**) The GIRK inhibitor Ba^{2+} concentration-dependently inhibited NA-induced hyperpolarization. Values are mean ± SEM.

NA-induced response. NA-induced hyperpolarization was also inhibited by a low concentration of Ba^{2+}, suggesting the involvement of GIRK channel activation (Fig. 4E). Because the α2 receptor is known to couple with Gi protein, GIRK would be directly activated like the 5-HT_{1A} receptor. These results suggest that NA-induced hyperpolarization of orexin neurons is mediated mainly by the α2 receptor and by subsequent activation of the GIRK channel. Although adrenaline (EC_{50}: 2.4 μ*M*) or a high concentration of dopamine (EC_{50}: 140 μ*M*) also hyperpolarized orexin neurons, it seems that these effects are mediated by an α2 receptor because idazoxan inhibited these effects.

2.2.3. Input From Acetylcholinergic Neurons

Acetylcholine is a major neurotransmitter in the brain. The cholinergic nuclei (laterodorsal tegmental nucleus [LDT] and peduncle pontine nucleus [PPN]) are implicated in rapid eye movement (REM) sleep and arousal, whereas cholinergic neurons in the basal forebrain (BF) have been reported to play an important role in the maintenance of wakefulness. Therefore,

cholinergic innervation of orexin neurons was examined. The effect of carbachol, a muscarinic agonist, on orexin neurons was tested using a patch clamp slice prepared from *orexin/EGFP* transgenic mice. Carbachol activated 27% of orexin neurons examined (Fig. 5A) and inhibited 6% of orexin neurons examined (Fig. 5A). These effects were concentration-dependently inhibited by a muscarinic antagonist, atropine, suggesting the involvement of a muscarinic receptor in these responses. The remaining population of orexin neurons did not detectably respond to carbachol. Cells that responded to carbachol were equally distributed in every section from rostral to caudal (Fig. 5B), although the middle region contained a relatively large number of cells that were activated by carbachol. Five subtypes of the muscarinic receptor (M_{1-5}) have been reported *(21)*. M_1, M_3, and M_5 coupled with the Gq/G_{11} type of G protein, whereas M_2 and M_4 coupled with the Gi/Go type of G protein. Thus, it is likely that the M_1, M_3, and/or M_5 receptors are involved in CCh-induced activation, whereas the M_2 and/or M_4 receptors are involved in CCh-induced inhibition of orexin neurons. The GIRK channel is stimulated by a Gi-coupled receptor, and activation of GIRK is possibly involved in CCh-induced hyperpolarization, like 5-HT and NA. Gq-type G protein increases intracellular calcium concentration via phospholipase C activation. This pathway is probably involved in CCh-induced activation of orexin neurons.

2.2.4. Input From Histaminergic Neurons

The tuberomammillary nucleus (TMN) is a histaminergic nucleus that highly expresses OX_2R, and receives many orexin nerve endings *(22–24)*. Histamine neurons are directly activated by orexins via OX_2R. Interestingly, however, histamine (100 μM) had little or no effect on any of the orexin neurons. This result suggests that the histaminergic neuron does not innervate orexin neurons directly.

3. CONCLUSIONS

Electrophysiological experiments using *orexin/EGFP* transgenic mice revealed that orexin neurons receive glutamatergic, GABAergic, serotonergic, noradrenergic, and acetylcholinergic input (Fig. 6). Orexin neurons showed homogeneous response to glutamate, GABA, 5-HT, and NA, that is, all orexin neurons tested were activated by glutamate, and inhibited by 5-HT and NA. On the other hand, orexin neurons showed a heterogeneous response to an acetylcholine receptor agonist. These neurotransmitters are implicated in the regulation of sleep/wakefulness, and these inputs to orexin neurons should play an important role in modulating the activity of orexin neurons in the behavioral or circadian state. Our next study should clarify the source of these inputs, in an attempt to understand the sleep/wakefulness regulation system. The mechanism might not be a simple one, since it is well known that a neuron releases more than one neurotransmitter. Further studies are needed to clarify the interactions between these neurons and orexin systems in vivo and their effects on sleep/wakefulness regulation.

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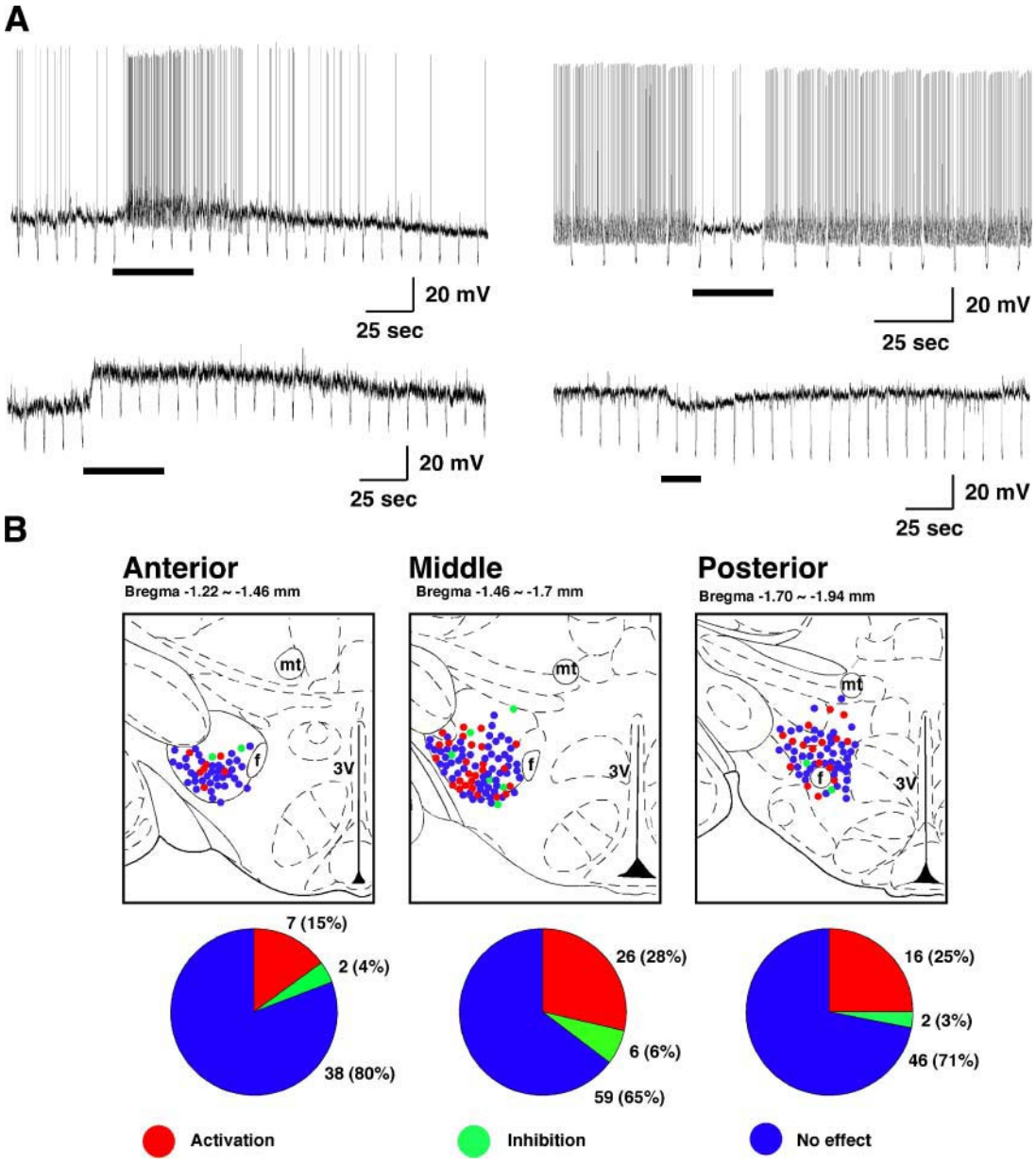

Fig. 5. The effect of acetylcholine on orexin neurons. (**A**) In the current clamp mode, carbachol (CCh, 100 μ*M*), an acetylcholine receptor agonist, was applied to orexin neurons in the absence (uppertrace) or presence (lowertrace) of TTX (1 μ*M*). Input resistance was monitored by the amplitude of electrotonic potentials generated by injection of a rectangular wave current pulse (–20 pA, 500 ms, 0.1 Hz). Orexin neurons showed a variety of responses to CCh: 27% showed depolarization (left panel), 6% of the neurons showed hyperpolarization (right panel), and 67% showed no effect. (**B**) Distribution of orexin neurons, which showed activated (red circles), inhibited (green circles), or no effect (blue circles) of carbachol application analyzed in several successive slice preparations prepared from rostral, middle, or caudal regions of hypothalamus.

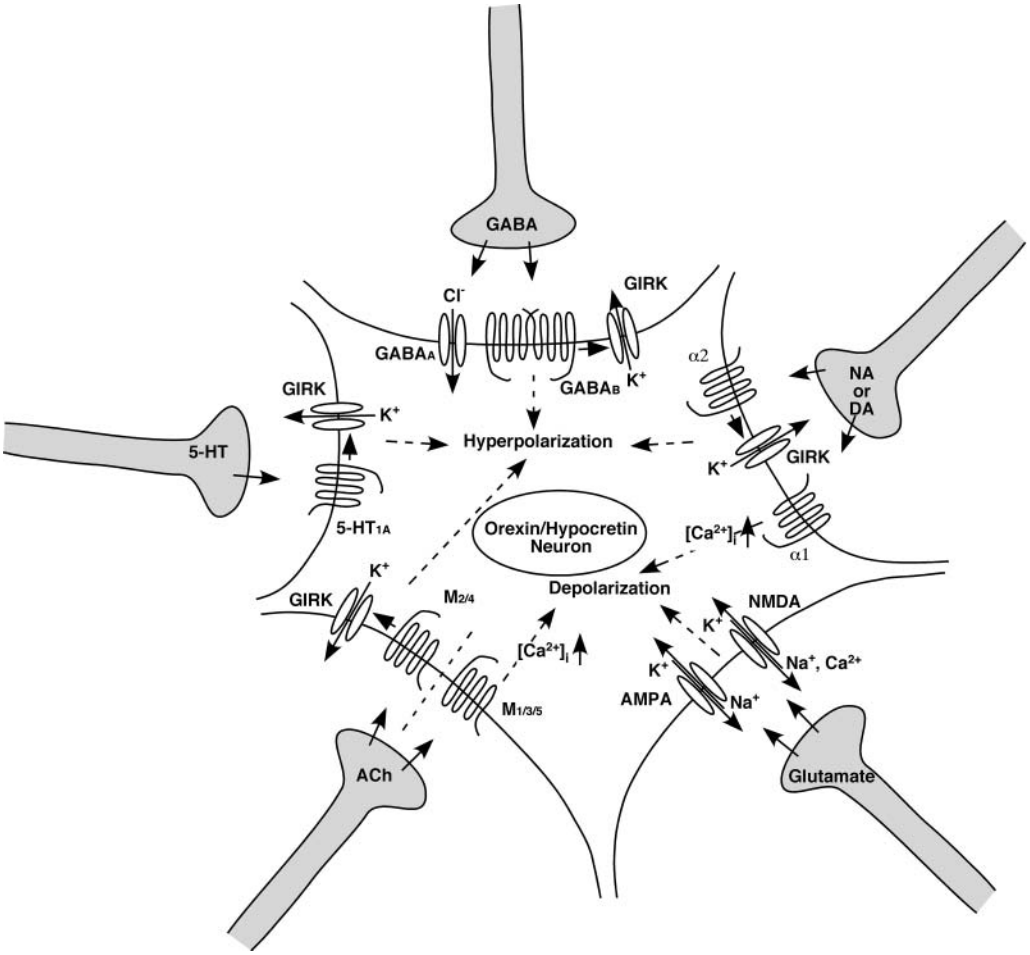

Fig. 6. Schematic drawing of the afferent system of orexin neurons revealed by electrophysiological experiments. NA, noradrenaline, DA, dopamine, ACh, acetylcholine, 5-HT, serotonin, GIRK, G-protein–coupled inward rectifier potassium channel, $[Ca^{2+}]_i$, intracellular calcium concentration; GABA, γ-aminobutyric acid; NMDA, *N*-methyl-D-aspartate; AMPA, α-amino-3-hydroxy-5-methyl-4-isoxazole-propionate.

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III

Assessment of Orexin/Hypocretin Functions in Tissue and Biological Fluids

6

Hypocretin Measurements in the CSF, and Blood and Brain Tissue

Basic and Clinical Applications

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1. INTRODUCTION

After the discovery of narcolepsy genes in dogs (hypocretin receptor 2) and mice (prepro-orexin) *(1,2)*, establishing functional assays for hypocretin/orexin status in human cases became a high priority. It is unlikely that these high-penetrant hypocretin-related genes found in animals are involved in most human narcoleptic cases (this was later confirmed by mutation screenings in human narcoleptic subjects including high-risk cases; *3*), but functional loss/impairment of hypocretin neurotransmission might be involved.

We therefore initiated hypocretin measures in the blood and cerebrospinal fluid (CSF) using commercially available ^{125}I radioimmunoassay (RIA) kits and found that hypocretin-1 can be reliably measured in human CSF but not in blood (*see* ref. *4*). The inhibitory concentration of 90% (IC_{90}) of the RIA was 4–8 pg/tube, and the antibody used did not crossreact to other neuropeptides such as growth hormone-releasing factor (GRF), glucagon, vasoactive intestine peptide (VIP), secretin for the secretin family peptides, bombesin, gastrin-releasing peptide (GRP), and neuromedin C and B for the bombesin family peptides (unpublished data). Using these RIA kits, we subsequently found that most human narcolepsy–cataplexy subjects (as well as sporadic cases of canine narcolepsy) had undetectably low CSF hypocretin levels *(4–8)*.

This finding in human CSF was immediately confirmed by several other investigators; undetectably low CSF hypocretin levels were observed in 90–95% of narcolepsy–cataplexy subjects in several ethnic groups *(7,9–11)*. Because the specificity of low CSF hypocretin levels in neurologic and sleep disorders is high *(7,8,12)*, CSF hypocretin measures will be included in the diagnostic criteria for narcolepsy in the second version of International Classification of Sleep Disorders (ICSD). Narcolepsy is currently diagnosed mostly by clinical observation and polysomnographic findings (shorter sleep latencies and sleep onset REM periods during multiple sleep latency tests) with the aid of human leukocyte antigen (HLA) typing (HLA DQb1*0602 positive; *see* ref. *13*). However, the sensitivity and specificity of these findings for narcolepsy are not high, and the final diagnosis is often delayed for several years after disease onset. With this new discovery, many patients are likely to receive immediate benefit from this new diagnostic test. The results of CSF hypocretin measures also addressed nosological issues regarding classification of narcolepsy/excessive daytime

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sleepiness (EDS) disorders, since most cases of "narcolepsy without cataplexy" (as well as idiopathic narcolepsy) were found to have normal CSF hypocretin levels *(7,9–11)*, suggesting an etiological difference between "narcolepsy–cataplexy" and "narcolepsy without cataplexy" and other primary EDS disorders. CSF hypocretin measures are also useful for selecting treatment, especially if hypocretin replacement therapies become available.

Hypocretin measures in the CSF and extracellular fluid are also useful in basic neuroscience research to study the roles of the hypocretin system in regulation of sleep and other hypothalamic functions. This is especially important since changes in mRNA signals are relatively slow and do not vividly reflect synaptic releases of hypocretin peptides (hypocretin neuronal activities; *see* Chap. 13, Hypocretin/Orexin Tonus and Vigilance Control).

In this chapter, technical concerns regarding hypocretin measures, especially their usage and limitations, are discussed.

2. GENERAL USAGE AND LIMITATIONS OF HYPOCRETIN MEASURES

Over the past 4 yr, about 50 papers have reported the results of CSF hypocretin measures, and 20 papers have reported on blood hypocretin levels in relation to hypocretin status in various physiological and pathological conditions. However, the results (especially hypocretin levels in the blood; *see* Subheading 5 below) vary significantly depending on the assay conditions used. This has made some of the major findings of the studies controversial (*see* refs. *9* and *14–16*). Therefore, it is strongly recommended that investigators (if they are not familiar with RIA/enzyme immunoassay [EIA] techniques) refer to specific handbooks, such as *Radioimmunoassay of Gut Regulatory Peptides* by Bloom and Long *(17)*, for details on the uses, limitations, and technical problems of RIA/EIA.

Currently, RIA and EIA are the two major methods for measuring hypocretin peptides. Most authors measure hypocretin peptides by ^{125}I RIA using polyclonal antibodies either with extraction (brain, CSF, and blood) or without extraction (CSF and blood). Bioassays, such as Ca^{2+} mobilization using a hypocretin receptor 2-expressing cell line, have also been used to quantify CSF hypocretin levels *(18)*, but the availability of these cell lines and the equipment needed for the assay are limited to a few laboratories. In any case, these microassays, including Ca^{2+} mobilization, are "relative measures" and do not give us the absolute values of the substances measured even if respective standards are included in each assay. The values reported by RIA/EIA depend on the characteristics of the antibodies, the radioligand (RIA), the reagents, and many other factors that also influence the immunoreaction and the final values *(17)*.

Tables 1 and 2 present a typical example of measured values of the same CSF samples using RIA and different antibodies and radioligands. Duplicates of 100 µL of CSF (without extraction) were applied to the respective RIA (i.e., direct assay), and the results clearly show the nature of the "relative measures" of the immunoassay. This reminds us that we cannot compare values if they are obtained with different assay settings. Although RIA is a well-established and reliable microassay that is widely used (quantification in the fmol range, with a small intraassay variation), the intraassay variation is sometimes large even if we use the same antibody, mostly because of variation in the specific activity of the radioligand and its quality (*see* paragraph below). Thus, appropriate adjustments should be made if the values are obtained from multiple assays.

Compared with ^{3}H labeling, the specific activity of ^{125}I (radioactivity that labels 1 mol of substance) is much larger, and thus ^{125}I RIA, in general, is more sensitive *(17)*. EIAs for hypocretin measures are also available commercially or in several laboratories. EIA, a less expensive and environmentally safer assay system (no radioactive ligand required, and thus no radioactive

Table 1
CSF Hypocretin-1 Measures in Four Different Samples Using Two Different Antibodies and Two Different Radioligands (^{125}I hypocretin-1)[a]

	Radioligand A		Radioligand B	
	Antibody A	Antibody B	Antibody A	Antibody B
Sample 1 (pg/mL)	182	87	133	42
Sample 2 (pg/mL)	320	131	258	58
Sample 3 (pg/mL)	781	190	632	78
Sample 4 (pg/mL)	951	258	862	166
Ratio over sample 1				
Sample 1	1.0	1.0	1.0	1.0
Sample 2	1.8	1.5	1.9	1.4
Sample 3	4.3	2.2	4.8	1.9
Sample 4	5.2	3.0	6.5	4.0

[a]The measurements varied significantly with respect to which antibody and radioligand were used. Based on the ratio over sample 1, however, each of the four samples fell into the same order with no exceptions.

Table 2
CSF Hypocretin-1 Levels in Narcoleptic Subjects, Initially Assessed Using Two Different Antibodies[a]

	Radioligand A	
	Antibody A	Antibody C
Detection limit (pg/mL)	40	70
Normal levels (pg/mL)	270	120
Hypocretin deficient narcolepy (pg/mL)	<40	<70

[a]Although CSF hypocretin-1 levels in most narcolepsy-cataplexy subjects were undetectable, the respective normal CSF hypocretin levels were varied, making the difference in CSF hypocretin-1 level between narcolepsy and controls obtained with antibody C less robust. We decided to use antibody A for our study thereafter.

wastes, and no expiration of radioligand due to its decay), generally produces a high sensitivity assay, similar to ^{125}I RIA. Unfortunately, the EIAs that are currently available for hypocretin-1 and -2 measures are less sensitive than most ^{125}I RIAs currently available (*see* ref. *19*).

3. PRINCIPLES AND PRACTICE OF CSF HYPOCRETIN MEASURES

We originally extracted hypocretins in the CSF using a disposable reverse phase column (Sep-Pak C 18) *(4,5)*. Because this method can concentrate the sample by applying a large initial volume, we could lower the detection limit and thus increase the sensitivity of the assay. However, there are three major drawbacks to the use of this extraction method:

1. Extraction requires additional costs (column and reagents).
2. The recovery rate may vary for each sample. Because our initial experiment using a Sep-Pak C 18 column revealed that the recovery rate of hypocretin in the CSF (1–20 mL) was small *(4)*, we decided not to add any internal standards to each sample. However, if this is not the case (the quality of the reverse phase column may vary depending on the brand, and the recovery rate may also vary depending on the specimen), adding an internal standard for each sample, such as

a small amount of ^{3}H hypocretin-2 (*see* ref. *20*) that does not interfere with the ^{125}I RIA of hypocretin-1, is suggested. The recovery rate for each sample can be calculated as recovered ^{3}H over added ^{3}H.

3. Owing to the additional extraction procedures, the intra- and interassay variations became higher compared with the direct assays, and thus the extraction method may not be suitable for clinical diagnostic testing (*see* below).

For establishing reliable assays, it is essential to evaluate the linearity between measured values and the initial volumes of the samples; the measured values should be linearly increased (decreased) with the initial volume applied (Fig. 1). It is also important to evaluate linearity between added (spiked) known substance concentrations and measured values (Fig. 1).

Even if a direct assay were used, it would be safer to test the linearity for a wide volume range by concentrating samples, since nonspecific interference may also occur in a linear fashion.

4. TECHNICAL CONSIDERATIONS IN CSF HYPOCRETIN MEASURES FOR THE DIAGNOSIS OF NARCOLEPSY

In our original CSF hypocretin measures, we have shown that hypocretin-1 can be reliably measured from as little as 1 mL of extracted CSF. In contrast, hypocretin-2 is not consistently detected even in 20 mL of CSF (Fig. 1). Because both peptides are cleaved from the same precursor, then the same amounts (mole) should be produced and released. The fact that hypocretin-2 levels are much lower in the extracellular fluid and CSF *(18,21)* is probably because of the difference in stability of these peptides in the biological fluid. Indeed, we found that hypocretin-1 is much more stable than hypocretin-2 in the CSF by intracerebroventricular injections of both peptides in rats *(18)*.

The hypocretin-1 signal in the CSF is very stable, and the levels measured by RIA were not altered in the CSF samples that were frozen and thawed up to six times as well as in samples kept at room temperature for 72 h *(4)*. However, it was not fully determined whether the levels measured by RIA correspond to the biological activity of hypocretin-1 in the CSF samples, especially if CSF samples were kept under varied conditions. Fortunately, for the diagnostic purpose of hypocretin deficient-narcolepsy, the "net" immunoreactive hypocretin signals (without consideration of biological activity) may be sufficient. There was also no concentration gradient in hypocretin-1 levels up to 12 mL in human lumbar CSF *(4)*, making strict selection of the CSF fraction for this diagnostic test impossible.

As mentioned earlier, CSF hypocretin-1 levels can also be reliably measured by direct assay without extraction. Immunoreactive hypocretin signals measured by direct assay are also very stable *(8)*. There was good linearity between measured and amount in the serial dilutions or added (known hypocretin amount) hypocretin measures with this assay *(8)*. We also found that such CSF hypocretin-1 values correlated well with those measured using Sep-Pak extraction in healthy subjects and in various neurological conditions *(8)*.

In the direct assay, certain substances in the CSF (that can be removed by the extraction procedure) may interfere with the immunoassay. However, we have not found any disease condition that caused a discrepancy between the results of direct assay and extraction, but this possibility cannot be completely ruled out and caution should be used if undetectable CSF hypocretin is observed in particular neurologic conditions (*see* ref. *22*).

The only drawback of the direct assay (100 µL applied volume) was its higher detection limit (2.5 times higher compared with that measured from 1 mL of CSF using extraction, at a 60% recovery rate *see* ref. *8*). By using a higher sensitivity antibody, however, this problem

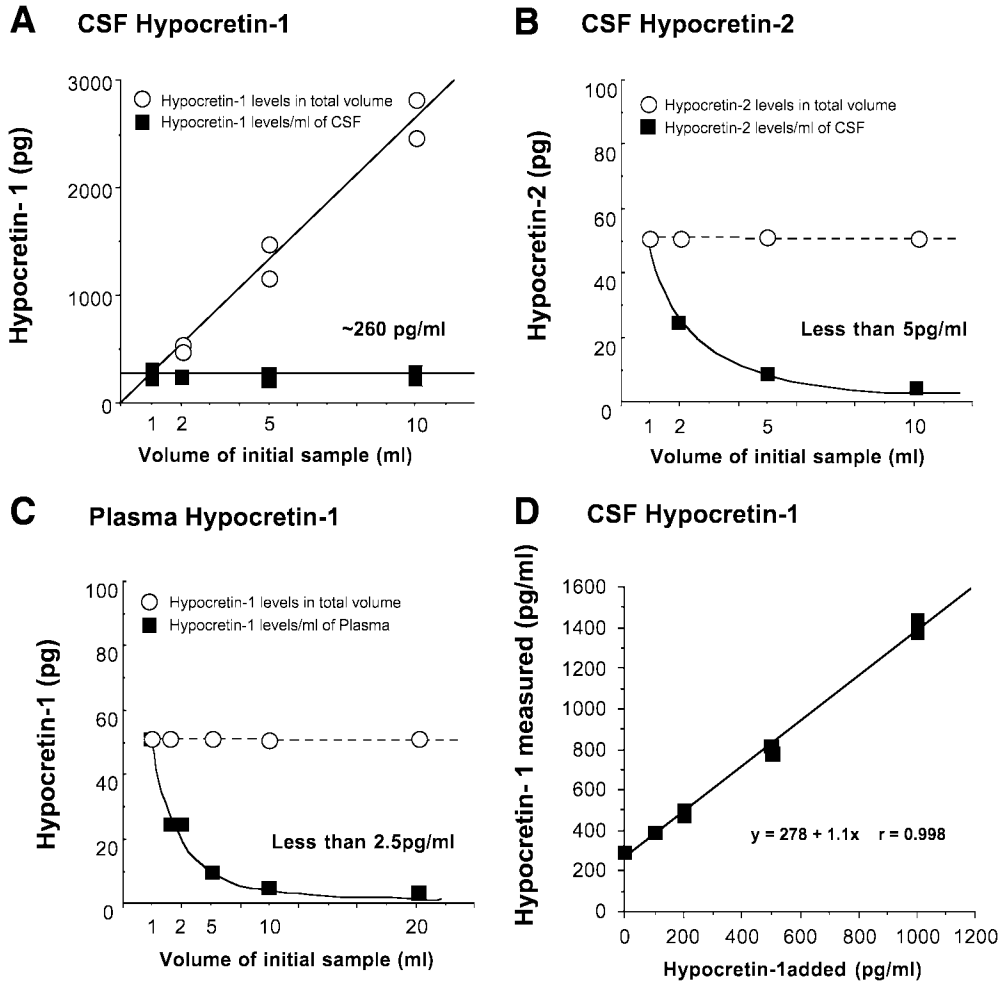

Fig. 1. Hypocretin-1 and -2 signals in cerebrospinal fluid (CSF) and hypocretin-1 signal in plasma in relation to sample volumes used for the measurements. **(A)** Hypocretin-1 can be reliably measured from 1 mL of human lumbar CSF using a commercially available antibody, and a good linearity between total hypocretin amounts and sample volumes used for the measurements can be obtained. **(B)** In contrast, hypocretin-2 signal is very low and could not be detected from 10 mL of CSF (<5 pg/mL). **(C)** Similarly, blood hypocretin-1 (and -2, data not shown) signal in the plasma could not be detected even from 20 mL plasma (<2.5 pg/mL). **(D)** In the CSF, a good linearity between added known amounts of hypocretin-1 and measured hypocretin amounts is obtained.

can be overcome. Considering that the extraction procedure has the disadvantage of causing much larger interassay variation, the direct assay may be the best we presently have for clinical diagnostic purposes.

Nevertheless, a large interassay variation is still one of the major concerns, regardless of the assay method. In our experience, the variation was as high as 20% even in short-term assessments, and up to 100% for long-term assessments. These variations are probably caused by many different factors. As noted earlier, (1) specific ligand activity often varies; (2) antibody batches may vary; for many commercially available polyclonal antibodies, blood may have been collected many times from a single immunized animal, thus creating multiple batches of antibodies from a single immunized animal; and (3) the standard peptide solution may degrade over time, and care should be taken when one is handling this solution.

Thus, standardization of assay conditions is critical especially for diagnostic purposes. The following measures are suggested: (1) use radioligands from the same vendor and request a specific activity range; (2) use the same antibody batch; there is also a possibility of antibody decay so take appropriate precautions (*see* ref. *3*); and (3) use up the standard solution (kept at 4°C) from the standard stock solution in a short period, and store aliquots of the standard stock at –80°C.

These precautions should always be taken to reduce intraassay variation. In addition, all comparative samples should be seen in a single RIA. If this is not possible, reference samples (such as CSF from healthy subjects), in addition to the standard, should be included in each assay and the values adjusted accordingly. Finally, we suggest using 30% of the normal CSF hypocretin value obtained in the same lab (rather than the published values) as the cutoff point for the diagnosis of a hypocretin-deficient condition.

At the 5th International Symposium on Narcolepsy (Ascona, 2004), a workshop for the diagnostic guideline for narcolepsy was held and the standardization CSF hypocretin measures were discussed. The panel discussed the possibility to provide (1) monoclonal antibody and RIA development kits, (2) hypocretin standards, and (3) reference CSF samples (ideally two different concentrations, such as 150 and 300 pg/mL) to the laboratories in the world that carry out the assay.

5. HYPOCRETIN MEASURES IN BLOOD

There is considerable controversy as to the usefulness of hypocretin measures in the blood. As stated earlier, we could not detect any hypocretin signals in human blood. (From 20 mL of plasma, the estimated level was less than 2.5 pg/mL). Similarly, reported findings based on blood measures are very controversial. In a typical example, a significant positive correlation between plasma hypocretin levels and apnea hypoxia index (AHI) was reported in obstructive sleep apnea patients *(16)*. Another group reported a completely opposite result (i.e., significant negative correlations between AHI and blood hypocretin levels) *(15)* (*see* also ref. *24*), whereas two other studies demonstrated no changes in CSF hypocretin levels in obstructive sleep apnea patients *(7,25)*.

Regarding blood hypocretin status in narcolepsy, one group reported that there were no changes in plasma hypocretin levels in narcolepsy–cataplexy subjects (with CSF hypocretin deficiency) *(9)*. Others reported significantly low blood hypocretin levels in narcolepsy (11/12 cases with cataplexy), although the sensitivity of low plasma hypocretin levels for narcolepsy was only 25% at 100% specificity *(14)*.

At the conceptual level, hypocretins are likely to exist in the blood. Small amounts of peptides can leak from the brain to the periphery through the blood–brain barrier. An increasing number of studies have demonstrated that hypocretin receptors are present in the periphery *(26)* and that low levels of hypocretins are likely to be produced peripherally *(27)*. All these factors can contribute to the existence of a small amount of hypocretin in plasma. If most of the hypocretin signal in the blood originates from the brain, and/or global (i.e., both central and peripheral) hypocretin deficiency exists in narcolepsy–cataplexy, then blood hypocretin measures may provide additional, but less invasive, diagnostic information.

We also observed reduced hypocretin signals in the blood in genetically produced hypocretin/orexin-deficient mice (i.e., orexin/ataxin-3 transgenic mice [TG] and prepro-orexin knockout [KO] *(28)*. Several investigators, including us, had noted that rodents have relatively high hypocretin signals in the blood. This may be partially related to higher hypocretin levels in the CSF in these species (CSF hypocretin levels in rats are about five times higher than those in humans or dogs) *(29)*.

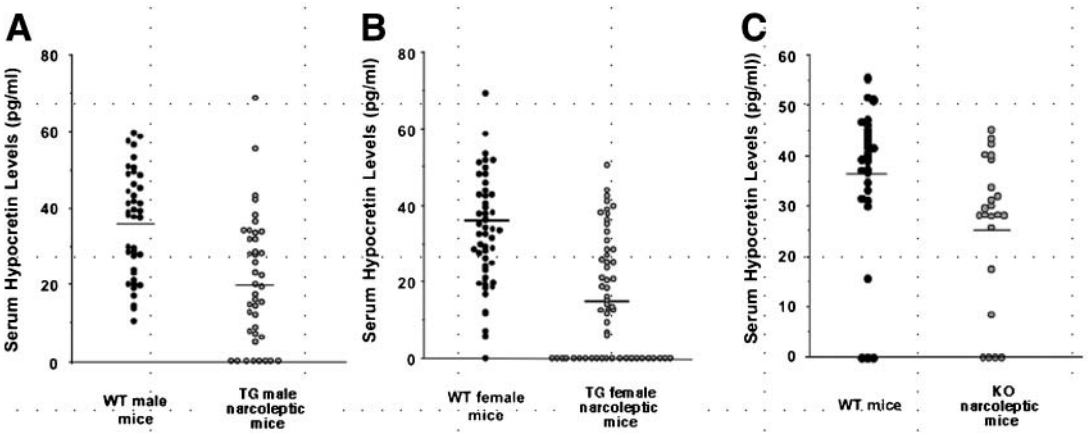

Fig. 2. Serum hypocretin levels in prepro-orexin/ataxin-3 transgenic (TG) and wild-type (WT) mice and prepro-orexin knockout (KO) mice. (**A,B**) Hypocretin-1 levels were similar in males and females. Blood hypocretin levels were significantly lower in TG narcoleptic mice compared with the WT mice group (17.6 ± 1.6 pg/mL vs 35.8 ± 1.8 pg/mL, $p < 0.0001$). Thirty of 100 TG mice had undetectably low blood hypocretin levels, whereas only 1 of 96 WT mice had undetectable levels. The specificity of undetectable blood levels in TG mice was high (99%), although the sensitivity was only 30%. (**C**) Similarly, blood hypocretin levels were significantly lower in KO narcoleptic mice compared with a WT mice group (26.1 ± 3.0 pg/mL vs 36.3 ± 2.6 [SEM] pg/mL, $p = 0.015$). However, the overlap in hypocretin values between KO and WT mice was larger than that of the TG mice comparison. Four of 23 KO mice and 3 of 21 WT mice showed undetectable blood hypocretin levels.

In these narcoleptic mice, serum hypocretin signals were measured by RIA without extraction using a rabbit antihypocretin-1 polyclonal antibody produced in house with high sensitivity (IC_{90} = 0.5 pg/mL). We found that blood hypocretin levels were significantly lower in both KO and TG narcoleptic mice compared with their respective wild-type mice groups (Fig. 2). Most strikingly, 30 of 100 TG mice had undetectably low blood hypocretin levels, whereas only 1 of 96 wild-type mice had undetectable levels (21% of TG mice overlapped with the mean ± 2SD of control level) (Fig. 2). The specificity of undetectable blood levels in TG mice was high (99%), although the sensitivity was only 30%. Notably, the overlap in hypocretin values between KO and WT mice (39% of KO mice overlapped, with a mean ± 2SD of the control level) was greater than that of the TG mice.

Therefore, hypocretin signals in the blood (detected with RIA using a polyclonal antibody) reflect hypocretin deficiency status in both hypocretin-deficient narcoleptic mice models (gene vs cell targeting model). Although hypocretin status in peripheral organs has not been investigated in orexin/ataxin-3 narcoleptic mice, no hypocretin production in peripheral organs (and brain) was assumed in KO mice. Thus the overlap observed in hypocretin signals between KO and WT mice is probably caused by large background signals in the blood. By reducing the background signals, e.g., by applying larger sample volumes or extraction methods together with more sensitive and specific assays, we may be able to diagnose narcoleptic mice reliably by blood hypocretin measures. Similarly, if major blood hypocretin signals come from the brain, or if peripheral organ hypocretin is also deficient in human narcolepsy we may be able to develop a blood assay for detecting hypocretin- deficient human narcolepsy.

Several blood preparation factors may also have affected the ability to detect hypocretins in the blood. It needs to be determined whether plasma or serum hypocretin levels and the

use of different types of protease inhibitors (aprotinin and protease cocktails) can be more appropriately measured. If we use plasma, the collection method (e.g., in EDTA, anticoagulant citrate dextrose [ACD], or heparin) is an important issue (*see* ref. *30*). The various extraction techniques (organic solvents vs Sep-Pak methods) and purification methods (HPLC and antibody-mediated affinity column) also need to be considered. Finally, the RIA conditions should also be varied, as some peptides are better detected under nonequilibrium conditions.

6. HYPOCRETIN MEASURES IN BRAIN TISSUE

Hypocretin measures in brain tissues also need to cope with high background signals. Therefore, appropriate extraction and purification methods are required before quantifying hypocretin levels. The samples should be diluted, and the final concentration(s) used in the RIA should be around the IC_{50} of the assay. It is important to note that hypocretin levels in the brain are not the same as hypocretin release (and hypocretin neuronal activity). Neuropeptides, once released to the synaptic cleft, rapidly degrade, and neuropeptides are not taken up *(31)*. Thus, the hypocretin projection area, where hypocretin release is active, is likely to have low hypocretin levels in the tissue homogenate.

7. IMMUNOBLOT/BIOASSAY FOR QUANTIFICATION OF HYPOCRETIN PEPTIDES

Because findings of low CSF hypocretin levels (i.e., less than one-third of the normal value by direct RIA) will be included in the diagnosis of narcolepsy in the second revised version of the ICSD, a simple EIA-based immunoblot can be developed for a diagnostic test (*see* ref. *23*). There is a no need for emergency bedside diagnosis for this chronic sleep disorder, and thus there is little merit in developing a one-step immunoblot test.

As described earlier, a Ca^{2+}-mobilizing bioassay using hypocretin receptor-expressing cell lines can also be used to quantify hypocretin peptides in the CSF *(18)*. In contrast to RIA/EIA, this bioassay can directly assess the biological activity of hypocretin peptides, and thus is useful in a certain experimental setting. However, availability of materials and equipment and standardization of the assay limit the use of this method.

8. CONCLUSIONS

Low CSF hypocretin levels (less than one-third of the normal value) are the first positive diagnostic finding (with a high specificity) for human narcolepsy–cataplexy. Hypocretin measures in various specimens are also useful for clinical and basic sciences.

Hypocretins are currently measured by RIA/EIA. These assay methods are not yet standardized, and reported values vary depending on the laboratory. This is partially because of the nature of "relative measures" of these assays; the investigators need to fully understand the usage and limitations of these measures. If these are not considered, the reported results will be too confusing.

Blood hypocretin measures may be informative for some physiological and pathological conditions, thus providing a less invasive diagnostic test for narcolepsy, but more systemic evaluations are needed.

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Hypocretin Receptor-Activated G Proteins Revealed by [^{35}S]GTPγS Autoradiography

René Bernard, PhD, Ralph Lydic, PhD, and Helen A. Baghdoyan, PhD

1. INTRODUCTION

The focus of this chapter is the hypocretin (orexin) ligand–receptor system as quantitatively characterized by [^{35}S]guanylyl 5´-(γ-thio) triphosphate ([^{35}S]GTPγS) autoradiography. We highlight future ways in which hypocretin-stimulated [^{35}S]GTPγS binding may provide insights into functional roles of the hypocretinergic system. For a comprehensive discussion of [^{35}S]GTPγS binding and autoradiography, the reader is referred to recent reviews (*1–4*).

For more than 2000 yr humans have been interested in understanding how environmental signals trigger physiological responses *(5)*. It was not until the beginning of the 20th century, however, that the concept of receptor molecules was first formulated by John N. Langley *(6)*. The antagonizing action of curare on nicotine-induced muscle contraction led Langley to conclude that the muscle must possess a "receptive substance." At about the same time, Paul Ehrlich *(7)* and A. J. Clark *(8)* independently hypothesized that biologically active substances must become bound to be effective, thus introducing the concept of receptor binding. The work of Langley, Ehrlich, and Clark provided the foundation for receptor studies in the 1960s that focused on the quantitative relationship among drug concentration, receptor occupancy, and stimulated response in tissues and organisms. The first radioactive ligands were used to label muscarinic uptake sites in the intestinal smooth muscle *(9)* and adrenergic uptake sites in the atria *(10)*.

The mid-1970s saw the development of receptor autoradiography, which permits visualization and quantification of radioactive ligands bound to receptors while preserving tissue anatomy *(11)*. About 20 yr later, autoradiography was first applied to the study of receptor-activated guanine nucleotide binding (G) proteins using [^{35}S]GTPγS *(12)*. With this approach it is possible to localize and quantify G proteins that are activated as a result of agonist binding to a specific receptor. Autoradiography with [^{35}S]GTPγS has been used to provide a unique combination of anatomical and functional data for many G-protein-coupled receptor systems in the brain, including opioid *(12,13)*, muscarinic cholinergic *(14–17)*, serotonergic *(18,19)*, histaminergic *(20)*, cannabinoid *(21)*, dopaminergic *(22)*, and adenosinergic *(23,24)* systems. Most recently, [^{35}S]GTPγS autoradiography was successfully applied to hypocretin signaling in the rat brainstem *(25,26)*. The results suggest for the first time that some hypocretin receptors may couple to inhibitory G proteins.

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2. ASSESSMENT OF RECEPTOR/G PROTEIN INTERACTIONS USING [^{35}S]GTPγS AUTORADIOGRAPHY

2.1. *In Vitro [^{35}S]GTPγS Autoradiography Mimics In Vivo G Protein Activation*

This section describes the process by which G proteins are activated when an agonist binds to a receptor. This process illustrates how [^{35}S]GTPγS is used in vitro to label functionally active receptor–G protein complexes. Figure 1A shows that in the absence of a receptor-bound agonist, receptor-associated G proteins are held in an inactive state owing to the high-affinity binding of guanosine diphosphate (GDP) to the Gα subunit (reviewed in ref. *1*). In this resting state the G protein forms a stable heterotrimeric complex composed of its three subunits, α, β, and γ (Fig. 1A). Binding of an agonist, such as hypocretin-1 or hypocretin-2, to the active site of the receptor induces a conformational change in the receptor-G protein complex. This conformational change destabilizes the interaction of GDP with the Gα subunit. GDP is then released from the Gα subunit, permitting GTP to bind to, and thus activate, the Gα subunit. The G protein dissociates into two active elements, Gα-GTP and the heterodimer Gβγ (Fig. 1B). Both the Gα-GTP and the Gβγ components are capable of interacting independently with downstream effectors. Prominent enzymes that interact with Gα-GTP in the hypocretin signaling transduction pathway are adenylyl cyclase and phospholipase C *(27)*. The Gβγ subunit can directly or indirectly interact with the same downstream effectors as the Gα subunit. Activation of the G protein is stopped by the intracellular GTPase activity of Gα, hydrolyzing GTP to GDP (Fig. 1C). The reformation of the heterotrimeric G protein complex completes the guanosine nucleotide exchange cycle, and the process of G protein activation can begin again (arrow back to Fig. 1A).

GTPγS is a molecule that closely resembles the conformation of bound GTP. The molecular difference between GTPγS and GTP is an oxygen atom that is exchanged for sulfur in the γ-phosphate group. The sulfur makes GTPγS nonhydrolyzable by Gα GTPase *(28)*. Thus, GTPγS stays bound to the Gα subunit upon agonist binding to the receptor–G protein complex (Fig. 1D). In vitro [^{35}S]GTPγS autoradiography takes advantage of the GDP–GTP exchange to radiolabel the event of G-protein activation. At the beginning of an in vitro assay, tissue sections undergo a washing process to remove endogenous ligands and guanine nucleotides. Subsequently, an agonist such as hypocretin is applied to the tissue sections in a buffer solution containing GDP and [^{35}S]GTPγS. This process mimics G-protein activation as it occurs in vivo. The radioactive sulfur isotope in [^{35}S]GTPγS allows detection of activated G proteins on radioactive-sensitive film. The resulting autoradiograms and calibrated radioactive standards are used to quantify activated G proteins localized to specific brain nuclei *(12,29)*.

2.2. *Advantages and Limitations of [^{35}S]GTPγS Autoradiography*

G-protein-coupled receptor complexes are functional in brain tissue sections *(30)*. Therefore, [^{35}S]GTPγS autoradiography can be used to localize and quantify activated G proteins while preserving brain anatomy *(1,4)*. Because [^{35}S]GTPγS autoradiography labels functionally active receptor–G protein complexes, this method can be used for receptor desensitization studies *(31)*. For example, chronic in vivo drug treatment decreases agonist-stimulated G-protein activation in brain regions known to be involved in the development of physical dependence and drug tolerance *(32)*. G-protein-coupled receptors comprise 30% of small-molecule drug targets *(33)*. Therefore, [^{35}S]GTPγS autoradiography can be used to analyze functional changes at the level of G proteins as a direct result of drug administration. A recent example is provided by a study using [^{35}S]GTPγS autoradiography to demonstrate that chronic consumption of ethanol in rats uncouples δ-opioid receptors from G proteins *(34)*.

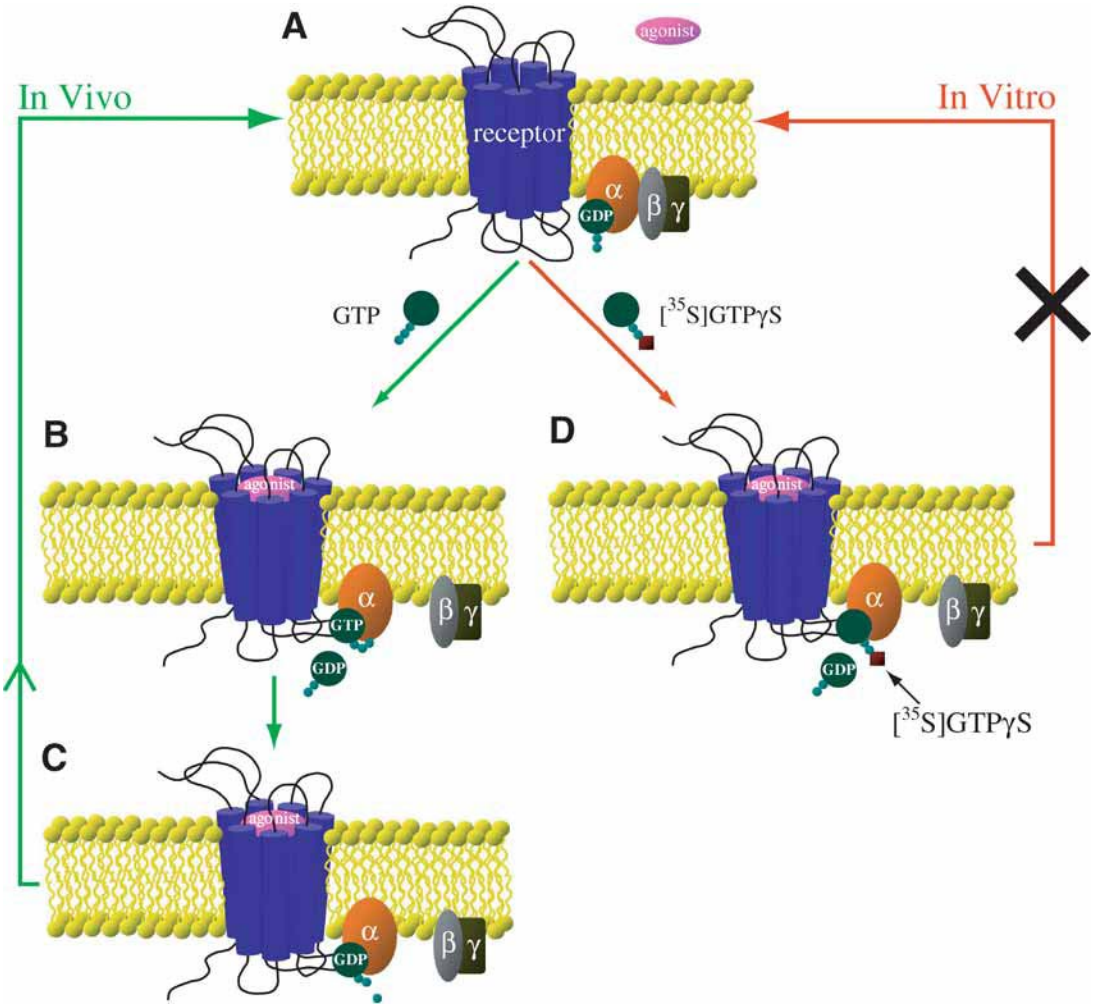

Fig. 1. In vivo and in vitro G-protein activation. **(A)** A cell membrane-spanning guanine nucleotide binding (G)-protein-coupled receptor is shown with an extracellular agonist not bound to the receptor. On the intracellular side of the membrane, guanosine diphosphate (GDP) is bound to the Gα subunit of a heterotrimeric G protein (αβγ). Each small green sphere represents one phosphate of the GDP molecule. In this configuration, the receptor–G protein complex is in the inactive state. **(A–C)** Schematization of G-protein activation caused by an agonist binding to its receptor. The green lines show in vivo activation (GDP–GTP exchange) and subsequent inactivation (hydrolysis of GTP to GDP) of G proteins. The arrow from (C) to (A) indicates the cyclic nature of inactivation and reactivation of G proteins in vivo. The red lines schematize the in vitro use of [^{35}S]GTPγS to radiolabel activated G proteins. The 3 on the arrow from **(D)** to (A) indicates that binding of nonhydrolyzable [^{35}S]GTPγS prevents the cycle of G-protein inactivation and reactivation. Activated G proteins labeled with [^{35}S]GTPγS can be localized and quantified by autoradiography. (Modified from Fig. 1 of ref. *29*.)

An additional advantage is that [^{35}S]GTPγS autoradiography provides a tool for studying G proteins activated by multiple receptors in the same brain region. Adjacent tissue sections from the same brain can be treated with agonists for different receptors or simultaneously with more than one agonist. Different G-protein-coupled receptor systems can converge on the same pool of G proteins *(35)*, and [^{35}S]GTPγS autoradiography can be used to determine

whether different receptor systems in the same brain nucleus activate G proteins by fully additive, partially additive, or nonadditive mechanisms. This approach was applied to brainstem nuclei known to regulate levels of behavioral arousal *(24)*. G-protein activation by a μ-opioid agonist and an adenosine A_1 agonist was shown to be partially additive, suggesting that in brainstem nuclei known to regulate arousal μ-opioid and adenosine A_1 receptors reside on the same neurons to activate some common G-protein pools *(24)*. Knowledge about activation of the same G proteins by different receptors provides unique information for optimizing pharmacotherapy with multiple drugs to maximize desired therapeutic drug actions and lower unwanted effects.

G-protein-coupled receptors are an attractive target for drug design. New receptor agonists can be screened for their ability to activate G proteins throughout the brain using [^{35}S]GTPγS autoradiography. Such screening is especially useful if receptor binding studies with radiolabeled ligands are not possible. The hypocretins, for example, show very high nonspecific binding; thus radiolabeled hypocretin is not useful for receptor assays or autoradiography in brain tissue *(36)*. In contrast, [^{35}S]GTPγS autoradiography with hypocretin can provide quantitative information, revealing concentration–response relationships and measures of potency (EC_{50}) for specific brain nuclei *(26)*. Such studies have the potential to identify brain regions in which agonist activity may result in the greatest efficacy.

One limitation of [^{35}S]GTPγS autoradiography is the inability to identify the subtype of activated G protein directly. Receptor systems that have low coupling efficacy or a low expression density or that exhibit a low GDP-GTP exchange rate are difficult to detect by [^{35}S]GTPγS binding *(3)*. Autoradiography with [^{35}S]GTPγS is thought to be most effective for labeling agonist-activated inhibitory G proteins of the Gi/o family (reviewed in ref. *4*). Recent modifications in cell culture expression systems permit the study of the Gq and Gs families of G proteins using [^{35}S]GTPγS binding (reviewed in refs. *1* and *3*). There is now one report of a Gq-coupled receptor that has been localized using [^{35}S]GTPγS autoradiography. This study labeled serotonin$_{2A/2C}$ receptors in membrane fractions and intact tissue sections *(37)*.

Another limitation of [^{35}S]GTPγS autoradiography is that it is labor intensive. Autoradiograms generated by each tissue section and the actual tissue sections are digitized before brain nuclei-specific [^{35}S]GTPγS binding can be quantified. Every tissue section is compared with a brain atlas to localize the nuclear boundaries precisely. Nonspecific binding is quantified for each brain nucleus of interest. These steps have been described in detail *(14,15,26)*.

Despite these limitations, the unique combination of functional information and high anatomical resolution makes [^{35}S]GTPγS autoradiography an invaluable technique for quantifying G-protein activation in specific brain nuclei. The assay also permits experimental designs that incorporate positive controls via additional agonists and negative controls such as analyses of fiber pathways or application of receptor antagonists *(26)*.

3. HYPOCRETIN-STIMULATED [^{35}S]GTPγS BINDING

The first reports of hypocretin-stimulated [^{35}S]GTPγS binding were published in 2002 *(25,36)*. Hypocretin-1 and hypocretin-2 were applied to homogenates from rat pons, cortex, midbrain, hypothalamus, and cerebellum in the presence of [^{35}S]GTPγS *(36)*. To demonstrate that hypocretin-stimulated [^{35}S]GTPγS binding resulted from receptor activation, one binding condition contained the hypocretin receptor antagonist NBI 3648710 in addition to hypocretin-1 or hypocretin-2. The results showed that hypocretin-1 (Fig. 2A, top panel) and hypocretin-2 (Fig. 2A, bottom panel) stimulated [^{35}S]GTPγS binding in the pons and midbrain, but not in hypothalamus, cortex, or cerebellum. In the pons, hypocretin-1 (Fig. 2B,

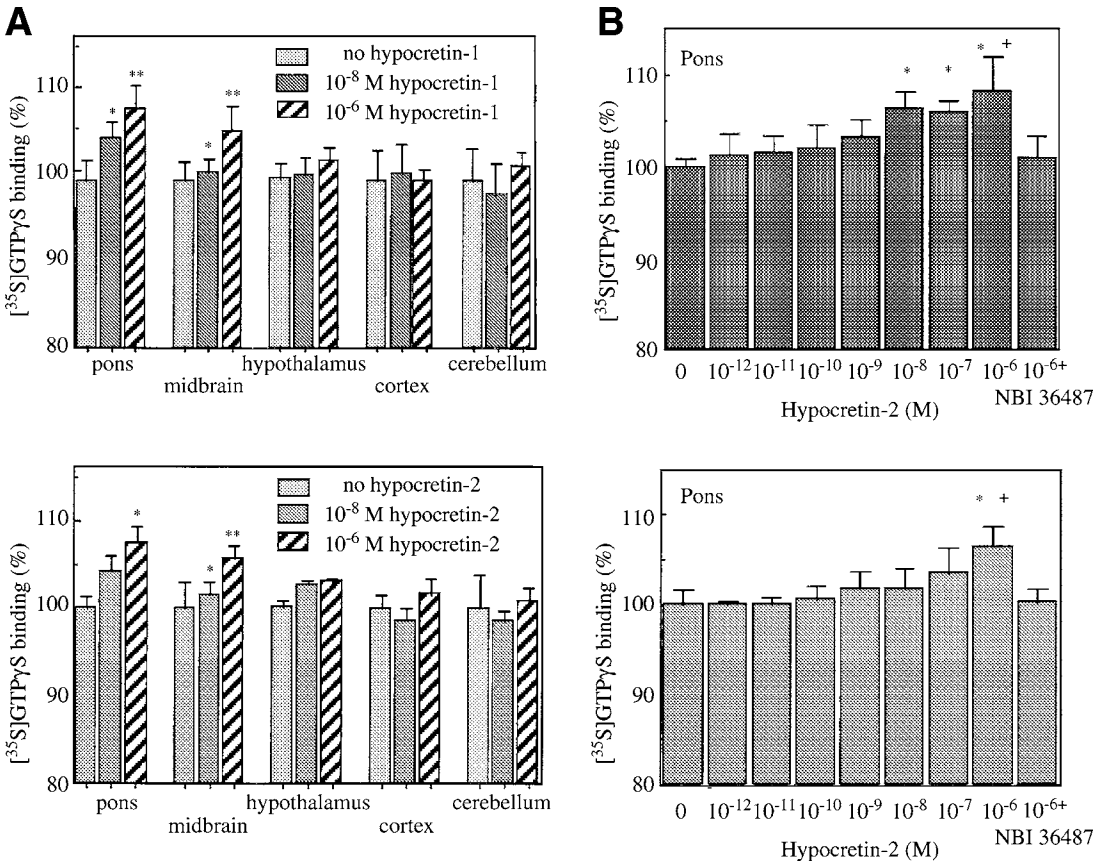

Fig. 2. Hypocretin-stimulated [^{35}S]GTPγS binding in rat brain homogenates. (**A**) Data are presented as means ± SEM of six rats in five brain regions (pons, midbrain, hypothalamus, cortex, and cerebellum) for stimulation by hypocretin-1 (top panel) and hypocretin-2 (bottom panel). *, $p < 0.05$; **, $p < 0.01$ from baseline, one-way ANOVA, and post hoc Fisher's test. (**B**) Blockade by a selective hypocretin receptor antagonist (NBI 36487, 10^{-8} *M*) of 10^{-6} *M* hypocretin-1 (top panel) and hypocretin-2 (bottom panel)-stimulated [^{35}S]GTPγS binding in the pons. Data are presented as means ± SEM of six rats. *, $p < 0.05$, from baseline; +, $p < 0.05$, from level of the coadministration of the antagonist, one-way ANOVA and post hoc Fisher's test. These findings are consistent with the interpretation that hypocretin-stimulated G-protein activation in pontine homogenates is mediated by hypocretin receptors. (Reprinted with permission from ref. *36*.)

top panel) and hypocretin-2 (Fig. 2B, bottom panel) increased [^{35}S]GTPγS binding over basal levels in a concentration-dependent manner. The hypocretin receptor antagonist NBI 36487 blocked [^{35}S]GTPγS binding stimulated by hypocretin-1 (Fig. 2B, top panel) and hypocretin-2 (Fig. 2B, bottom panel). Taken together, these results indicated that G-protein activation by hypocretin is receptor mediated.

In vitro binding studies performed using tissue homogenates lack the anatomical resolution provided by in vitro autoradiography. Considerable information is now available about the arousal-related roles of specific nuclei in the pons (reviewed in refs. *38* and *39*), and hypocretin has opposite effects on sleep and wakefulness depending on the pontine site of administration. Microininjection of hypocretin-1 into the locus coeruleus of rat *(40,41)* or the laterodorsal tegmental nucleus of cat *(41)* increases wakefulness, whereas direct administration

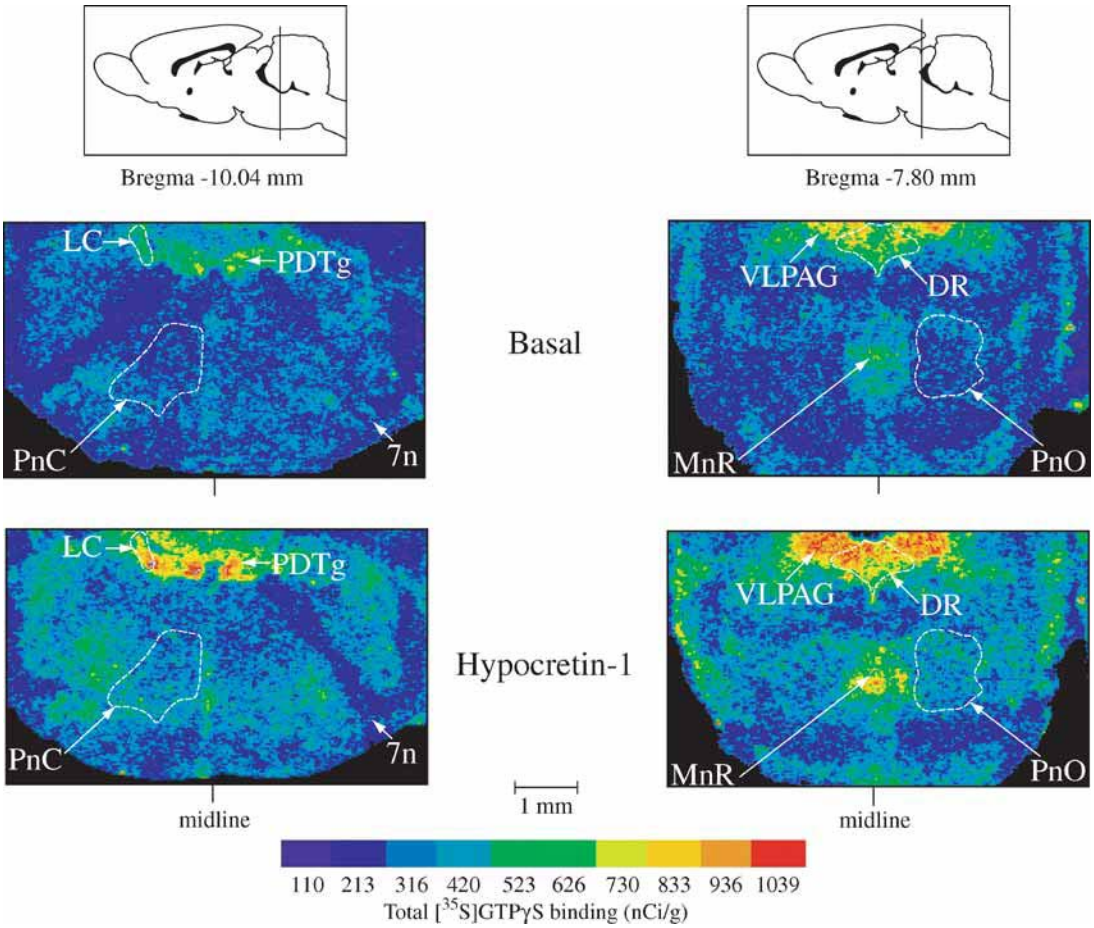

Fig. 3. Hypocretin-1-activated G proteins in rat pons. Boxes at the top of each column show a sagittal schematic of the rat brain (modified from ref. *63*). The vertical line in each schematic indicates the anterior–posterior level (relative to bregma) of the coronal autoradiograms shown below. The color-coded autoradiograms illustrate total [^{35}S]GTPγS binding under basal conditions and following treatment with hypocretin-1 (2000 n*M*). The boundaries of nuclei where [^{35}S]GTPγS binding was quantified and found to be significantly increased by hypocretin-1 are outlined in white. Quantitative data for these nuclei are shown in Fig. 4. Anatomical abbreviations: 7n, facial nerve; DR, dorsal raphe nucleus; MnR, median raphe nucleus; LC, locus ceruleus; PDTg, posterodorsal tegmental nucleus; PnO, pontine reticular nucleus oral part; PnC, pontine reticular nucleus caudal part; VLPAG, ventrolateral periaqueductal gray. (Reprinted with permission from ref. *26*.)

of hypocretin-1 into the pontine reticular formation of cat enhances rapid eye movement (REM) sleep *(42)*. Because of these site-specific effects of pontine hypocretin on sleep and wakefulness in vivo, it was of great interest to determine whether hypocretin activates G proteins in a site-specific manner within the pons.

An initial in vitro [^{35}S]GTPγS autoradiography study showed that hypocretin-1 activates G proteins in the locus ceruleus, dorsal raphe nucleus, and pontine reticular formation of rat *(25)*. Figure 3 illustrates hypocretin-1-stimulated [^{35}S]GTPγS binding in these arousal-related nuclei of the pons. In vitro [^{35}S]GTPγS autoradiography studies also demonstrated that hypocretin-1-stimulated G-protein activation in the dorsal raphe, locus ceruleus, and

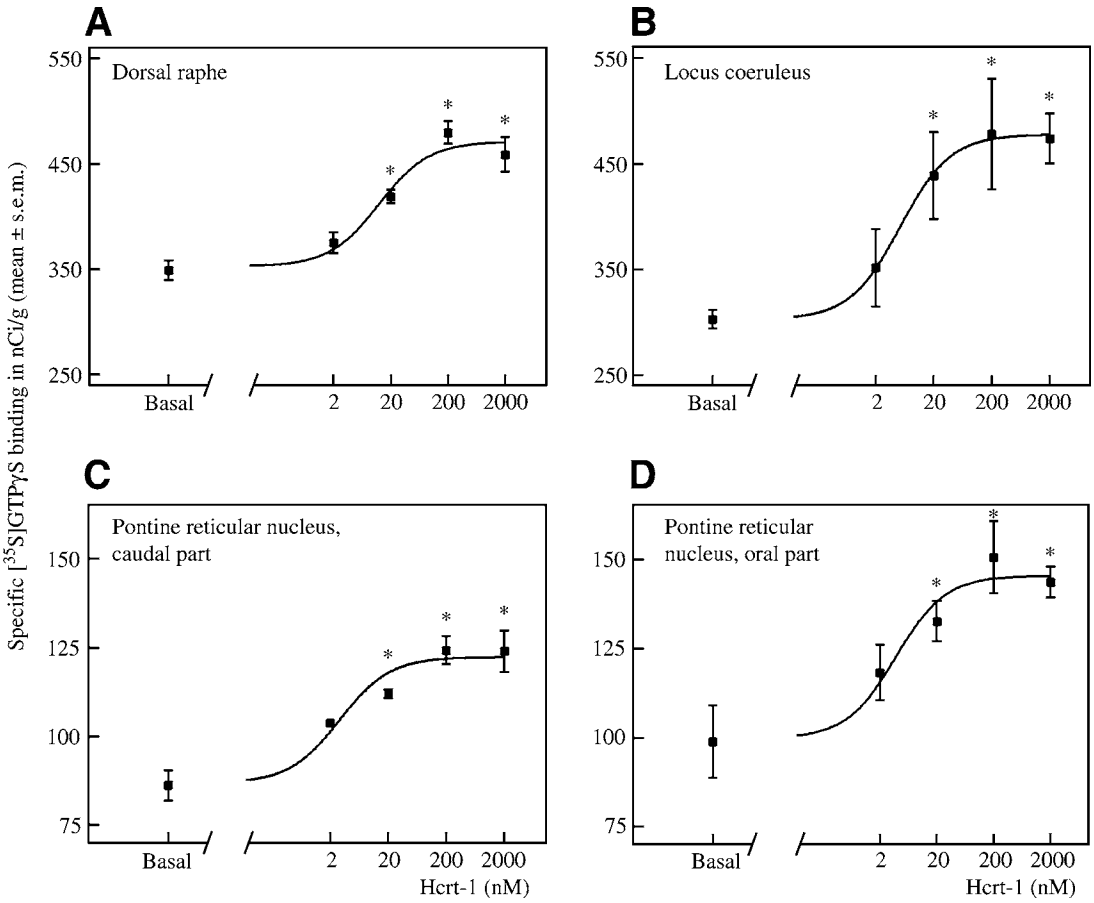

Fig. 4. (A–D) Hypocretin-1-stimulated G-protein activation is concentration dependent in four arousal-related nuclei of the pons. Specific [^{35}S]GTPγS binding following in vitro treatment with 20, 200, and 2000 n*M* hypocretin-1 was significantly increased (*, $p < 0.05$) over basal G-protein binding levels. A sigmoid concentration response curve was fitted to the [^{35}S]GTPγS binding data for each nucleus. The resulting coefficients of determination (r^2) were 0.83 (dorsal raphe), 0.56 (locus ceruleus), 0.79 (pontine reticular nucleus, caudal part), and 0.69 (pontine reticular nucleus, oral part). Thus, depending on the brain region, 56–83% of the variance in [^{35}S]GTPγS binding was accounted for by the concentration of hypocretin-1. The EC_{50} values (n*M*) calculated from these concentration response data were 12.6 (dorsal raphe), 5.3 (locus ceruleus), 2.9 (pontine reticular nucleus, caudal part), and 3.8 (pontine reticular nucleus, oral part). This finding indicates that hypocretin-1 was less potent in the dorsal raphe nucleus than in the other nuclei studied. (Reprinted with permission from ref. *26*.)

oral and caudal parts of the pontine reticular formation is concentration dependent and reaches saturation (Fig. 4) *(26)*. Construction of concentration response curves for specific brain nuclei permits between-nuclei comparisons of the potency (EC_{50}) of hypocretin-1 for activating G proteins. The Fig. 4 data revealed that hypocretin-1 was less potent in the dorsal raphe nucleus than in the locus ceruleus, pontine reticular nucleus, caudal part, or pontine reticular nucleus, oral part. The Fig. 4 data suggest that direct administration of hypocretin-1 into the dorsal raphe nucleus may be less effective in altering arousal than hypocretin-1 administration into the locus ceruleus. This suggestion arising from in vitro studies can be tested in vivo.

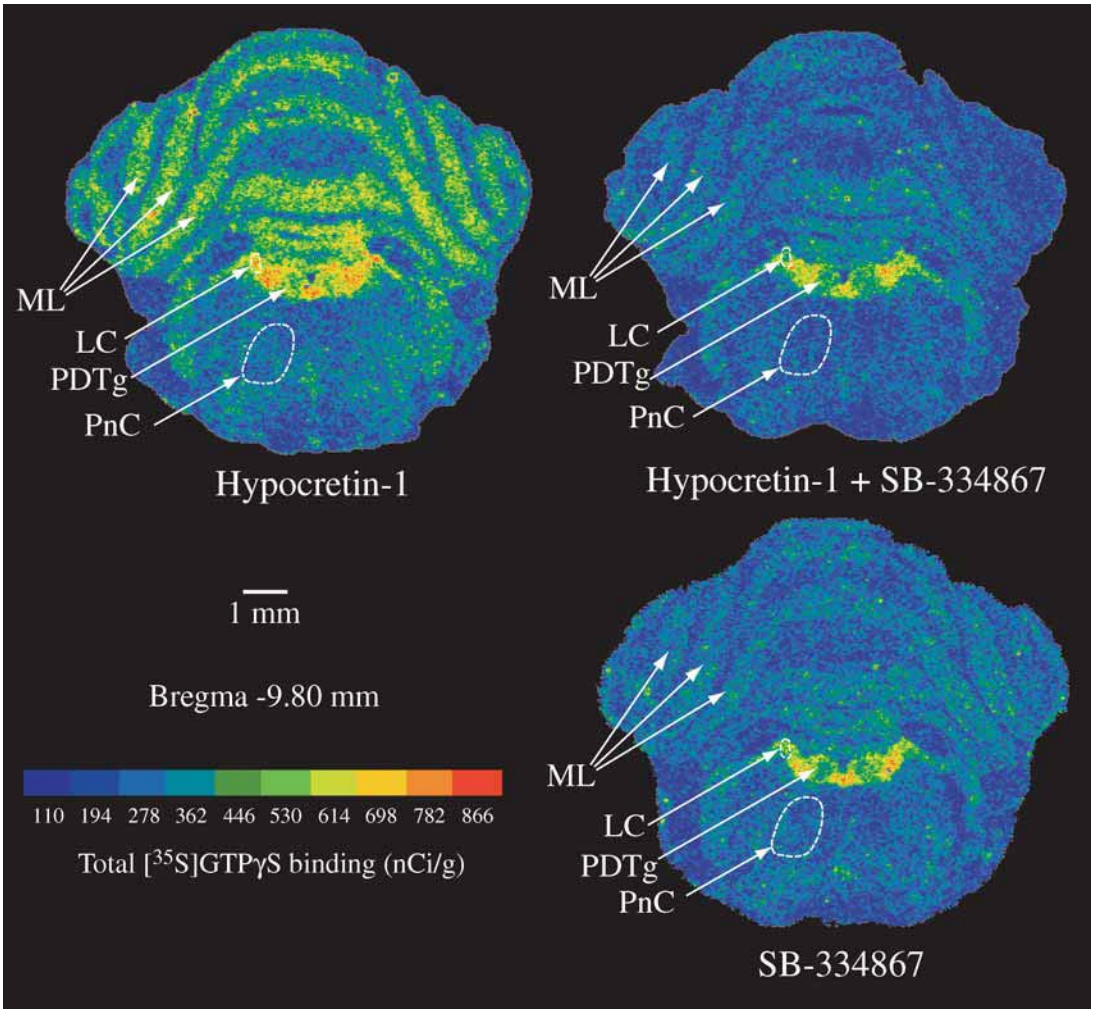

Fig. 5. Antagonist (SB-334867) blocking of hypocretin-1-stimulated G-protein activation. Color-coded autoradiograms of coronal brainstem sections show total [^{35}S]GTPγS binding after in vitro treatment with hypocretin-1 (200 n*M*), hypocretin-1 (200 n*M*) + SB-334876 (2 μ*M*), or SB-334867 (2 μ*M*). The enhancement of [^{35}S]GTPγS binding caused by hypocretin-1 was blocked by adding the hypocretin receptor antagonist SB-334867 to the assay buffer. Quantitative analyses showed that SB-334867 significantly blocked hypocretin-1-stimulated G protein activation in the LC and PnC (*see* Fig. 6). Visual examination indicates that hypocretin-1-stimulated G protein activation in the ML and PDTg was also blocked by the hypocretin receptor antagonist. Anatomical abbreviations: LC, locus ceruleus; ML, molecular layer of the cerebellum; PDTg, posterodorsal tegmental nucleus; PnC, pontine reticular nucleus caudal part.

Establishing receptor mediation of a response requires demonstration of antagonist blocking as well as concentration dependence. Figures 5 and 6 show that the competitive hypocretin receptor antagonist SB-334867 *(43)* blocked hypocretin-1 stimulated [^{35}S]GTPγS binding *(26)*. Taken together with the Fig. 4 results, these data provide the first autoradiographic demonstration that hypocretin-1-induced G-protein activation in the dorsal raphe, locus ceruleus, and pontine reticular formation is receptor mediated. Because [^{35}S]GTPγS autoradiography is thought to label activated inhibitory G proteins preferentially *(4)*, these studies

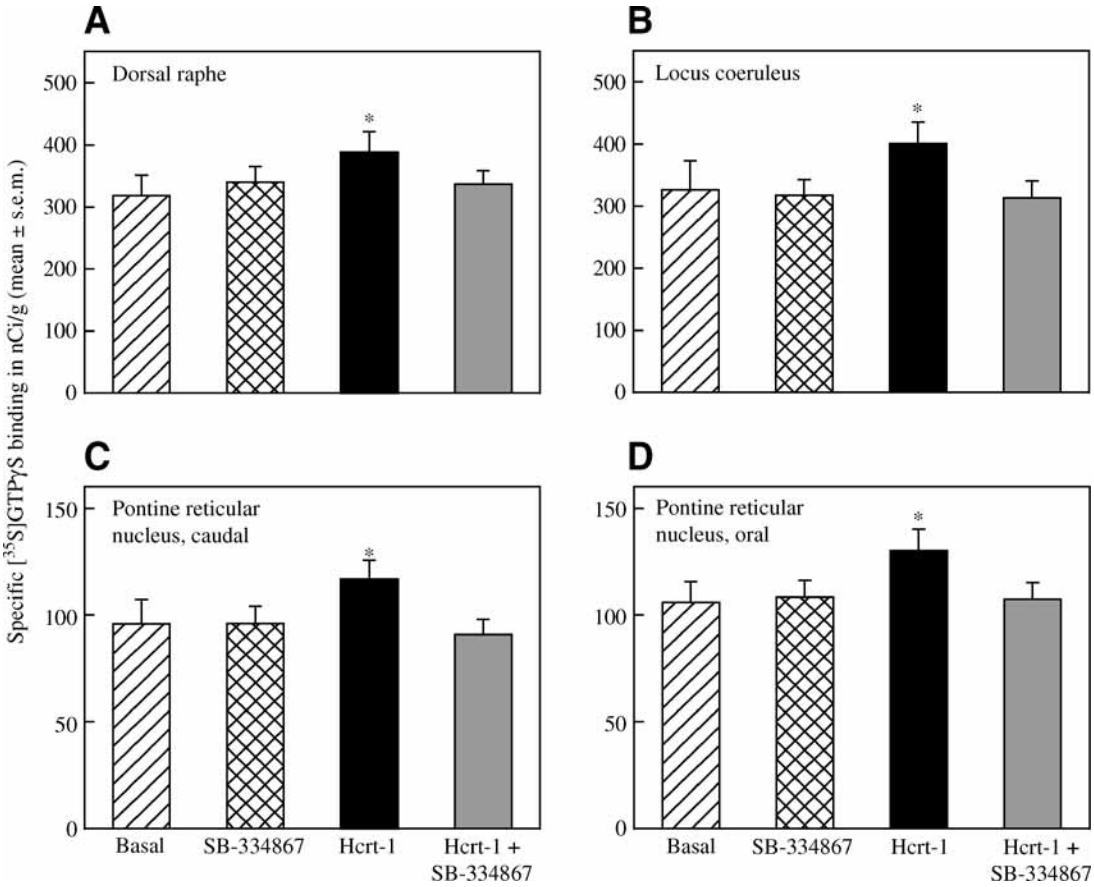

Fig. 6. Hypocretin-1-stimulated [^{35}S]GTPγS binding was blocked by the hypocretin receptor antagonist SB-334867. The abscissa indicates the in vitro treatment condition: basal binding (no agonist or antagonist), SB-334867 (2 μ*M*), hypocretin-1 (200 n*M*), or hypocretin-1 (200 n*M*) + SB-334867 (2 μ*M*). *, $p < 0.05$ vs basal [^{35}S]GTPγS binding. (Reprinted with permission from ref. *26*.)

suggested that at least some hypocretin receptors in the pontine brainstem may couple to Gi/o proteins *(25,26)*.

The inference that some hypocretin receptors in rat pons couple to inhibitory G proteins is supported by preliminary data showing that hypocretin-1-stimulated [^{35}S]GTPγS binding in the oral pontine reticular nucleus is blocked by pertussis toxin *(44)*. In cell lines transfected with hypocretin receptors, responses to hypocretin can also be blocked with pertussis toxin *(27,45)*. Pertussis toxin selectively inactivates inhibitory G proteins *(46)* and thus is a useful tool for identifying Gi/o-mediated responses. Pertussis toxin blocking of hypocretin-1-stimulated G protein activation does not prove that hypocretin receptors couple to Gi/o proteins. An alternative interpretation is that hypocretin-1-stimulated increases in [^{35}S]GTPγS binding are caused by an indirect activation of inhibitory G proteins. As discussed elsewhere *(24)*, receptor-activated G protein signaling is complex, and different receptors can converge on the same G proteins or diverge to activate multiple G proteins *(47)*. G-protein-coupled receptors are known to form dimers *(48–50)*, and indirect activation of inhibitory G proteins could result from heterodimerization of hypocretin receptors with other G-protein-coupled receptors known to activate inhibitory G proteins. Future studies are required to determine the extent

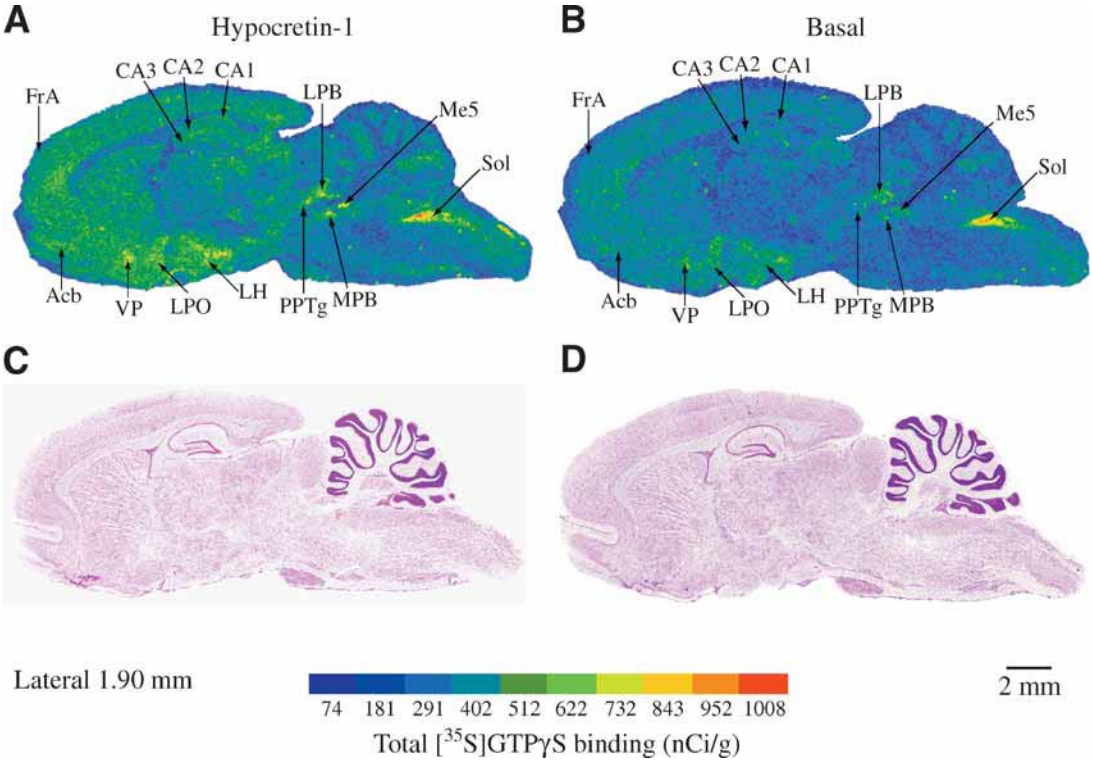

Fig. 7. Sagittal sections reveal that hypocretin-1 (200 n*M*) activates G proteins in different sites along the anterior-posterior axis of the rat brain. Each color-coded autoradiogram **(A,B)** shows total [^{35}S]GTPγS binding and was generated from the corresponding cresyl violet-stained tissue sections **(C,D).** In vitro [^{35}S]GTPγS autoradiography provides an ideal approach for identifying brain regions in which binding of hypocretin-1 to its receptors activates G proteins. Activated brain regions, identified on these autoradiograms by arrows, are candidates for future autoradiographic studies to characterize G protein activation by hypocretin-1 pharmacologically. Anatomical abbreviations: Acb, nucleus accumbens; CA1, CA2, CA3, fields of hippocampus; FrA, frontal association cortex; LPB, lateral parabrachial nucleus; LH, lateral hypothalamus; LPO, lateral preoptic area; MPB, medial parabrachial nucleus; Me5, mesencephalic 5 nucleus; PPTg, pedunculopontine tegmental nucleus; Sol, nucleus of solitary tract; VP, ventral pallidum.

to which hypocretin-stimulated [^{35}S]GTPγS binding reflects activation of hypocretin receptors coupled to Gi and/or Gq proteins in specific brain nuclei.

4. FUTURE DIRECTIONS

By the middle of 2004, only two studies had been published using in vitro [^{35}S]GTPγS autoradiography to quantify hypocretin-stimulated G-protein activation in the brain *(25,26)*. The pons was the focus of both of these studies. Localization and quantification of hypocretin-stimulated G-protein activation outside the pontine brainstem is an important future direction. Preliminary data show that hypocretin-1 stimulates [^{35}S]GTPγS binding in many brain regions outside the pons, including the midbrain, forebrain, cortex, and cerebellum (Fig. 7). These findings are consistent with the expression pattern of the hypocretin peptides *(51)*, receptor proteins *(52,53)*, and receptor mRNA *(52,54)*.

Future studies combining in vitro [^{35}S]GTPγS autoradiography for hypocretin-1 with in vivo pertussis toxin administration *(44)* can identify populations of hypocretin receptors that may couple to pertussis toxin-sensitive inhibitory G proteins in specific brain nuclei. Functional interactions between hypocretin and other arousal-related neurotransmitters also remain to be studied using [^{35}S]GTPγS autoradiography. Many drugs used clinically to modulate arousal (e.g., modafinil [*55*] and gabapentin [*56*]) act through unknown mechanisms. The [^{35}S]GTPγS binding assay is likely to provide insights into the mechanisms of drug action.

Pathophysiological changes at the level of G proteins may occur as a result of neurotoxins, hypoxic/ischemic damage, pathogens, and secondary to trauma. Human narcolepsy is a neurodegenerative disease thought to be caused, in part, by a loss of hypocretinergic neurons *(57)*. The effects of diminished hypocretin peptide levels on the plasticity and function of hypocretin receptor-G protein complexes in brain tissue from narcoleptic patients have not been investigated. Agonist-stimulated [^{35}S]GTPγS binding can be measured in postmortem human brain samples *(58)*, suggesting future experiments using [^{35}S]GTPγS autoradiography to test the functional activity of hypocretin receptor-G protein complexes in brains from narcoleptic patients. Cell lines expressing hypocretin 2 receptors from narcoleptic dogs showed no G-protein activation upon treatment with hypocretin *(36)*, consistent with the known receptor defect *(59)* and demonstrating the utility of [^{35}S]GTPγS binding for evaluating normal receptor function.

The orexin/ataxin-3 transgenic mouse is characterized by a developmental ablation of hypocretin-containing neurons and a narcoleptic-like phenotype *(60)*. Preliminary data show an increase in hypocretin-1-stimulated [^{35}S]GTPγS binding in the pontine reticular formation of 8-wk-old transgenic mice compared with wild-type litter mates *(61)*. This finding demonstrates that hypocretin receptors are functionally active in the orexin/ataxin-3 transgenic mouse and suggests that hypocretin receptors initially may be upregulated in response to the loss of hypocretinergic neurons. Future studies can quantify the effects of diminished hypocretinergic input on the functional activity of hypocretin receptors at different times during development using hypocretin-stimulated [^{35}S]GTPγS autoradiography. Application of [^{35}S]GTPγS autoradiography to transgenic mouse models may contribute information about the plasticity of the hypocretin ligand-receptor system and indicate whether hypocretin replacement therapy may be useful for narcoleptic patients *(62)*.

ACKNOWLEDGMENTS

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IV

PHYSIOLOGY

A. Orexin/Hypocretin System and Hypothalamic Function

8
Orexin System and Feeding Behavior

Takeshi Sakurai, MD, PhD

1. INTRODUCTION

The physiological roles of the the lateral hypothalamic area (LHA) have been shown historically by lesioning and complementary electrical stimulation studies. Animals with lesions of the LHA exhibit hypophagia and an increased metabolic rate, whereas electrical stimulation studies of the LHA showed that acute stimulation causes hyperphagia and that chronic stimulation can cause obesity. Therefore, it has long been thought that the LHA is a feeding center and an important region for the regulation of body weight homeostasis *(1–3)*. Because orexin/hypocretin neurons are localized exclusively in the LHA, orexins have been thought to participate in the regulation of feeding behavior. Many studies have now shown the physiological importance of orexins in the regulation of feeding behavior. This chapter explores the roles of orexin in the regulation of feeding behavior and energy homeostasis.

2. PHARMACOLOGICAL EFFECTS OF OREXIN ON FEEDING BEHAVIOR

The striking localization of orexin-containing neurons in the LHA and some of its adjacent areas suggests that orexins may be involved in the regulation of food intake. Therefore, the initial studies investigating the physiological role of the orexins focused mainly on feeding behavior.

When administered into lateral ventricles of rats in the early light phase, orexin-A stimulated food consumption in a dose-dependent manner within 1 h (Fig. 1). The effect persisted at 4 h; the amount of food consumed during the interval from 2 to 4 h post- injection (30 nmol) was increased approx three-fold with either dose compared with vehicle control. Human orexin-B also significantly augmented food intake. The effect of orexin-B did not last as long as that of orexin-A; there was little stimulation of food intake after 2 h even with the higher dose *(4)* (Fig. 1). The difference in time-course of pharmacological activities between orexin-A and orexin-B might be due to the relative instability of orexin-B in cerebrospinal fluid compared with orexin-A, because the N terminus of orexin-A is protected by a pyroglutamyl residue and two disulfide bridges. Many studies have also confirmed that intracerebroventricular (icv) administration of orexin-A or orexin-B in rodents during the early light phase dose-dependently stimulates food consumption *(4–8)*. Increase of food intake by icv orexin administration was also reported in goldfish *(9)*.

The physiological relevance of the effects of orexin on feeding is supported by the finding that central administration of a neutralizing anti-orexin antibody significantly and dose-dependently suppressed spontaneous feeding in fasted rats *(7)*. A selective orexin receptor type-1 antagonist also consistently, inhibited natural feeding over several days *(10)*.

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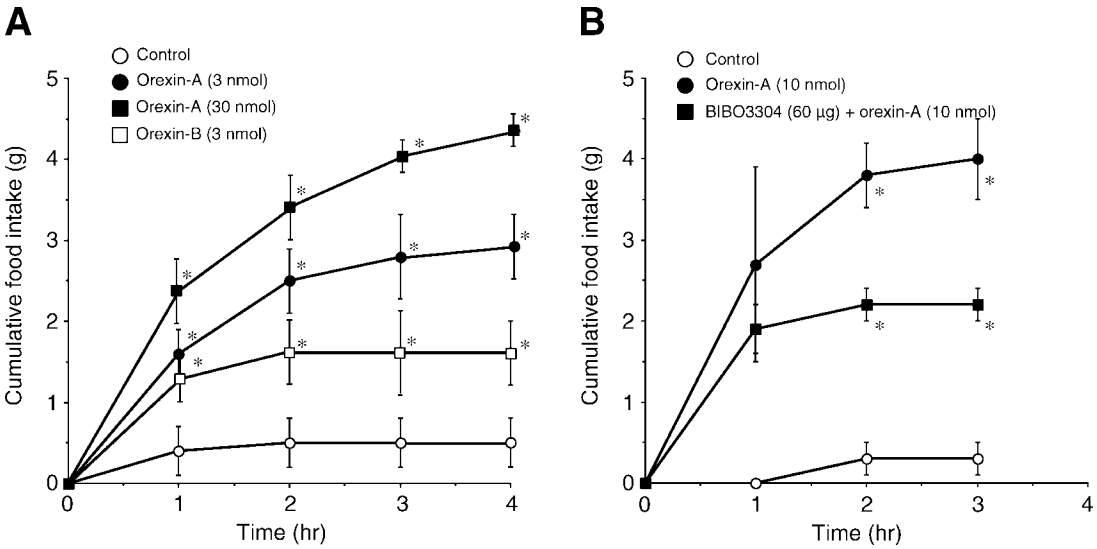

Fig. 1. Stimulation of food consumption by intracerebroventricular injection of orexin-A and -B in freely fed rats. **(A)** Designated amounts of synthetic human orexin-A or -B were administered in a 5-µL bolus through a catheter placed in the left lateral ventricle in early light phase. Cumulative food consumption was plotted over a 4 h after injection. Data are mean + SE. Asterisks (*) indicate significant difference from vehicle controls ($p < 0.05$, $n = 8–10$, ANOVA followed by Bonferroni-Dunn test.) **(B)** Effect of a selective NPY1 receptor antagonist, BIBO3304, on orexin-A (10 nmol) induced food intake. Lines show the cumulative food intake in rats for 3 h after icv injection of orexin-A. BIBO3304 was administered icv 15 min before administration of orexin-A. The experiment was started in the early light phase (10:00 AM). Data are mean + SE. Asterisks (*) indicate significant difference from vehicle controls ($p < 0.05$, $n = 8–10$, ANOVA followed by Bonferroni-Dunn test).

The feeding response to acute icv administration of orexin is dependent on the time of day and the energy balance of the animal *(8)*. The largest increase in orexin-stimulated food intake occurred in the early light phase and 6 h into the dark phase, when normal food intake is slowing. Administration of orexin-A at the beginning of the dark period, when the normal feeding rate is at its highest, had no effect. Similarly, orexin-A did not increase food intake in the first hour of refeeding after a fast. This phenomenon might be explained by the fact that at the beginning of the dark phase and immediately after a fast, the orexin-stimulated feeding pathways are already maximally activated and therefore unresponsive to additional exogenous pharmacological stimulation by orexin-A. In fact, the activity of orexin neurons might have diurnal fluctuation; activity might be higher during the active period and decreased during the inactive period *(11,12)*. A similar phenomenon was also observed when orexin-A was administered chronically. Chronic administration of orexin-A (0.5 nmol/h) for 7 d resulted in a significant increase in food intake in the daytime, which increased to 180% of the control value *(13)*. However, it resulted in a slight decrease in nighttime food intake. Thus, chronic infusion of orexin-A over several days disrupted the normal circadian feeding pattern in rats by increasing daytime and decreasing nighttime food intake *(13)*. The total food intake per day was almost the same as that of vehicle-administered rats. The gain in body weight and blood glucose, total cholesterol, and free fatty acid levels also remained normal. Therefore, chronic orexin-A treatment did not cause obesity in rats. These observations suggest that continuous administration of orexin-A could not overcome the regulation of energy homeostasis and

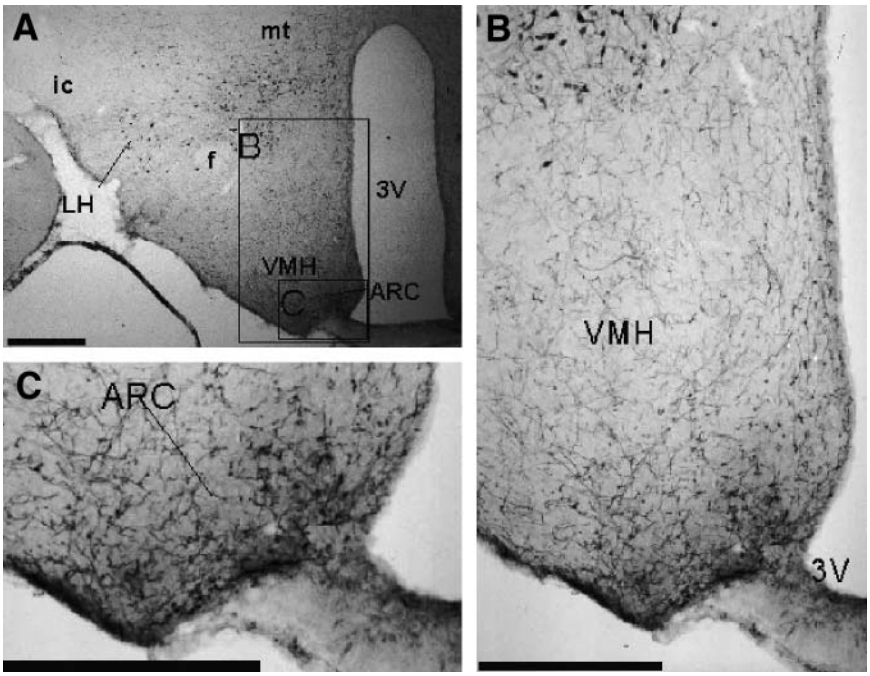

Fig. 2. Orexin neurons in the LHA innervate the arcuate nucleus. **(A)** An adult rat brain section stained by anti-orexin antiserum. B_5 bregma, 3.3 mm. LHA, lateral hypothalamic area; VMH, ventromedial hypothalamus; ARC, arcuate nucleus; 3V, third ventricle; mt, mammillothalamic tract; ic, internal capsule; f, fornix. Orexin-immunoreactive cell bodies are distributed within the LHA. **(B,C)** High-power views of the region indicated by rectangles in **(A)**. Note that abundant orexin-immunoreactive fibers are observed in the ARC and VMH. Scale bars = 500 μm.

body weight. These observations suggest that orexins are involved in the regulation of short-term feeding rather than long-term body weight homeostasis.

3. MECHANISMS THAT MEDIATE OREXIN-INDUCED FEEDING

Increases in adiposity result in higher circulating leptin levels, which cross the blood–brain barrier to suppress feeding via neurons that express the signal-transducing leptin receptor Ob-Rb *(14,15)*. The arcuate nucleus (ARC), which contains neuropeptide Y (NPY)/agouti-related protein (AgRP)-coexpressing neurons as well as pro-opiomelanocortin (POMC)/cocaine and amphetamine-regulated transcript (CART)-coexpressing neurons, is a major site of leptin's action and is regarded as an important region that regulates feeding behavior *(16)*. NPY/AgRP neurons stimulate feeding, whereas POMC/CART neurons suppress feeding. Leptin-mediated inhibition of NPY/AgRP neurons and excitation of POMC/CART neurons are thought to be the major mechanisms of suppression of feeding by leptin *(16,17)*. Orexin neurons densely project to the ARC *(5,18,19)* (Fig. 2), and Fos expression was induced in NPY neurons of the ARC by icv injection of orexin, suggesting that orexin-stimulated feeding may occur at least partly through NPY pathways *(5)* (Fig. 1B). Indeed, the orexin-A-induced increase in food intake was partly inhibited by prior administration of BIBO3340, an NPY-Y1 receptor antagonist, in a dose-dependent manner (Fig. 1B) *(5)*. These experiments suggest that orexin-stimulated food intake is at least partially mediated by activation of NPY neurons. However, because 60 μg of NPY antagonist (which completely abolishes NPY-induced feeding)

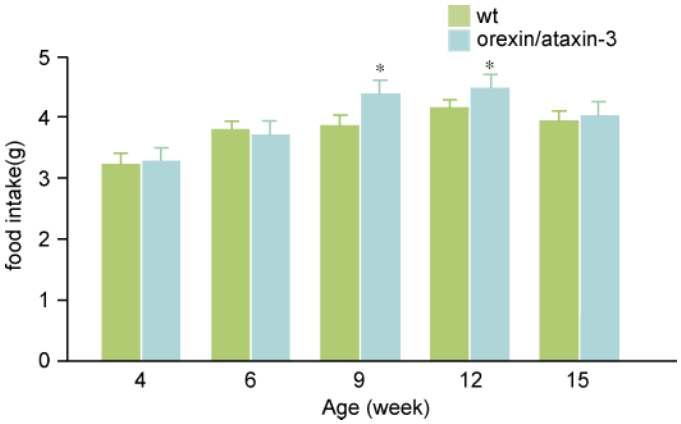

Fig. 3. Food consumption in wild-type and *orexin/ataxin-3* mice at 4–15 wk of age; asterisks (*) indicate significant difference between genotypes ($p < 0.01$; $n = 8$).

only partially (50%) abolished orexin-induced feeding, other pathways by which orexin induces feeding might exist. One pathway might include inhibition of glucoreceptive, POMC-expressing neurons *(20)* (Fig. 3). Orexin-mediated arousal might also be important in supporting feeding behavior. The relationship between arousal regulation and feeding is discussed in other chapters of this book.

Both NPY- and galanin-induced feeding behavior were completely inhibited by preadministration of leptin, whereas the same or a higher dose of leptin only partially inhibited the orexin-A or orexin-B-induced increase of food intake in rats *(21)*. Preadministration of anorectic peptides (α melanin-stimulating hormone [MSH] and glucagon-like peptide-1 [GLP-1]), which are regulated by leptin, almost completely abolished NPY-induced feeding; however, orexin-induced feeding was only partially inhibited by these anorectic peptides. These observations suggest that NPY- and galanin-induced increases in feeding involve a leptin-sensitive pathway, whereas orexin-induced feeding involves both leptin-sensitive and -insensitive pathways. It is possible that the leptin-insensitive pathway might be responsible in part for the "leptin resistance" found in obese humans.

4. MOLECULAR GENETIC STUDIES

Destruction of orexin neurons in human narcolepsy is accompanied not only by abnormal sleep/wake regulation but also by metabolic alterations *(22,23)*. The finding of decreased caloric intake *(24)* combined with an increased body mass index *(23)* in narcolepsy suggests that narcolepsy patients have a feeding abnormality with reduced energy expenditure or low metabolic rate. Because narcolepsy is caused by orexin deficiency, the altered energy homeostasis in human narcolepsy patients suggests roles of orexin in regulation of energy homeostasis *(22,23)*. Consistently, orexin neuron-ablated mice show hypophagia and late-onset obesity *(25)*. Complex disruptions of energy homeostasis in *orexin/ataxin-3* transgenic mice are indicated by hypophagia (Fig. 3), obesity, and inactivity relative to control litter mates *(25,26)*. Orexin knockout mice were also showed to be hypophagic *(27)*. The decreases in food intake observed in orexin knockout or orexin neuron-ablated mice suggest the physiological roles of orexins in support of feeding behavior.

Studies from orexin knockout mice, orexin neuron-ablated mice, and narcolepsy patients showed that chronic deficiency of orexins could result in obesity despite hypophagia *(23,25,27)*. Although we have found that the development of obesity observed in orexin-deficient mice

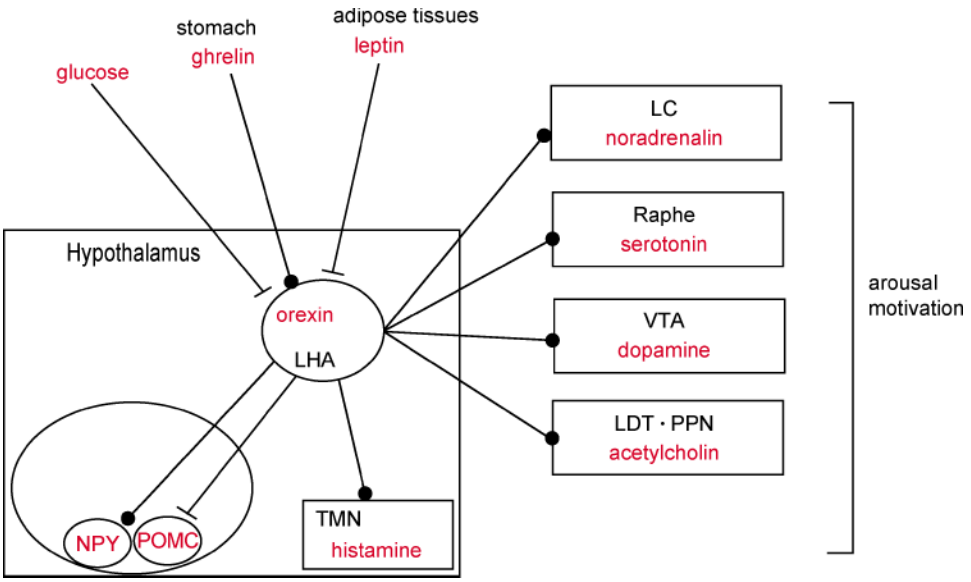

Fig. 4. A schematic drawing of the mechanisms by which orexins induce food intake. Orexin neurons activate neuropeptide Y (NPY) ergic neurons and inhibit pro-opiomelanocortin (POMC) neurons in the arcuate nucleus to increase food intake. Orexin neurons also regulate monoaminergic/cholinergic nuclei in the brainstem to maintain arousal and support motivation for reinforcing feeding behavior.

is dependent of genetic background (our unpublished observation), these observations suggest altered energy homeostasis in orexin-deficient narcolepsy. On the other hand, acute pharmacological blockade of orexin-1 receptor (OX_1R) results in hypophagia and decreased body weight *(10)*. This paradoxical phenomenon might suggest that receptor blockade is necessary for developing obesity. In fact, we observed that OX_1R knockout mice do not develop obesity (our unpublished observation). It is also possible that chronic disruption of orexin signaling by gene targeting and acute or semichronic blockade of orexin receptor might have different impacts on the orexin-mediated feeding pathway and an orexin-mediated increase in metabolic rate.

5. OREXIN AND OTHER FEEDING BEHAVIOR-RELATED FUNCTIONS

Orexin might also participate in other feeding-related functions. Tuberomammillary nucleus (TMN) histaminergic neurons were shown to be activated by mastication owing to a signal originating from oral proprioceptors and initiated by chewing *(28)*. Reciprocally, histaminergic neurons densely innervate the mesencephalic trigeminal sensory nucleus. This pathway was shown to be involved in the regulation of mastication. Because orexin neurons densely innervate the mesencephalic trigeminal sensory nucleus and the TMN, and orexin receptor mRNAs exist in these nuclei, it might be possible that orexin neurons are also involved in the regulations of mastication. In fact, we usually observed a chewing-like movement of the mandible in rats after orexin was administered centrally (our unpublished observation).

Orexins might also be involved in the cephalic phase of gastric acid secretion. Intracisternal injection of synthetic orexin-A dose-dependently stimulated gastric acid secretion in rats *(29)*. Vagotomy or atropine administration abolished the action of orexin-A. These observations suggest that orexin-A may act in the brain to stimulate gastric acid secretion by modulating vagal activity.

6. ROLES OF OREXIN IN THE REGULATION OF FEEDING BEHAVIOR AND BODY WEIGHT HOMEOSTASIS

Upregulation of prepro-orexin mRNA suggests that orexin neurons monitor the animal's nutritional status *(4)*. Indeed, our electrophysiological studies on orexin neurons showed that leptin robustly inhibited most orexin neurons, causing hyperpolarization and a decrease in firing rate. Furthermore, leptin administration depresses *orexin* expression in the hypothalamus of normal mice. We also showed that ghrelin activates a population of isolated orexin neurons by depolarization with increases in action potential frequency. Circulating ghrelin, as well as ghrelin-containing neurons *(30)*, may in part mediate the activation of orexin neurons such as that occurring during food restriction. Induction of food intake by ghrelin, which counteracts reduction in body weight, may be mediated in part by orexins *(31)*.

There are two distinct classes of hypothalamic neurons that are modulated by physiological alterations of extracellular glucose concentrations. So-called glucose-responsive neurons of the medial hypothalamus are activated by low glucose levels and may function to suppress feeding behavior. The others are glucose-sensitive neurons in the LHA, which are inhibited under high glucose concentrations and may participate in stimulating food intake. Our electrophysiological study on orexin neurons suggested that orexin neurons are glucose-sensitive; we found that extracellular glucose concentrations affect orexin neuronal activity; orexin neurons are activated when extracellular glucose concentration is low *(32)*.

These regulatory mechanisms of orexin neurons by feeding behavior-regulating factors suggest that orexin neurons are activated upon fasting and that orexin is involved in the system that regulates feeding behavior and body weight. Orexin might regulate motivational and emotional aspects of appetite, because orexin neurons interact with dopaminergic neurons and the limbic system, as well as arousal pathways *(33)*. When orexins are administered centrally to rodents, they elevate sympathetic tone, plasma corticosterone levels *(34)*, metabolic rate *(35)*, food intake *(4)*, locomotor activity *(33)* and wakefulness *(34)*. Most of these effects resemble those observed in fasted animals *(36–38)*. These observations suggest that orexin neurons are activated during fasting and elicit appropriate behaviors, including feeding. Orexin receptor agonists and antagonists might also be useful in novel treatments for obesity, eating disorders, or other autonomic/metabolic disorders *(10)*.

Regarding body weight homeostasis, orexins have opposite impacts on feeding and metabolic rates; orexins increase food intake, and also increase metabolic rate *(35)*. Therefore, orexis do not act simply as a system that maintains long-term body weight homeostasis. Normal feeding behavior is accompanied by an increase in metabolic rates (specific dynamic action). Also, in nature, animals must be aware and active when they seek and intake foods. Orexins functions might be necessary for food seeking and feeding behaviors, especially when animals are faced with scarcity.

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9
Orexins and the Autonomic Nervous System

Tetsuro Shirasaka, MD, PhD, and Masamitsu Nakazato, MD, PhD

1. INTRODUCTION

Hyperphasia (overeating) is often associated with energy overstorage and obesity, which may lead to a myriad of serious health problems, including heart disease, hypertension, and type 2 diabetes. The hypothalamus, in which a number of neuropeptides have been demonstrated to stimulate or suppress food intake, is considered an important organ for the regulation of appetite and energy homeostasis *(1)*. Recently, a novel hypothalamic peptide family (subsequently termed orexins) was discovered in a cytoplasmic calcium-level assay on several cells expressing individual orphan G-protein–coupled receptors *(2)*. The mRNA for the precursor of these peptides is abundantly and specifically expressed in the lateral hypothalamus (LH) and adjacent areas, a region classically implicated in the regulation of feeding and energy homeostasis. The LH also participates in the reciprocal relation of sympathetic activity and feeding. The neuropeptides, monoamines, and many drugs involved with modulating food intake and fat stores have reciprocal effects on cardiovascular response, sympathetic nerve activity, and thermogenesis *(3–5)*. Within the hypothalamus, orexin/hypocretin nerve fibers *(6)* and orexin/hypocretin receptors (OX_1R and OX_2R), especially OX_2R *(2,7)*, are found extensively in the hypothalamic paraventricular nucleus (PVN), which is thought to be involved in control of the autonomic nervous system, cardiovascular function, and neuroendocrine system *(8,9)*. On the other hand, OX_1R is most abundant in the ventromedial hypothalamic nucleus (VMH). Thus, orexins may have a functional role in regulation of the cardiovascular and autonomic nervous systems. The aim of this chapter is to summarize our recent studies, in which we used direct recording of sympathetic nerve activity in conscious rats and an in vitro whole cell patch-clamp technique to examine the direct effect of orexins on PVN neurons using a hypothalamic slice. These studies were performed to elucidate the central actions of orexins on cardiovascular functions and the autonomic nervous system.

2. OREXINS AND CARDIOVASCULAR AND SYMPATHETIC FUNCTIONS IN VIVO

Several studies have reported that central administration of orexins induces *c-fos* expression in the locus coeruleus, arcuate nucleus, central gray, raphe nuclei, nucleus tractus solitarius (NTS), supraoptic nucleus (SON), and PVN in rats *(6,10)*, indicating that central administration of orexins activates specific nuclear groups in the hypothalamus and brainstem known to regulate autonomic and neuroendocrine functions. Accordingly, we hypothesized that orexins may affect cardiovascular and sympathetic functions mediated via a central nervous system (CNS) site of action.

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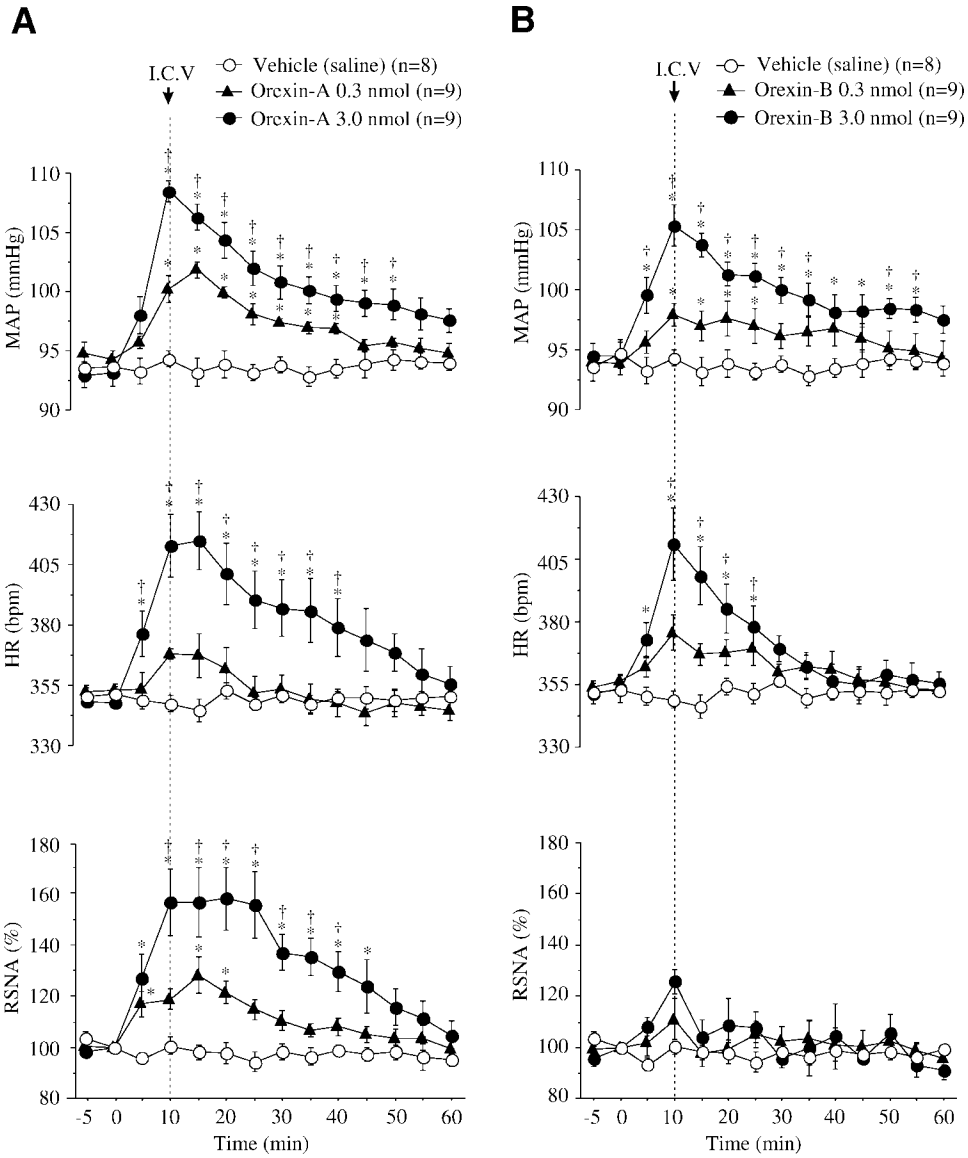

Fig. 1. Time-course of changes in mean arterial pressure (MAP), heart rate (HR), and renal sympathetic nerve activity (RSNA) during the 60 min following icv administration of orexin-A (0.3, 3.0 nmol) **(A)** or -B (0.3, 3.0 nmol) **(B)** in conscious rats. Vertical dotted line indicates time 0 min; bpm, beats per min. All data are mean ± SEM; *n* is no. of animals, $^{*}p < 0.05$ vs. vehicle, $^{\dagger}p < 0.05$ vs orexin A or -B (0.3 nmol). (Results are from ref. *11*.)

To examine this possibility, the cardiovascular and renal sympathetic nerve responses produced by intracerebroventricular (icv) administration of orexin-A and -B were studied in conscious, unrestrained Wistar rats *(11)*, since there is overwhelming evidence that autonomic and endocrine responses are profoundly influenced by anesthetics, even to the extent that, using the same intervention, opposite results may be produced in conscious and anesthetized animals *(12,13)*. Administration of orexin-A icv provoked a dose-related increase in the mean arterial pressure (MAP), heart rate (HR), and renal sympathetic nerve activity (RSNA) in

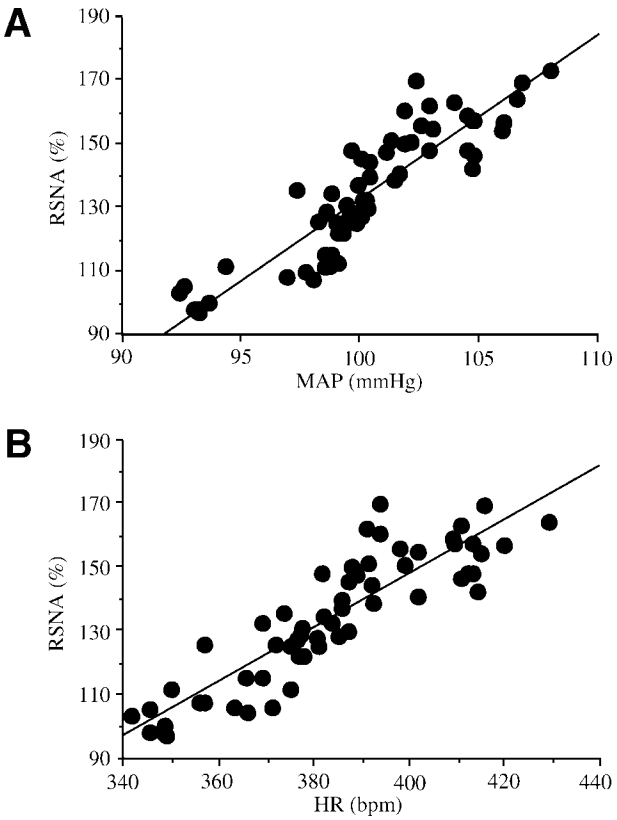

Fig. 2. The relationship of renal sympathetic nerve activity (RSNA) and mean arterial pressure (MAP) (**A**) or heart rate (HR) (**B**). There was a statistically significant correlation between RSNA and MAP or HR ($r = 0.83$ and $r = 0.89$, respectively; both p values < 0.001) in the orexin A (3.0 nmol)-injected group. (Results are from ref. *11*.)

conscious rats (Fig. 1A) *(11)*. MAP and HR increased rapidly and reached a peak value of 10–15 min after orexin-A administration. These pressor effects induced by orexin -A and -B were also demonstrated in conscious SD rats *(14)* and rabbits *(15)*. In addition, in urethane- or pentobarbital-anesthetized rats, central administrations of orexin-A or -B dose-dependently increased MAP and HR *(16,17)*. On the other hand, iv injection of the same dose of orexin-A or -B used in the icv injection experiment failed to cause any cardiovascular or sympathetic nervous activity (SNA) change *(11,15)*.

These findings suggest that the pressor effects induced by orexin-A and -B were mediated via a CNS site of action. A high dose of orexin-A additionally produced a significant increase in RSNA 10 min after injection, which persisted for approx 15 min. RSNA also increased transiently at a low dose (0.3 nmol) of orexin-A. There was a statistically significant correlation coefficient (r) between RSNA and MAP ($r = 0.69$ and $r = 0.83$, respectively; both p values < 0.001) or HR ($r = 0.76$ and $r = 0.89$, respectively; both p values < 0.001) at 0.3- and 3.0-nmol doses in the orexin-A-injected group (Fig. 2).

There was also a statistically significant correlation between MAP and plasma norepinephrine (NE) concentration under icv administration of orexin-A (5.0 nmol) *(17)*. Orexin-B (3.0 nmol) administered icv also produced a significant increase in MAP; this

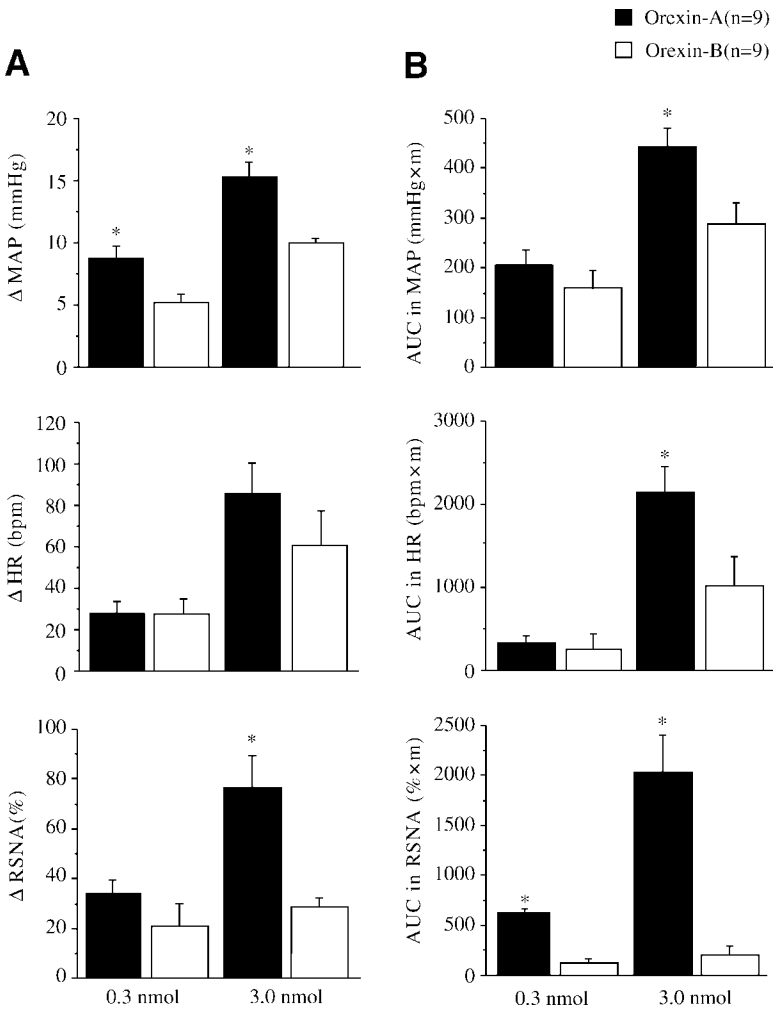

Fig. 3. Bar graphs showing maximal changes from control values (**A**) and the area under the curve (AUC; **B**) for mean arterial pressure (MAP), heart rate (HR), and renal sympathetic nerve activity (RSNA) during the 60 min following icv administration of orexin-A and -B (0.3, 3.0 nmol, respectively) in conscious rats; bpm, beats per min. All data are mean ± SEM; *n* is no. of animals.* $p < 0.05$ vs orexin-B for each dose. (Results are from ref. *11*.)

response pattern was similar to that observed for orexin-A administration (Fig. 1B). HR also rapidly increased and returned to control level within 30 min of the administration of orexin-B (3.0 nmol). In contrast to the results with orexin-A, RSNA did not increase significantly at 0.3- and 3.0-nmol doses of orexin-B. However, a 30-nmol dose of orexin-B resulted in an approx 40% ($p < 0.001$) increase in RSNA 20 min after injection in our study (unpublished data). For each dose, the maximum changes from control values during the recording time (60 min) were compared for orexin-A and -B (Fig. 3). The increase in MAP induced by central orexin-A was 1.5-fold larger than that of orexin-B for both doses, but no significant differences were observed in HR. Our results showing that pressor response induced by orexin-A is larger than that induced by -B agree with those of another study *(16)*.

The increase in RSNA produced by icv administered orexin-A was larger than that of orexin-B at 3.0 nmol. In addition, the area under the curve (AUC) in MAP and HR was significantly larger in orexin-A than orexin-B at only 3.0 nmol (Fig. 3). The AUC in RSNA was larger in orexin-A than orexin-B at each dose. In almost all orexin icv administered rats, increases in locomotor activities, such as chewing and grooming, which are known to be related to a stress response *(18)*, were observed *(19)*. Stress, muscle exercise, and postural change are well known to induce the activation of sympathetic outflow *(20)*. To exclude the effects of stress and/or locomotion on these parameters, orexin-A and -B (3.0 nmol) were administered centrally in rats anesthetized with pentobarbital (50 mg/kg ip). Orexin-A administered icv evoked a significant increase in MAP, HR, and RSNA, and orexin-B increased MAP and HR significantly in anesthetized rats, indicating that the increases in these parameters were not owing to the rats' activated locomotion and/or stress.

The existence of regional differences in sympathetic outflow has been demonstrated *(21)*. Thus, to examine systemic sympathetic outflow induced by central orexins, plasma CA was measured under similar conditions to record nerve activity (Fig. 4). High doses of orexin-A and -B increased plasma norepinephrine (NE), the effect being larger and longer lasting with orexin-A. Therefore, it is likely that the orexin-induced increase in sympathetic nerve outflow leads to the increase in plasma NE, which produces cardiovascular responses. Orexin-A administered icv also significantly increased the plasma epinephrine (Epi) level 10 min after injection. Rapid increases in plasma concentrations of adrenocorticotropic hormone (ACTH) and corticosterone, and the mRNA levels of corticotropin-releasing factor (CRF) and argine vasopressin (AVP) in the parvocellular neurons of the PVN *(22)* induced by orexin-A were demonstrated.

These results suggest that orexin-A acts centrally to activate the hypothalamic-pituitary-adrenal (HPA) axis involving the stimulation of both CRF and AVP expression. In addition, central orexin-A has been reported to increase plasma Epi, glucose, and AVP levels in conscious rabbits *(15)*. The elevated circulating levels of Epi as well as NE after injections of a high dose of orexin-A suggest activation of the sympatho-adrenomedullary (SA) system. In contrast to orexin-A, central orexin-B did not produce an increase in plasma Epi. The large pressor response induced by central orexin-A, compared with that induced by orexin-B, may be owing to the activation of the SA system in addition to the sympathetic outflow. These results indicate that icv administered orexin-A and -B produce cardiovascular responses via different central mechanisms.

Orexin-A and -B are endogenous neuropeptide agonists for orexin receptor-1 (OX_1R) and orexin receptor-2 (OX_2R) *(2)*. Orexin-A has equal affinity for OX_1R and OX_2R, whereas orexin-B displays higher affinity for OX_2R *(2)*. The novel OX_1R antagonist SB-334867 *(23)*, which alone did not significantly change baseline hemodynamic variables and plasma catecholamine levels, markedly attenuated increases in MAP, HR, and plasma NE concentration induced by orexin-A in rats *(17)*. These results suggest that orexin-A may regulate sympathetic and cardiovascular activity mainly through OX_1R. On the other hand, orexin-containing neurons in the hypothalamus or PVN neurons project to the intermediolateral (IML) cell column of the thoracolumbar spinal cord *(6,24)*, which is the site of sympathetic preganglionic motor neurons involved in the regulation of HR and blood pressure (BP). The increases in MAP induced by central orexin-A were abolished by a ganglion-blocking agent, pentolinium *(15)*. The α_1-adrenergic receptor antagonist prazosin and the β-adrenergic receptor antagonist proranolol markedly diminished the orexin-A-induced increase of MAP and HR, respectively *(25)*. These results support the contention that the pressor and tachycardic

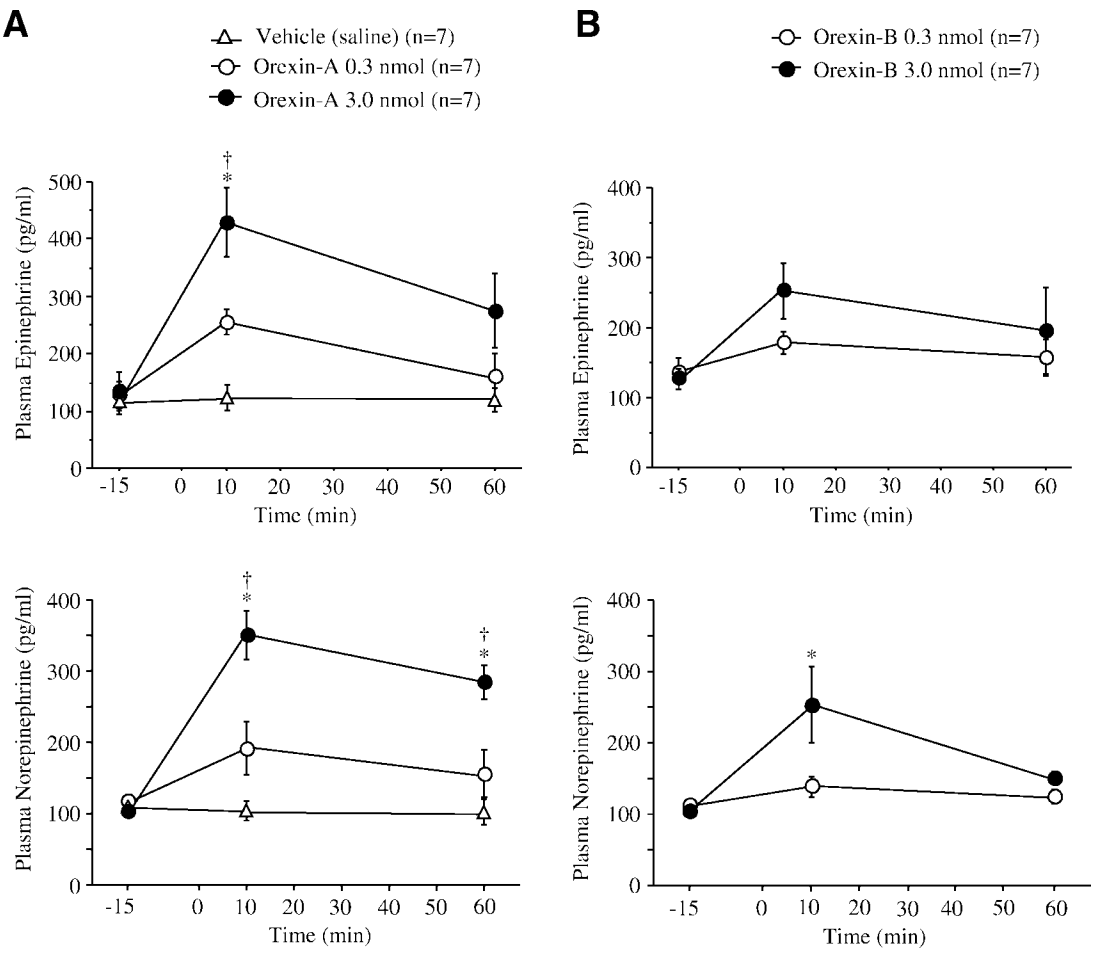

Fig. 4. Effect of icv administration of vehicle (saline), or orexin-A (0.3, 3.0 nmol) **(A)** and orexin-B (0.3, 3.0 nmol) **(B)** on plasma concentration of epinephrine and norepinephrine in conscious rats. 0 min, time of administration. All data are mean ± SEM; *n* is no. of animals, $^{*}p < 0.05$ vs preadministration values, $^{\dagger}p < 0.05$ vs orexin-A (0.3 nmol). (Results are from ref. *11*.)

responses induced by orexin-A are mediated by activation of the sympathetic nervous system. Moreover, intrinsic orexin participates in BP maintenance at basal conditions probably through activation of the sympathetic vasoconstrictor outflow, as demonstrated using orexin knockout mice *(26)*. The results indicate that endogenous orexins play an important role in cardiovascular and sympathetic regulation in the CNS.

3. OREXINS AND CARDIOVASCULAR AND SYMPATHETIC FUNCTIONS IN VITRO

The mRNA of two known orexin receptors (OX_1R and OX_2R) belongs to the G-protein-coupled receptor superfamily and has a proposed seven-transmembrane topology that is observed exclusively in the rat brain *(2)*. The OX_1R and OX_2R mRNAs are differentially distributed, with OX_1R mRNA being most abundant in the VMH, which plays an important role in the homeostatic regulation of body metabolism mediated through sympathetic nerves *(27)*, and OX_2R mRNA being predominantly expressed in the hypothalamic PVN. The preautonomic

parvocellular neurons of the PVN send long descending projections to several areas within the CNS that are known to be important in cardiovascular function *(28,29)*. These regions include the NTS, where baroreceptor and chemorecepor afferents terminate, and the vagal complex present in the dorsomedial medulla, the RVLM, which is probably the major site for generation of sympathetic tone for the vasculature, and the IML cell column of the thoraco lumbar spinal cord, which is the site of sympathetic preganglionic neurons (SPNs) involved in the regulation of HR and BP.

To examine whether orexins affect PVN neurons directly, the changes in membrane potential induced by application of orexins on the PVN neurons of a rat hypothalamic slice were studied *(30,31)*. About 70–80 % of magnocellular and parvocellular neurons in the PVN responded to orexin-A or -B. Orexin-A produced a depolarization accompanied by an increase in action potential firing in a dose-dependent manner and also produced increases in the excitatory postsynaptic current (EPSC) frequency and amplitude in magnocellular neurons *(31)*. The depolarizing effects of orexin-A on magnocellular neurons were blocked by tetrodotoxin (TTX) and the broad-spectrum glutamate antagonist kynurenic acid, indicating that these effects were the result of activation of a glutamate interneuron. Depolarization of parvocellular neurons induced by orexin-A were maintained in TTX, indicating the direct effects of orexin-A on this type of neuron *(31)*. A voltage-clamp study revealed that orexin-A induced a nonselective cationic conductance with a reversal potential of –40 mV in parvocellular neurons. Orexin-B also depolarizes both types of PVN neurons in a concentration-dependent manner *(30)* (Fig. 5). The depolarizing responses were greater in parvocellular than in magnocellular neurons. The effects of orexin-B persisted in the presence of TTX, indicating that these were direct effects. Addition of Cd^{2+} (1 m*M*) to artificial cerebrospinal fluid (ACSF) containing TTX significantly reduced the depolarizing effect in parvocellular neurons. The excitation of magnocellular neurons induces secretion of AVP from the posterior pituitary, antidiuresis, and vasoconstriction (Fig. 6). On the other hand, parvocellular neurons activate autonomic centers in the brainstem and spinal cord, increasing the HR and BP or causing the release of CRF. Secretion of ACTH from the posterior pituitary is controlled by CRF and AVP, synthesized by the parvocellular neuron.

These studies suggest that orexin-A and -B depolarize PVN neurons and increase the firing rate, leading to modulation of autonomic, cardiovascular, and neuroendocrine functions. In addition, dense orexinergic projections innervate the IML of the spinal cord. Orexin-A or -B directly and reversibly depolarized SPNs in spinal cord slices *(25)*. These findings indicate that orexins also excite sympathetic activity at the spinal level.

4. CONCLUSIONS

Orexins and hypocretins *(32)* were first characterized as stimulators of appetite and food consumption. Recently a number of reports have suggested additional roles for these peptides in the control of narcolepsy *(33)* and diverse autonomic functions, including cardiovascular control, hormone secretion, and energy metabolism. Orexins appeared to be involved in the modulation of autonomic and cardiovascular functions. Double-label immunohistochemistry confirmed that orexin-A and dynorphine-A peptides were highly colocalized in the LH *(34)*. Further experiments are needed to define the exact signal transduction of the orexin system in the CNS and the interaction with other appetite-regulating peptides, such as leptin *(35)* and dynorphin *(34)*.

The functional significance of the activation of cardiovascular and sympathetic function has not been established; several lines of evidence suggest that orexins may be activated

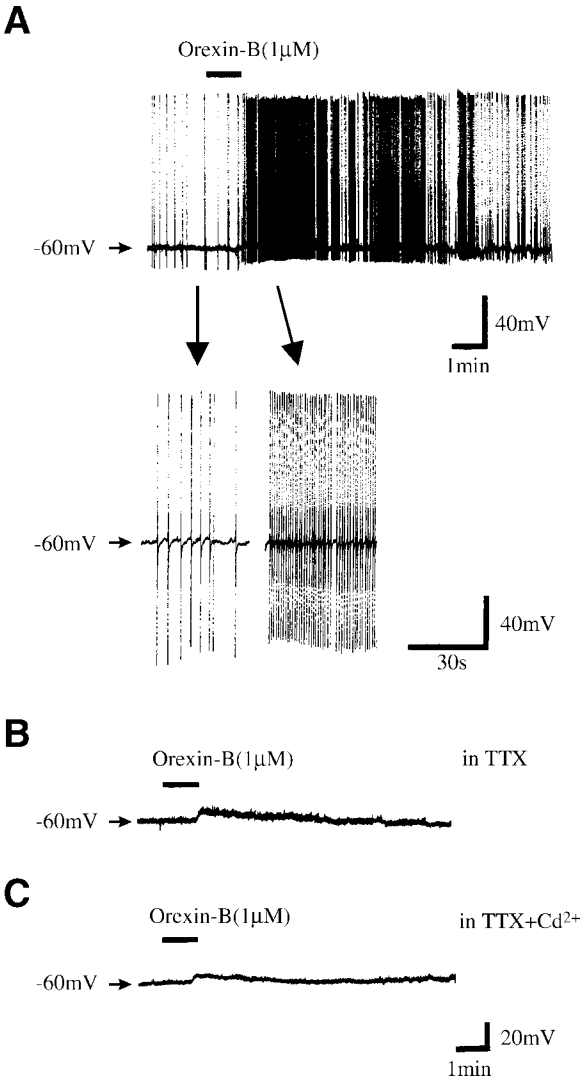

Fig. 5. The effects of orexin-B on membrane potential in hypothalamic paraventricular nucleus (PVN) neurons. Horizontal bars indicate the peptide application time (1 min). Arrows indicate the resting membrane potential (RMP). **(A)** Upper trace: The application of orexin-B (1μ*M*) induced a transient depolarization in magnocellular neurons in the PVN in normal artificial cerebrospinal fluid (ACSF). Lower left trace: Baseline firing at the RMP. Lower; right: Increased action potential frequency at the peak of the response to 1μ*M* orexin-B. **(B)** In the presence of tetrododoxin (TTX) (1 μ*M*), orexin-B (1 μ*M*) depolarized a parvocellular neuron in the PVN. **(C)** Addition of Cd^{2+} (1 m*M*) in ACSF containing TTX (1 μ*M*) significantly reduced the depolarization induced by orexin-B (1 μ*M*) in the same parvocellular neurons. (Results are from ref. *30*.)

under stress conditions and cause an increase in AP, HR, and SNA as an autonomic adaptive response. Orexins have a direct excitatory postsynaptic effect on PVN neurons, leading to diverse pathophysiological consequences, including autonomic and cardiovascular functions associated with the stress reaction (Fig. 6) *(36)*. Hypothalamic prepro-orexin mRNA expression has been found to be decreased in grossly obese animals with a defective leptin system *(37)* and increased by a high-fat diet, especially in those rats that become most obese *(38)*.

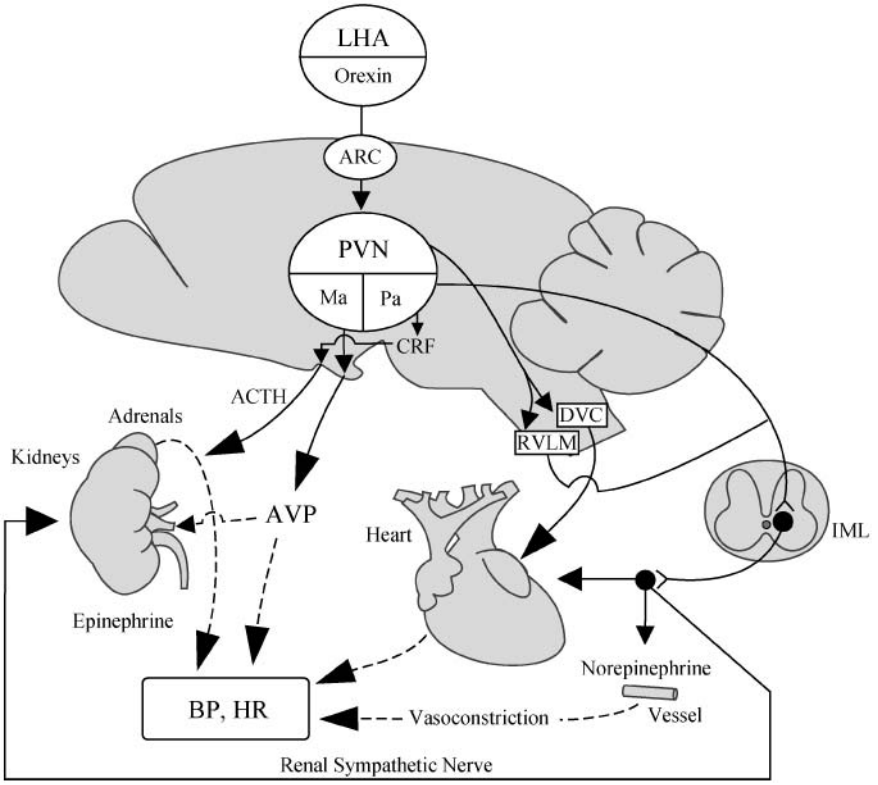

Fig. 6. Schematic diagram of possible mechanisms for the action of central orexins in cardiovascular, neuroendocrine, and sympathetic outflows. Orexins bind to their receptors of the magnocellular or parvocellular neurons of the hypothalamic paraventricular nucleus, or arcuate nucleus neurons, causing their depolarization. Excitation of magnocellular neurons induces secretion of arginine vasopressin (AVP) from the posterior pituitary, antidiuresis, and vasoconstriction. Conversely, parvocellular neurons activate autonomic centers in the brainstem and spinal cord, increasing the heart rate (HR) and blood pressure (BP), or causing the release of corticotropin-releasing factor (CRF). Secretion of adrenocorticotropic hormone (ACTH) from the anterior pituitary is controlled by CRF and AVP, synthesized by the parvocellular neurons. The right lower vessel indicates that norepinephrine released from the sympathetic nerve ending induces vasoconstriction. Activation of the renal sympathetic nerve induces antidiuresis and secretion of renin. The solid lines indicate a neural or humoral pathway. The dotted lines indicate a functional influence. ARC, arcuate nucleus; DVC, dorsal vagal complex; IML, intermediolateral cell column; LHA, lateral hypothalamus; PVN, paraventricular nucleus; Ma, magnocellular neuron; Pa, parvocellular neuron; RVLM, rostral ventrolateral medulla. (Reprinted from ref. *36*.)

These results are consistent with the orexin system participating in a counterregulatory response to obesity. Although the pathophysiological role of the sympathoexcitatory effects of orexins is not clear, the close relationship among obesity, hypertension, and altered cardiovascular responses has been documented in a number of studies *(39)*. Therefore, orexins may be the chemical mediators in the brain responsible for the generation and maintenance of hypertension that is associated with conditions of energy imbalance, such as obesity.

ACKNOWLEDGMENTS

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10
Neuroendocrine Role of the Orexins (Hypocretins)

Shahrad Taheri, MD, PhD

1. INTRODUCTION

Although the orexin (hypocretin) neuron cell bodies are exclusively located in the lateral perifornical hypothalamus (the classical "feeding center"), they send projections throughout the neuraxis *(1,2)*. This suggests that orexin neuropeptides and their receptors have functions beyond their originally observed effects on food intake and their currently accepted pivotal role in the regulation of sleep and wakefulness *(3–6)*. In particular, orexin fibers have been observed to innervate several brain, and specifically hypothalamic, regions that are intimately involved in the regulation of pituitary hormone secretion. Therefore, a link between the orexins and pituitary hormone secretion appeared highly likely from the outset. This view was underscored by the observed role of orexins in sleep regulation, since the secretion of some hormones is closely linked to circadian rhythms (e.g., corticotrophin, [ACTH]) and sleep (e.g., growth hormone).

The hypothalamus regulates the activity of endocrine organs through the release of releasing and release-inhibitory factors that act on the anterior pituitary to regulate hormone secretion (Fig. 1). The hypothalamus also directly releases the hormones vasopressin (antidiuretic hormone [ADH]) and oxytocin into the circulation through the posterior pituitary. The anterior pituitary hormones include the pro-opiomelancortin (POMC)-derived peptide ACTH, the glycoprotein hormones (luteinizing hormone [LH] follicle-stimulating hormone [FSH] and thyroid-stimulating hormone [TSH]), and the somatomammotrophin hormones (growth hormone [GH] and prolactin [PRL]). The orexins (hypocretins) have been reported to alter the secretion of all these hormones through various mechanisms. This chapter presents our current knowledge of the role of orexins in pituitary hormone secretion and alterations in orexin neurotransmission with changes in the hormonal milieu.

2. THE REPRODUCTIVE HORMONE AXIS AND LACTATION

Like feeding, the hypothalamic regulation of reproduction involves multiple circuits. Unlike circuits involved in feeding, however, the final target (hypothalamic luteinizing hormone-releasing hormone [LHRH] neurons) is more clearly defined. Control of the estrous cycle is complex and requires orchestration of multiple neurons (Fig. 2) and alterations in receptor number and receptor sensitivity. Patterns of neurotransmitter secretion are also important. The major event is the pituitary LH surge resulting in ovulation. Negative and positive feedback loops are involved. During most of the cycle, negative feedback from estradiol and progesterone on LHRH secretion predominates. At proestrous, positive feedback occurs to generate the pituitary LH surge and hence ovulation. This has been proposed to be an

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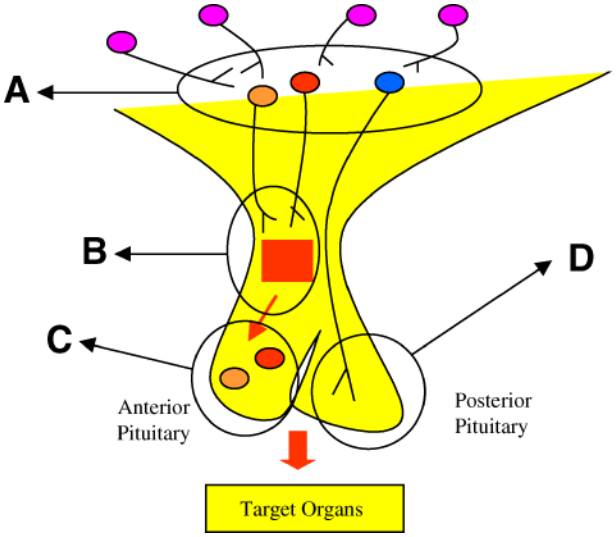

Fig. 1. Regulation of pituitary hormone release by the hypothalamus. The hypothalamic neurons involved in the release of pituitary control factors receive inputs directly from the circulation (e.g., where the blood-brain barrier is deficient or by changes in osmolality and temperature) or from neurons throughout the brain (A). Hypothalamic neurons release releasing and inhibitory hormones into the portal circulation (B) through which these factors act on pituicytes to influence pituitary hormone release (C). Neurohypophysial neurons project directly to the posterior hypothalamus, where they release oxytocin, arginine vasopressin (AVP; antidiuretic hormone [ADH]) and neurophysins directly into the circulation (D). Target organs for anterior pituitary hormones include the thyroid gland, adrenal gland, gonads, and liver.

indirect effect of estradiol feeding into other neurons that synapse with LHRH neurons. LHRH neurons are loosely located in the preoptic and septal regions, and, compared with other neurons, little of their surface area is occupied by synapses *(7)*. This suggests that any neuron synapsing on these cells, such as the orexin neuron, is likely to exert powerful effects.

Orexin neurons project to, and orexin immunoreactivity is high in, the hypothalamic and brain regions involved in the regulation of the reproductive hormonal axis *(1,8)*. The preoptic area and the septum, areas where LHRH neurons are predominantly located, are clear targets for orexin action. Orexin immunoreactivity is also high in the brainstem, where monoaminergic neurons that influence the hypothalamo-pituitary-gonadal (HPG) axis are densely located *(8)*. Administration of orexin-A into the lateral cerebral ventricle stimulated plasma LH in ovariectomized (OVEX) steroid-replaced rats, but inhibited plasma LH in OVEX unreplaced rats *(9)*. This ovarian steroid-dependent bimodal LH response to orexin-A is similar to that of other orexigenic neuropeptides, such as neuropeptide Y (NPY). Orexin-A suppressed the pulsatile secretion of LH via β-endorphin *(10)*. Orexin-A also restores the preovulatory LH surge suppressed by fasting *(11)*. Sex differences in regional hypothalamic orexin immunoreactivity suggest that the orexins are influenced by the gonadal steroid milieu *(8)*.

The orexins are therefore likely to play a role in the regulation of the HPG axis, and the gonadal hormone milieu may influence orexin action on the HPG axis. Changes in orexin immunoreactivity throughout the female estrous cycle and with gonadal hormone manipulation in both females and males have been studied *(12)*. Lower orexin-A peptide content was observed in hypothalami harvested from female rats at late proestrus (when estradiol levels

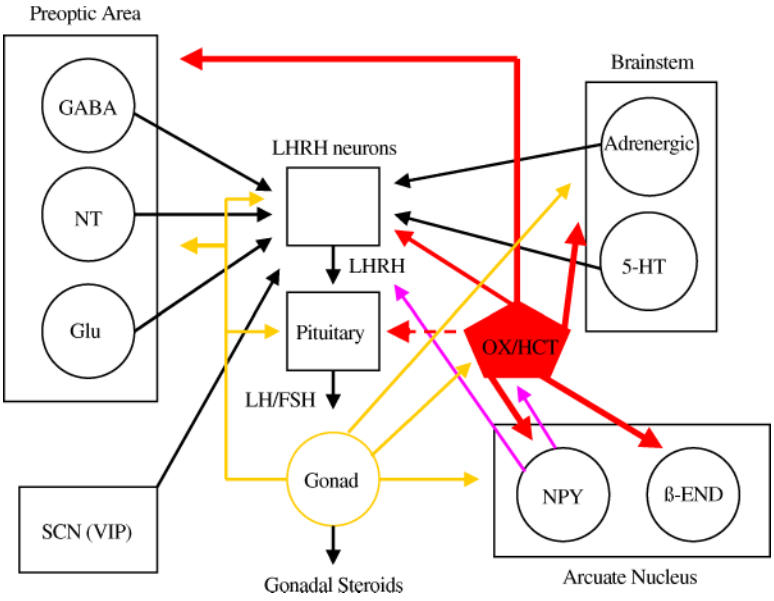

Fig. 2. Control of the hypothalamo-pituitary-gonadal (HPG) axis. The control of the HPG axis is complex and involves multiple neuronal circuits. The orexins may influence luteinizing hormone-releasing hormone (LHRH) secretion directly or indirectly via other neural pathways. These peptides may also act at the pituitary levels. Gonadal steroids are able to regulate orexin neurotransmission as orexin-A immunoreactivity changes throughout the estrous cycle and is altered by gonadal hormone manipulation. OX/HCT, orexin/hypocretin; SCN, suprachiasmatic nucleus; VIP, vasoactive intestinal peptide; NT, neurotensin; GABA, γ-aminobutyric acid; Glu, glutamate; 5-HT, serotonin; NPY, neuropeptide tyrosine (Y); β-END, β-endorphin.

are high) relative to all other stages of the cycle. These observations may reflect greatest release of orexin-A at proestrus, resulting in lower hypothalamic levels at late proestrus. Chronic hyperestrogenization also resulted in significantly lower orexin-A immunoreactivity in the hypothalamus relative to OVEX rats *(12)*. Compared with OVEX animals, no changes in peptide immunoreactivity were noted in any brain region after chronic progesterone replacement in OVEX rats. Progesterone action requires an estrogen background for progesterone receptor synthesis *(13)*. Hypothalamic orexin-A immunoreactivity was suppressed by castration and chronic testosterone treatment (Taheri, unpublished data). Therefore, both male and female gonadal hormones influence orexin neurotransmission.

Orexin-A increases LHRH release from hypothalamic explants at proestrous *(12)*. This provides further support for a role for orexins in stimulation of the LH surge. The effect on LHRH release could be mediated directly via LHRH neurons and through NPY, possibly via the NPY Y1 receptor, but this may be only one of the mechanisms involved. It is of interest that no changes in prepro-orexin mRNA were noted with gonadal hormone manipulation *(12)*. The discrepancy between orexin message and peptide content has been observed in multiple systems on multiple occasions.

Most studies investigating the role of orexins have concentrated on female rats, with a suppression of plasma LH being observed in ovariectomized rats, whereas a stimulation is observed in estrogen-primed female rats. It is possible that the orexins influence LH secretion in male rats, but this requires further investigation, including studies determining end-organ effect by measuring testosterone.

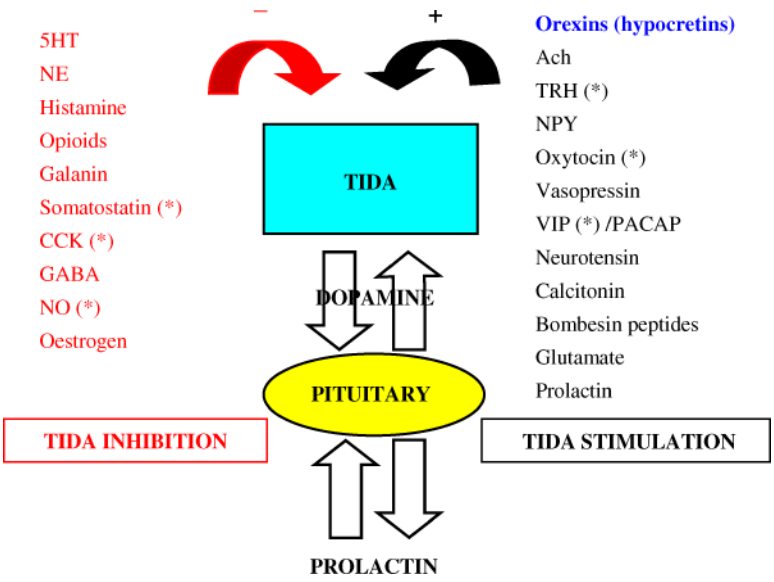

Fig. 3. Central factors that either stimulate or inhibit prolactin release via the tuberoinfundibular dopamine (TIDA) system. Pituitary prolactin release is under tonic inhibition from TIDA dopamine. (*) indicates independent effects at the pituitary levels that may be opposite to central effects. To date, direct innervation of the TIDA has not been reported. The inhibition of prolactin secretion by the orexins is not entirely reversed by the administration of the D_2 antagonist domperidone, suggesting that other pathways are involved. Orexins may affect multiple conflicting pathways. For example, they densely innervate adrenergic, histaminergic, and serotoninergic neurons that can increase prolactin secretion via the TIDA, while acting on neuropeptide Y (NPY) neurons may stimulate the TIDA and thus decrease prolactin secretion. Orexins may stimulate prolactin-inhibitory factors (PIFs) or inhibit prolactin-releasing factors (PRFs) that are independent of the TIDA. 5HT, serotonin; NE, norepinephrine; CCK, cholecystokinin; GABA, γ-aminobutyric acid; NO, nitric oxide; Ach, acetylcholine; TRH, thyroid-releasing hormone; VIP, vasoactive intestinal peptide; PACAP, pituitary adenylate cyclase-activating peptide.

Intracerebroventricular (icv) administration of orexins profoundly suppresses plasma prolactin in male rats *(14–16)*. Orexin-A was selected to investigate whether this effect occurred through the tuberoinfundibular dopaminergic (TIDA) system, which tonically inhibits prolactin secretion from pituitary lactotrophs *(15)*. The dopamine D_2 receptor antagonist domperidone was used to block pituitary D_2 receptors, and thus the inhibitory effect of the TIDA system on prolactin release. A high dose of domperidone (9 mg/kg) was chosen, aiming for full blockade of D_2 receptors. Even so, orexin-A still suppressed plasma prolactin. Therefore, at least part of the effect of orexin-A on plasma prolactin may be independent of the TIDA system.

Domperidone is a competitive antagonist; thus it is possible that the effect of domperidone could be overcome by orexin-A-induced dopamine release. However, domperidone-induced hyperprolactinemia activates the TIDA neurons as part of a negative feedback loop *(17)*. The TIDA may therefore already be fully activated to overcome the distal receptor blockade. Therefore, there may be no scope for orexin-A to suppress plasma prolactin through further activation of the TIDA. Unlike orexin-A, in the presence of dopamine antagonists, centrally administered NT, AVP, TRH, and atrial natriuretic peptide (Fig. 3) lose their prolactin-lowering effect, suggesting that these peptides act predominantly through the TIDA system. Hagan et al. *(14)* did not demonstrate any changes in hypothalamic dopamine turnover by orexin-A in vitro.

However, Hsueh et al. *(18)* observed that icv injection of NPY (10 μg) or orexin-A (1 μg) concomitantly increased median eminence DOPAC and decreased serum prolactin concentrations in estrogen-primed ovariectomized rats. A Y1 receptor antagonist, BIBP3226, blocked the effect of orexin-A, suggesting that the orexins act through NPY to suppress prolactin secretion.

An anatomical interaction between the orexins and the TIDA has not been reported. The orexins are likely to act at multiple levels to influence pituitary prolactin secretion. A direct effect at the pituitary level is possible, but orexin A does not influence TRH-stimulated pituitary prolactin release in vitro. Since suppression of plasma prolactin by orexins still occurs in the face of TIDA inhibition, orexins may also act through other prolactin inhibitory factors (PIFs) that are independent of TIDA. One candidate is γ-aminobutyric acid (GABA). Lactotrophs express GABA receptors, and GABA is inhibitory to PRL release from the pituitary *(19)*. Since most reports have suggested that the orexins are stimulatory, inhibition of a PRL-releasing factor is less likely.

Two aspects of the action of the orexins on plasma PRL should be highlighted. One is the interaction with sleep and the other is lactation. It is well established that the secretion of PRL is highly dependent on sleep *(17)*. Plasma PRL concentrations are highest during sleep and lowest during wakefulness. Thus, sleep and circadian rhythmicity interact in determining the temporal organization of PRL secretion during the day. High PRL levels, in turn, increase the duration and frequency of REM sleep. The reduction in plasma PRL with orexin administration may therefore be related to its effects on increasing arousal and reducing REM sleep.

Lactation is an important period in terms of metabolic demands. During lactation, energy demands are increased, initiating increased food intake *(20,21)*. Despite increased food consumption, animals are placed marginally in a state of negative energy balance. The mechanism for the increased food intake in lactation is unknown, but leptin is suppressed and may thus influence hypothalamic mechanisms *(22)*. NPY neurons in the arcuate nucleus are an important target for leptin. One mechanism whereby NPY neurons may affect lactation is through Y4 receptors in the lateral hypothalamus. Interestingly, orexin, but not melanin-concentrating hormone (MCH) neurons, have been shown to have Y4 receptors and may be substrates for NPY's role during lactation *(23)*. Cai et al. *(24)* studied the effects of lactation and food restriction in lactating rats on prepro-orexin mRNA and immunoreactive peptide levels. No significant difference was observed in mRNA levels in these states compared with controls. However, food restriction in lactating rats resulted in significantly elevated hypothalamic levels of orexin-B. They suggested that that orexin-B may be more important in fasting during lactation than orexin-A.

The effects of knocking out/overexpressing the prepro-orexin gene or orexin receptor genes on reproductive function have not been reported. No breeding problems have been reported in the colony of narcoleptic dogs at Stanford University that have mutations in the orexin-2 receptor (OX_2R) receptor gene. There are no reports of reduced reproductive function, delayed puberty, or infertility in patients with narcolepsy despite evidence that most of these patients have undetectable orexin-A in their cerebrospinal fluid (CSF). The significance of lower LH in narcoleptic men is at present unclear *(25)*. The extent of involvement of the orexins in human reproductive function therefore remains to be determined.

3. THE THYROID HORMONE AXIS

The effects of central orexins on TSH secretion have not been investigated in detail. Mitsuma et al. *(26)* administered orexin-A (50 μg/kg iv) and observed a dose-dependent decrease in plasma TSH. They also observed a reduction in hypothalamic TRH secretion in

response to orexin-A and suggested a hypothalamic mechanism for the action of systemic orexin-A since no pituitary effect was observed. No significant changes in TRH secretion were observed when orexin-A was administered to hypothalamic explants *(27)*. It is possible that the effects of icv orexins on TSH secretion are time dependent, since there was suppression of plasma TSH by icv orexin-A at 10 min, but at no other time point, until 90 min, when there was again a suppression in plasma TSH *(28)*. There were no effects on pituitary hormone secretion with chronic intra-paraventricular nucleus (iPVN) injections of orexin-A. Since orexins also stimulate the stress axis, it is possible that any suppression of plasma TSH by orexins is mediated by glucocorticoids. Jones et al. *(16)* administered increasing doses of icv orexins and studied plasma TSH 40 min after. They did not observe any effects on TSH by orexin-A but observed increases in plasma TSH induced by orexin-B. This is surprising because most feeding studies fail to show any effect of icv orexin-B on feeding, and kinetic studies suggest that orexin-B is unstable when administered icv *(29)*. It would be unexpected that orexin-B will last long enough to produce an effect at 40 min, unless the TSH changes observed are a consequence of a more immediate effect. In this case, it would be expected for orexin-A to produce a more pronounced effect based on receptor potency and greater stability.

TRH neurons are located in the paraventricular nucleus where the presence of the orexin-2, but not orexin-1 receptor mRNA has been reported *(30)*. The presence of OX_2R or OX_1R receptors specifically on TRH neurons and orexin (hypocretin) projections to TRH neurons have not been reported. Nevertheless, an interaction between orexins and the thyroid axis is intriguing and requires further study since thyroid hormones are important in the regulation of energy expenditure and catecholaminergic responses and, the orexins also influence these systems. No changes in prepro-orexin mRNA or orexin receptor levels with medical thyroidectomy or hyperthyroidism, however, have been observed *(31)*. The effects of thyroid hormone manipulations on peptide content and release have not been reported.

Since the orexins have been shown to have profound effects on sleep, wakefulness, and locomotor activity, changes in orexin levels may parallel or oppose the effects of low thyroid hormone status on these parameters. A reduction in spontaneous motor activity has been observed in hypothyroid rats, which agrees with low orexin action *(32)*. In humans, however, sleep stages 3 and 4 and REM sleep may be markedly shortened or absent in hypothyroidism, which agrees with increased orexin action *(33)*. The sleep effects of hypothyroidism in humans have not been observed in the rat; however, there were more frequent awakenings in slow-wave sleep in hypothyroid rats *(34)*. Interestingly, one severely hypothyroid patient whose CSF orexin-A levels were measured by Mignot et al. *(35)* had low levels. A larger human CSF study is necessary to substantiate this observation. There is no known association between narcolepsy and thyroid function, but subtle abnormalities cannot be excluded. The interaction between the orexins and the thyroid axis are therefore complex and require further investigation.

4. THE HYPOTHALAMUS-PITUITARY–ADRENAL (HPA) AXIS

The stress response mediated through activation of the HPA axis is essential to meet physical and psychological challenges. The HPA axis consists of corticotrophin-releasing factor/hormone (CRF/CRH) and arginine vasopressin (AVP) neurons in the hypothalamus, which stimulate adrenocorticotrophic hormone (ACTH) secretion from corticotrophs in the anterior pituitary through CRF-R1 and V1b receptors, respectively. ACTH, in turn, stimulates adrenal cortical glucocorticoid secretion. Negative feedback from glucocorticoids completes

the classic endocrine feedback loop. CRH and AVP neurons are located in the parvocellular hypothalamic PVN. CRH appears to be more involved in acute stress, whereas AVP is more important in chronic stress *(36)*. The HPA axis is under control of the hypothalamic suprachiasmatic nucleus (SCN), which produces the circadian rhythm in cortisol (corticosterone in rats) secretion. Stress is relayed to the PVN from peripheral nerves via neurons in the brainstem.

A number of data suggest that the orexins influence the HPA axis; a direct interaction on CRH and AVP neurons is likely because:

1. The orexin-2 but not orexin-1 receptor is expressed in the PVN *(30)*. Interestingly Backberg et al. *(37)* have reported that OX_1R immunoreactivity is present in CRH and AVP neurons. The reason for the discrepancy between receptor gene expression and immunoreactivity is unclear.
2. Administration of orexins icv dose-dependently increased plasma ACTH and corticosterone *(14,38)*. This was blocked by pretreatment with the CRH antagonist α-helical CRH *(38)*. The actions of icv orexins on the HPA axis were also blocked by NPY antiserum *(39)*. The stimulation of ACTH by icv orexin-A was observed to peak at 30 min and was maintained over 120 min, returning to baseline at 240 min post injection *(40)*.
3. Orexins administered icv induced Fos mRNA in the PVN *(41)*.
4. Orexin-A increased CRH release from hypothalamic explants. This was blocked by the NPY Y1 receptor antagonist BIBP3226 *(27)*. No effects of orexin-A on AVP release from hypothalamic explants was observed.
5. Orexin-A depolarized and increased spike frequency in magno- and parvocellular PVN neurons in hypothalamic slice preparations *(42)*.
6. The behavior effects of orexin-A such as grooming were reversed by the CRH antagonist α-helical CRF *(43)*.
7. Orexin-A administered icv significantly increased CRH and AVP mRNA in the PVN *(40)*.

The orexins are therefore likely to be involved in the stress response, through direct effects on PVN CRH and AVP neurons, but also indirectly through brainstem and hypothalamic neurons (Fig. 4). It is well known that different stressors activate the stress axis through different mechanisms. Ida et al. *(44)* have reported that immobilization stress, increased prepro-orexin mRNA levels in 2-mo-old rats, and cold stress increased prepro-orexin mRNA levels in 6-mo-old rats *(44)*. Zhu et al. *(45)* have reported increased Fos protein in orexin neurons after noxious stimuli, but not after conditioned fear stimuli.

It is possible that the orexins affect the HPA axis by stimulating arousal or influencing circadian input to the HPA axis. Part of the arousal response to orexins may be mediated by activation of the HPA axis. The temporal basis for the orexins to influence plasma corticosterone physiologically has been little studied, but changes in cortisol were not correlated with CSF orexin-A in the squirrel monkey model (*46*).

Most patients with narcolepsy-cataplexy have a deficiency in orexin neurotransmission *(2,47,48)*. Higuchi et al. *(49)* studied four male narcoleptics and found that the circadian periodicity of cortisol secretion was normal. The numbers in this study were, however, small, and it is unknown whether the patients were orexin deficient. Kok et al. *(50)* studied cortisol secretion in seven male orexin-deficient patients. They reported a blunted basal and total ACTH production in narcoleptics, but no difference in basal pulsatile cortisol secretion. The early morning rise in ACTH and cortisol was not different between patients and controls, suggesting an intact circadian control of the HPA axis. Whether these observations, have any practical implications in narcolepsy and other disorders remains to be studied. However, they suggest that the observed effects of the orexins on the HPA axis are not through alterations in circadian control mechanisms.

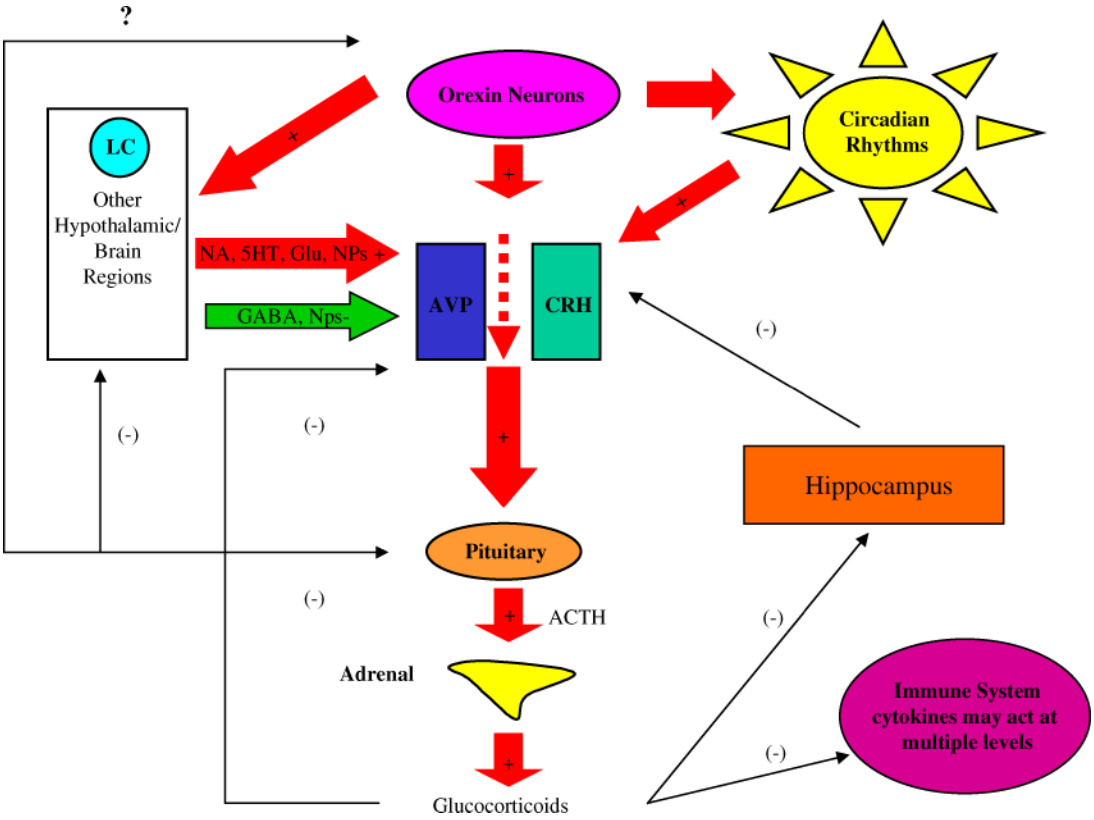

Fig. 4. Control of the hypothalamo-pituitary-adrenal (HPA) axis and the role of the orexins. Corticotropin-releasing hormone (CRH) and arginine vasopressin (AVP) neurons release their hormones into the portal circulation. CRH and AVP then stimulate adrenocorticotropic hormone (ACTH) secretion by pituitary corticotrophs, which, in turn, stimulates adrenocortical glucocorticoid secretion. Glucocorticoids feed back at pituitary and hypothalamic levels to reduce ACTH, and CRH and AVP secretion respectively. Glucocorticoids also act on the hippocampus, which acts on the hypothalamus to reduce CRH and AVP. Other influences include stimulation by noradrenergic (NA), serotoninergic (5HT), and glutamatergic (Glu) inputs. Inhibition occurs via γ-aminobutyric (GABA) ergic inputs. A number of neuropeptides may stimulate or inhibit HPA axis activity. The orexins may influence HPA activity directly or indirectly via brainstem (e.g., locus coeruleus [LC] noradrenergic) and hypothalamic (e.g., NPY) circuits, and/or via altering the circadian influences on HPA axis activity. The role of negative feedback onto orexin neurons by glucocorticoids is not completely determined. The immune system can influence the stress axis at multiple junctions.

Adrenalectomy did not alter orexin immunoreactivity in the hypothalamus (Taheri, unpublished data). Stricker-Krongrad and Beck *(51)* have, however, reported that with adrenalectomy there is a 50% decrease in lateral hypothalamic prepro-orexin mRNA detected by *in situ* hybridization. This returned to normal with dexamethasone administration. The reason for the discrepancy between peptide and mRNA levels is unclear.

5. GROWTH HORMONE SECRETION

Orexins administered icv significantly suppress plasma GH *(14)*. The major control of GH secretion from pituitary somatotrophs is via hypothalamic growth hormone-releasing hormone (GHRH; stimulatory) and hypothalamic somatostatin (inhibitory). The mechanisms of the

inhibitory effect of the orexins on GH have not been studied but are likely to involve GHRH and somatostatin *(52)*. One study reported increased somatostatin release from hypothalamic explants in vitro with administration of orexin-A *(15)*. GH is normally released during stage 4 sleep in humans. Therefore, the suppression in GH secretion may be owing to the induction of wakefulness by the orexins. Another possibility is that GH suppression is secondary to activation of the stress axis. Unlike humans, in rats GH is suppressed with stress. Paradoxically, Higuchi et al. *(49)* reported suppression of sleep GH secretion in narcolepsy. Also, Clark et al. *(53)* have reported a marked reduction in GH secretion in response to oral L-DOPA in narcolepsy and reduced sleep-related PRL release in women with narcolepsy. In patients with documented orexin deficiency, basal and pulsatile GH secretion rate and secretagogue-induced GH release were not different from controls. As expected, with increased daytime sleepiness, significant secretion of GH occurred during the daytime in subjects with narcolepsy *(54)*.

Preliminary data from orexin-overexpressing mice are available albeit in abstract form *(55)*. These mice, which have the orexin gene overexpressed in the CNS and also peripherally, are smaller than wild-type controls. This is consistent with the inhibitory effects of icv orexins on GH secretion, suggesting that there may be an important interaction requiring further investigation.

6. DIRECT PITUITARY ACTIONS

Date et al. *(56)* have detected orexin immunoreactivity in pooled pituitaries by radioimmunoassay. They also observed immunoreactivity in the median eminence. Using immunocytochemistry, immunoreactive orexin fibers were detected in the pituitary. It is likely that the orexins play a role in pituitary function because of the presence of both immunoreactivity and receptors in rodents and humans *(57)*. Interestingly, studies looking at the effect of the orexins on pituitary function have reported inhibitory actions such as inhibition of LHRH-stimulated LH release in females at proestrous and inhibition of CRH-stimulated (but not basal) ACTH release. Samson and Taylor *(58)* did not observe any effects on basal or stimulated GH, PRL, TSH, or LH in vitro. The observed inhibitory effects are opposite to the central effects of the orexins, which are mainly excitatory *(58)*.

7. CONCLUSIONS AND FUTURE DIRECTIONS

The orexins play multiple roles in the central control of pituitary hormone secretion. Orexin neurotransmission is also altered by changes in the organism's hormonal milieu. Some of the endocrine effects of the orexins are clearly related to their actions in sleep and appetite regulation. More studies are necessary to further define the physiological neuroendocrine roles of the orexins. A large number of gene-manipulated animal models are now available for study *(2)*. Investigation of these models in combination with the use of specific receptor agonists and antagonists will clarify the neuroendocrine role of the orexins. Human studies will determine the contribution of orexin abnormalities to sleep disorders associated with endocrine dysfunction.

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11

The Orexin/Hypocretin System and Stress and Emotion

Yoichi Ueta, MD, PhD, and Hiroaki Fujihara, PhD

1. INTRODUCTION

Stress induces changes in higher nervous functions such as depression, sleep/wakefulness, and feeding behaviour. It also induces emotional changes such as fear as well as changes in autonomic nervous functions such as increased heart rate and arterial blood pressure and endocrine responses, in particular activation of the hypothalamo-pituitary-adrenal (HPA) axis.

Many investigators have convincingly reported that orexin-A and -B (also known as hypocretin-1 and -2) are involved in stress-induced responses in the whole body. Orexin-A and -B-producing neurons in the lateral hypothalamic area (LHA) and surrounding areas project their axons to various brain regions, for example the paraventricular nucleus (PVN), the locus coeruleus (LC), and the raphe nucleus, which are involved in stress-induced physical and psychological responses *(1–3)*. This review explores the role of orexin-A and -B as stress-response mediators in the central nervous system (CNS).

2. EFFECTS OF STRESS ON OREXIN-A-PRODUCING NEURONS

Accumulating evidence suggests that various kinds of stressors activate orexin-A-containing neurons with increased transcripts of the *prepro-orexin* gene *(4–9)*. Central administration of orexin-A and -B regulates feeding behavior *(4)*. Thus, the effects of feeding-related stressors such as fasting and hypoglycemia on orexin-A-containing neurons were examined by using molecular techniques such as Northern blot analysis and *in situ* hybridization histochemistry for *prepro-orexin* mRNA or immunohistochemistry for Fos; Fos protein and c-*fos* mRNA are widely used as markers of neuronal activity in the CNS.

In rats, Northern blot analysis showed that fasting for 48 h upregulated hypothalamic *prepro-orexin* mRNA *(4,10)*. Hypoglycemia induced by intraperitoneal (ip) administration of insulin increased *prepro-orexin* mRNA in the LHA *(5)*. On the other hand, glucodeprivation induced by ip administration of 2-deoxy-D-glucose (2-DG) either decreased *prepro-orexin* mRNA levels *(11)* or had no effects *(10,12)*. Dual immunohistochemical staining for Fos and orexin-A was performed after similar stimuli. Hypoglycemia induced by ip administration of insulin induced Fos expression in up to 33% of orexin-A-containing neurons *(6)*. Administration ip or icv of 2-DG induced Fos expression in orexin-A-containing neurons (*7,13,14*; Fig. 1). Similar Fos expression induced by food restriction was also observed in orexin-A-containing neurons (*7*; Fig. 2), suggesting that orexin-A-containing neurons in the LHA are glucose sensitive.

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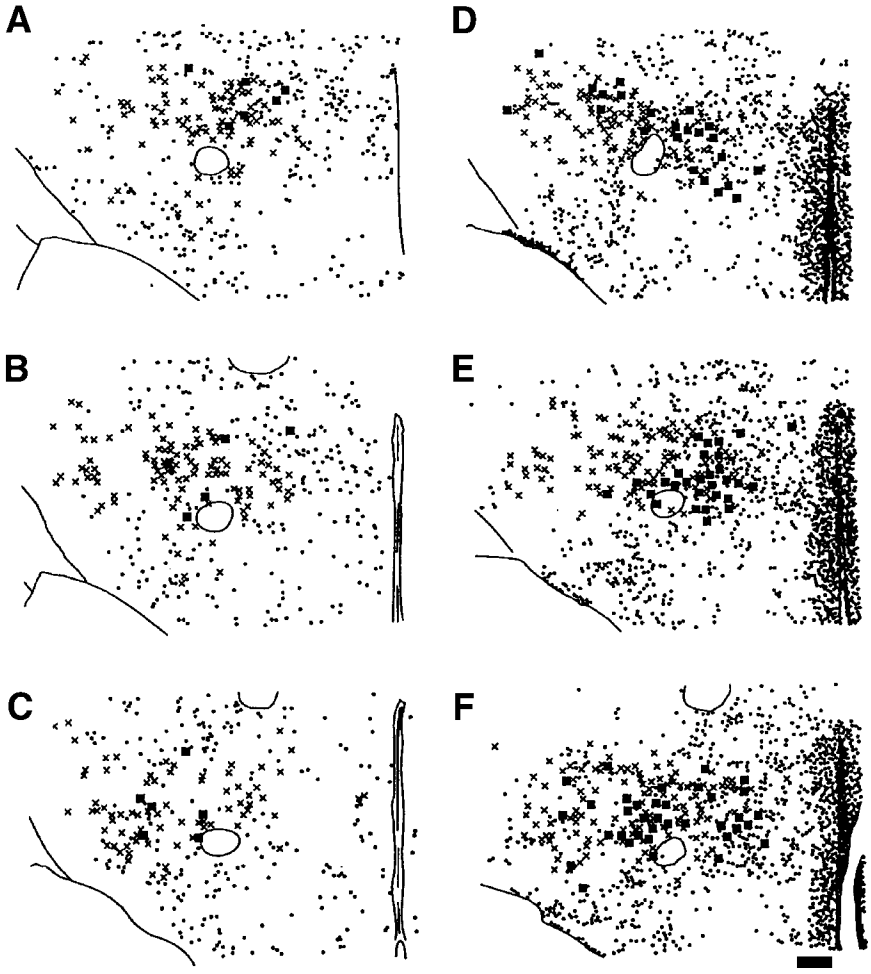

Fig. 1. Localization of neurons containing Fos-like immunoreactivity (LI) and orexin-A-LI 90 min after intracerebroventricular (icv) administration of vehicle (**A–C**) or 2-deoxy-D-glucose, (8 mg/rat; **D–F**). Fos-LI, orexin-A-LI cells, and colocalized cells are indicated by dots, crosses, and filled squares, respectively. Sections were taken from the rostral to caudal regions of the hypothalamus, including the lateral hypothalamic area. Scale bar = 200 µm. (Data from ref. *7*.)

It is well known that stress stimuli modulate food intake *(15)*. For example, noxious stimuli cause increased food intake, and emotional stimuli suppress feeding. Zhu et al. *(16)* clearly demonstrated that noxious but not conditioned fear stimuli caused Fos expression in almost all orexin-A-containing neurons. In addition to feeding-related stress, it has been reported that other stressors cause changes in *prepro-orexin* mRNA levels and induction of Fos expression in orexin-A-containing neurons. Cold and immobilization stress caused elevation of *prepro-orexin* mRNA levels in the LHA *(8)*. Sakamoto et al. *(9)* demonstrated that cold exposure at 4°C for 30 min or immobilized stress for 20 min caused Fos expression in approximately 15 and 24% of orexin-A-containing neurons, respectively (Figs. 3 and 4).

In general, stress activates the HPA axis, and orexins may be involved in regulation of this axis because adrenectomy (ADX) strongly reduced *prepro-orexin* mRNA levels in the LHA, which were restored to normal by peripheral replacement with dexamethasone in ADX *(17)*.

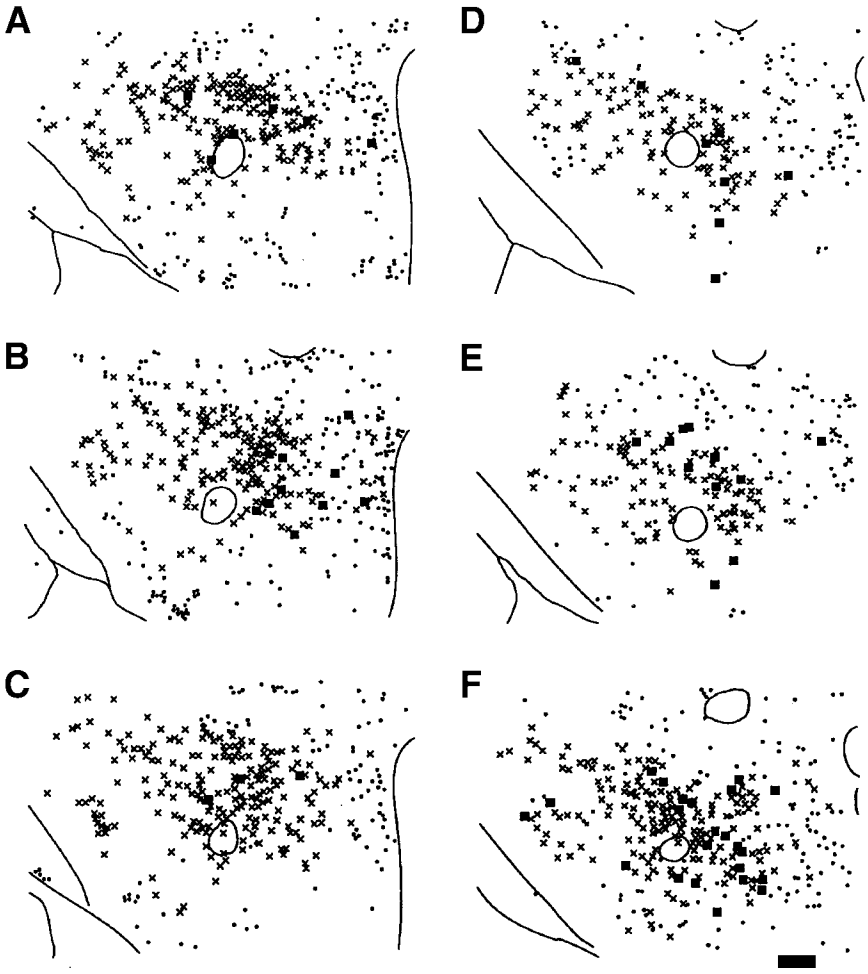

Fig. 2. Localization of neurons containing Fos-like immunoreactivity (LI) and orexin-A-LI in control rats (**A–C**) or rats food-restricted for 3 wk (**D–F**). Fos-LI, orexin-A-LI cells, and colocalized cells are indicated by dots, crosses, and filled squares, respectively. Sections were taken from the rostral to caudal levels of the hypothalamus, including the lateral hypothalamic area. Scale bar = 200 μm. (Data from ref. *7*.)

Expression of the *prepro-orexin* gene was unaffected by short-term sleep deprivation in both rats and mice *(18)*. Selective REM sleep deprivation also did not affect expression of the *prepro-orexin* gene in rats *(19)*. However, orexin-A-containing neurons exhibited Fos during the night period. Sleep deprivation or treatment with methamphetamine increased Fos expression in orexin-A-containing neurons *(20)*, and modafinil, a wake-promoting drug used for narcolepsy, induced marked increases of Fos expression in orexin-A-containing neurons *(21,22)*. There is no doubt that activation of orexin-A-containing neurons is involved in promoting or maintaining wakefulness.

3. POSSIBLE INVOLVEMENT OF OREXINS IN STRESS RESPONSE

Anatomical studies have demonstrated that the orexin type-2 receptor gene is abundantly expressed in the parvocellular division of the rat PVN, which is known to localize neurons that produce corticotropin-releasing hormone (CRH) *(23,24)*. There are also abundant

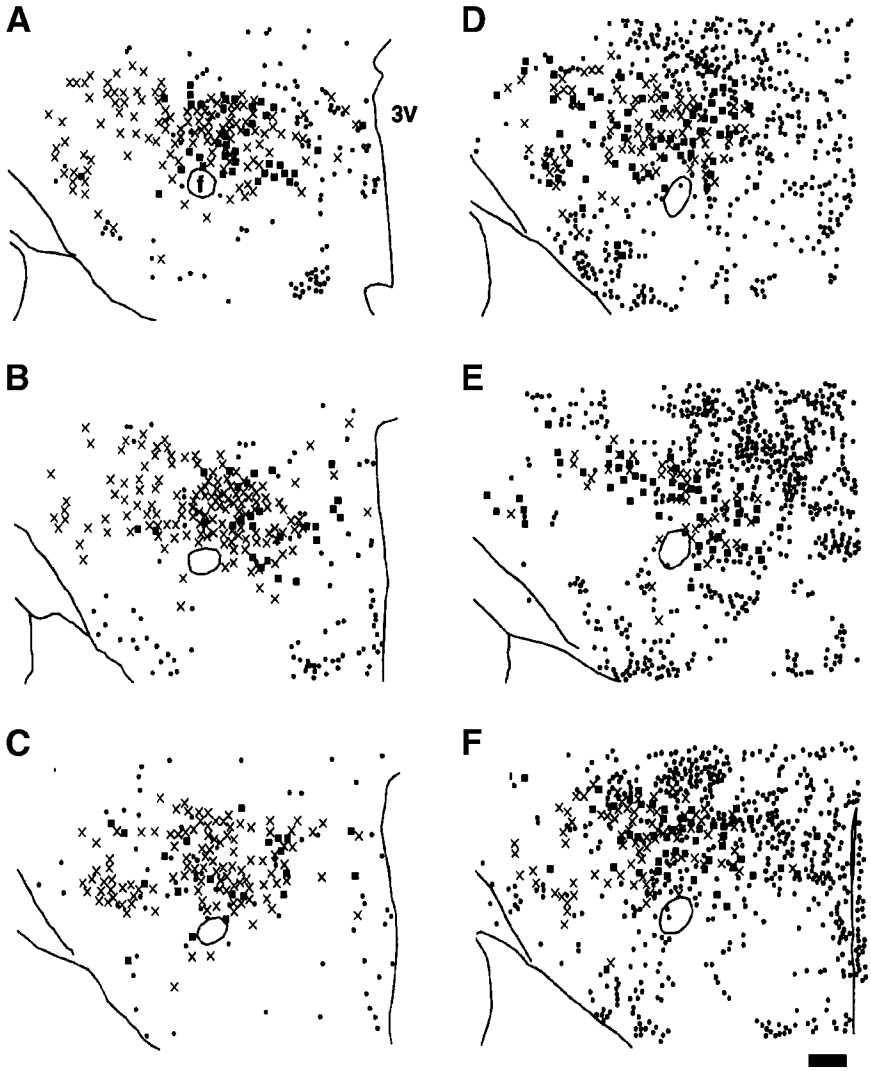

Fig. 3. Localization of neurons containing orexin-A-like immunoreactivity (LI) and Fos-LI in control rats (**A–C**) or rats 90 min after cold exposure for 30 min (**D–F**). Fos-LI, orexin-A-LI, and these colocalized cells are indicated by dots, crosses, and filled squares, respectively. Sections were taken from the rostral to caudal levels of the hypothalamus, including the lateral hypothalamic area. Scale bar = 200 μm. 3V, third ventricle; f, fornix. Coronal sections were cut at 30 μm thickness. (Data from ref. *9*.)

orexin-A-immunopositive fibers around the PVN (*1–3*). In vitro electrophysiological studies have demonstrated that bath application of orexin-A and -B caused depolarization in magno- and parvocellular neurons in the rat PVN *(25,26)*. In vivo studies have confirmed that central administration of orexin-A and -B activates stress-related responses such as the HPA axis, cardiovascular responses, and gastrointestinal reactions.

Intracerebroventricular (icv) administration of orexin-A and -B caused significant secretion of adrenocorticotropic hormone (ACTH) and elevation of plasma corticosterone *(8,26–32)* and produced marked induction of Fos in the parvocellular neurons that are putative CRH-containing neurons *(1,33,34)*. These findings were confirmed by *in situ* hybridization

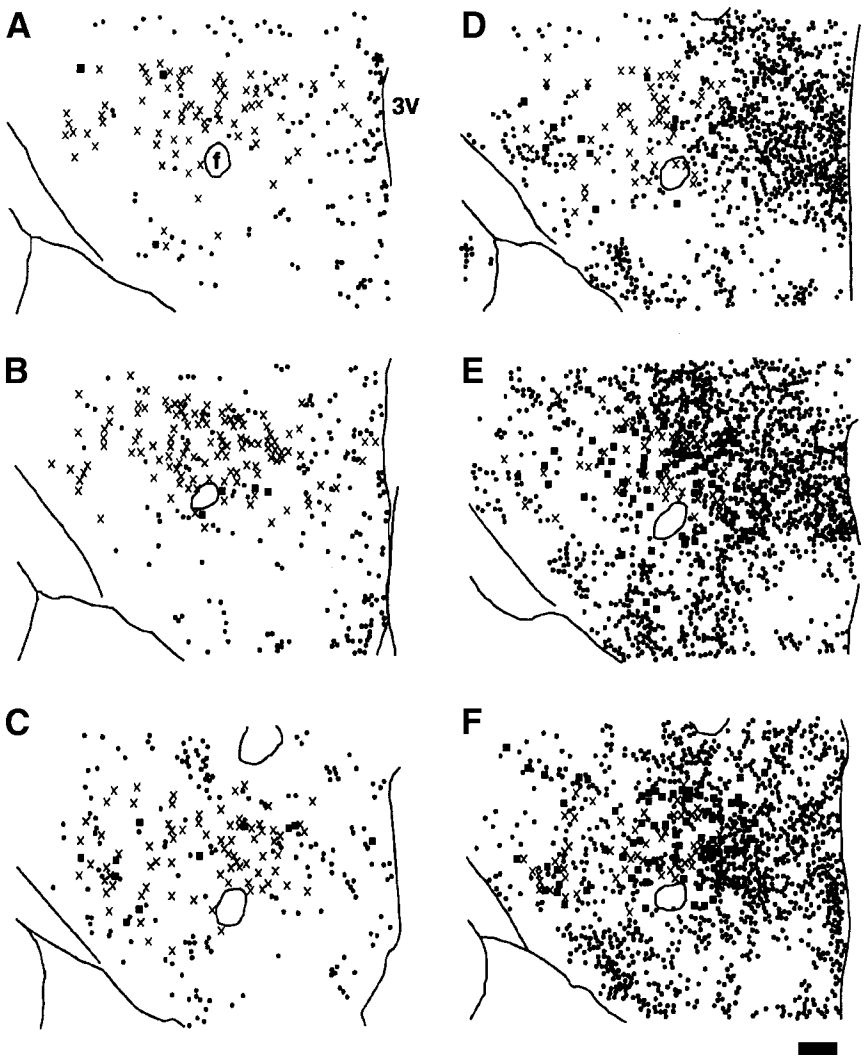

Fig. 4. Localization of neurons containing orexin-A-like immunoreactivity (LI) and Fos-LI in control rats (**A–C**) or rats 90 min after immobilized stress for 20 min (**D–F**). Fos-LI, orexin-A-LI, and these colocalized cells are indicated by dots, crosses, and filled squares, respectively. Sections were taken from the rostral to caudal levels of the hypothalamus, including the lateral hypothalamic area. Scale bar = 200 μm. 3V, third ventricle; f, fornix. Coronal sections were cut at 30 μm thickness. (Data from ref. *9*.)

histochemistry for c-*fos* mRNA: icv administration of orexin-A induced expression of the c-*fos* gene in the parvocellular division of the PVN in a dose- and time-dependent manner. The pattern of induction of the c-*fos* gene was similar to that of ACTH release *(27)*. To identify determine whether Fos-positive cells are CRH-containing neurons in the parvocellular division of the PVN, dual immunostaining for Fos and CRH was performed *(9)*. The results revealed that almost all CRH-containing neurons in this region exhibited Fos-like immunoreactivity (LI) after icv administration of orexin-A (*9*; Fig. 5). Interestingly, 45% of CRH-containing neurons in the central amygdaloid nucleus (CeA) also expressed Fos-LI after administration of orexin-A (*9;* Fig. 5). It should be noted that the CeA as well as the PVN have been shown to be involved in the neural circuits that cause stress-induced responses.

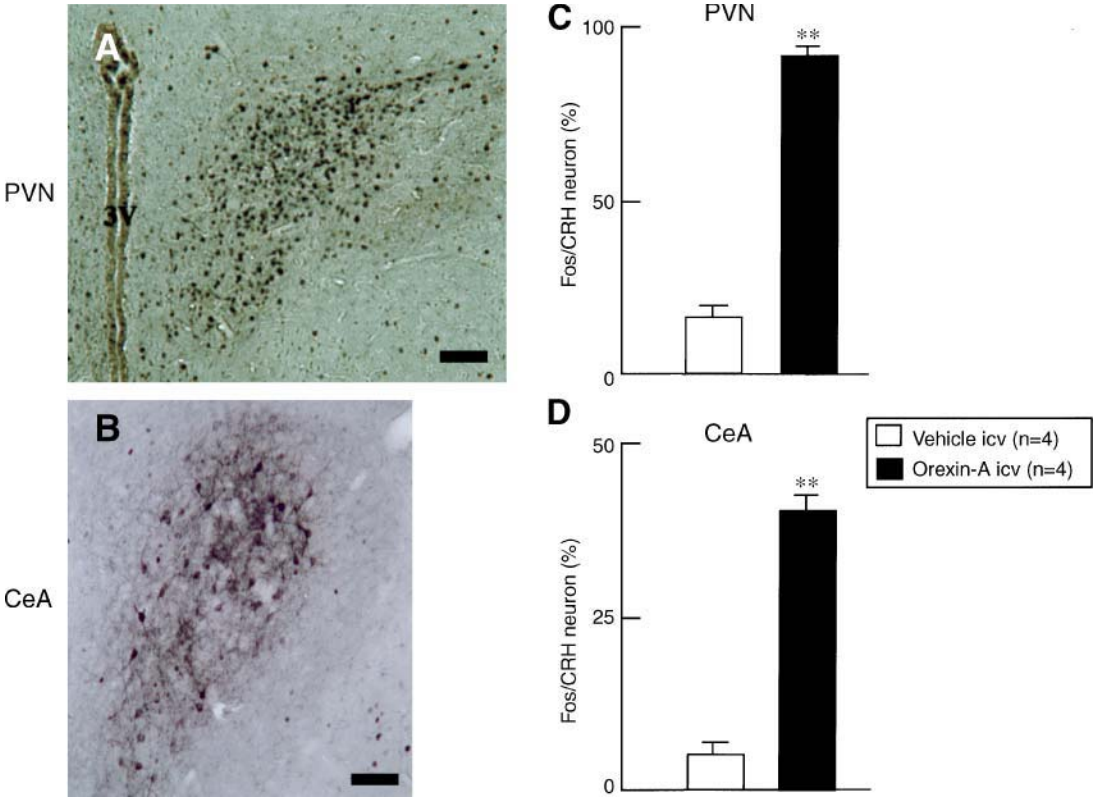

Fig. 5. (A) Dual immunostaining of corticotrophin-releasing hormone (CRH)-like immunoreactivity (LI) and Fos-LI in the rat paraventricular nucleus (PVN) 90 min after icv administration of orexin-A (30 μg/rat). **(B)** Dual immunostaining of CRH-LI and Fos-LI in the rat central amygdaloid nucleus (CeA) 90 min after icv administration of orexin-A (30 μg/rat). Scale bar = 50 μm. **(C),(D)** Percentage of Fos-LI cells in CRH-LI cells 90 min after icv administration of saline (vehicle) or orexin-A (30 μg/rat) in the PVN and the CeA. n = number of rats. Significance level(:) **, $p < 0.01$. (Data modified from ref. *9*.)

Studies have been performed to examine the mediators that activate the HPA axis after central administration of orexin-A and -B in rats. The secretory responses of ACTH and corticosterone were significantly suppressed by preadministration of the CRH antagonist α-helical CRH9-41 *(26,28)*. They were also attenuated by preadministration of neuropeptide Y (NPY) receptor antagonists and anti-NPY antibodies *(29,32,35)*. These results suggest that centrally administered orexin-A and -B activate CRH-producing neurons directly or through NPY and that secreted CRH stimulates the release of ACTH from the anterior pituitary.

According to stress-related behavioral changes, some studies have reported that icv administration of orexin-A and -B in conscious rats increased face-washing, grooming, and exploratory behavior *(8,30)*. These behaviors are significantly inhibited by preadministration of α-helical CRH9-41 *(8,35)*.

4. OREXIN-A AND -B: STRESS-RESPONSE MEDIATORS

Recent studies have indicated that central orexin-A and -B may mediate neuroendocrine, autonomic, and behavioral changes in response to stress. Centrally administered orexin-A and -B activate CRH-producing neurons and the HPA axis *(1,8,25–33,36)*. In rats, icv

Table 1
Effects of Various Stressors on Orexin-A-Containing Neurons and *Prepro-orexin* mRNA

Stressor (ref.)	Fos-LI/orexin cells (%)	Prepro-orexin mRNA level
Fasting *(4,10)*		Increased
Insulin-induced hypoglycemia *(5)*		Increased
2-DG-induced hypoglycaemia *(10–12)*		Decreased or no change
Insulin-induced hypoglycaemia *(6)*	8–33	
2-DG-induced hypoglycaemia *(7,13,14)*	26–55	
Food restriction *(7)*	22	
Noxious stimuli *(16)*	90	
Conditioned fear stimuli *(16)*	No induction	
Cold stress *(8,9)*	15	Increased
Immobilization stress *(8,9)*	24	Increased
Adrenectomy *(17)*		Decreased
Short-term sleep deprivation *(18)*		No change
Selective REM sleep deprivation *(19)*		No change
Sleep deprivation *(20)*	32–36	
Methamphetamine treatment *(20)*	50	
Modafinil treatment *(21,22)*	64	

administration of orexin-A and -B causes changes in autonomic functions such as sympathetic outflow *(36–38)*, cardiovascular function *(36–38)*, and gastric acid secretion *(39,40)*. Central administration of orexin-A and -B causes behavioral changes in conscious rats *(8,30,35)*. Some studies have suggested that these behavioral changes may be involved in dopaminergic *(41,42)*, serotonergic *(43–45)*, and noradrenergic systems *(30,46)*.

A straightforward study to prove that orexin-A and -B are stress-response mediators would be to block the stress response by pretreatment with orexin receptor antagonist or observation of stress responses in orexin gene knockout (KO) animals. Although orexin receptor antagonists have been developed *(47–50)*, a direct involvement of orexin-A and -B in the inhibition of the stress response is still unclear. However, a recent study has demonstrated that orexin KO mice showed attenuated defense responses *(51)*. The defense response is known to be a "fight-or-flight" response that occurs when animals encounter stressors. This response is elicited by stimulation of the perifornical area of the hypothalamus or dorsomedial hypothalamus. These areas are coincident with the localization of orexin-A-containing neurons.

5. CONCLUSIONS

Recent findings indicate that the orexin system in the CNS is closely related to physiological changes that occur in response to stress. The effects of various stressors on orexin neurons are summarized in Table 1. It would be of great interest to change the focus of orexin-related studies from feeding behavior to other physiological functions such as sleep/wakefulness, behavior, neuroendocrine, autonomic, and higher nervous functions.

Taken together, stress responses are known to be mainly mediated by classical neurotransmitters such as noradrenaline, dopamine, serotonin, and stress hormones (CRH) in the CNS. An understanding of the contribution of orexin-A and -B to various physiological stress-related functions will provide new insights into the crucial relationships among stress, emotion, and feeding.

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IV
PHYSIOLOGY
B. Orexins/Hypocretins and Sleep Regulation

12
Hypocretin as a Wakefulness Regulatory Peptide

Luis de Lecea, PhD and J. Gregor Sutcliffe, PhD

1. INTRODUCTION

In the past few years, the hypocretins (also known as orexins) have been shown to be critical components of the brain circuitry that modulates the states of vigilance *(1–3)*. Recent advances are yielding a clearer picture as to the mechanism of action of these peptides and how they control multiple circuits to produce a coherent behavioral output. Here we review the interactions of the hypocretinergic system with major neurotransmitter networks and discuss the role of the neurons that contain hypocretin in integrating information that dictates the state of arousal.

2. DISCOVERY AND PROPERTIES OF THE HYPOCRETINS

Analysis of the expression patterns of subtracted hypothalamus-enriched sequences *(4)* revealed that one of these was expressed exclusively by a bilaterally symmetric structure within the posterior hypothalamus. Its nucleotide sequence *(5)* encoded a 130-residue putative secretory protein (preprohypocretin) with an apparent signal sequence and three additional sites for potential proteolytic maturation. Two of the four putative products of proteolysis had 14 amino acid identities across 20 residues. This region of one of the peptides contained a 7/7 match with secretin, suggesting that the prepropeptide gave rise to two peptide products that were structurally related both to each other and to secretin. Thus, these peptides were named hypocretin-1 and -2 to reflect their hypothalamic origin and their similarity to secretin, which also extends to the secondary structure *(6)*.

Immunocytochemical mapping using antisera against chemically synthesized hypocretin peptides has shown that hypocretin neurons project their terminals throughout the brain *(7)*. Within the synaptic terminals of these fibers, hypocretin immunoreactivity is associated with dense core secretory vesicles *(5)*. Afferents of hypocretin neurons include an ascending pathway that projects to the basal forebrain, septum, and cerebral cortex; a very dense intrahypothalamic network; and a descending pathway that connects the lateral hypothalamus with brainstem nuclei and the spinal cord *(7)*. Both hypocretin peptides (1 and 2) are neuroexcitatory *(5,8)* and bind to postsynaptic hypocretin receptors (1 and 2) with different selective affinities *(9)*. The distribution of hypocretin fibers matches that of the hypocretin receptors described *(10)* and suggests that the hypocretins interact with multiple neurotransmitter networks involved in different functions. The studies showing that hypocretin mRNA is absent from narcoleptic brains *(11)* and that hypocretin immunoreactivity is highly decreased in

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narcoleptic hypothalami *(12)* provide compelling evidence that the main function of the hypocretinergic system is the regulation of arousal circuits.

3. AROUSAL CIRCUITS ACTIVATED BY THE HYPOCRETINS

3.1. The Noradrenergic Loop

The densest projection of hypocretin fibers terminates in the locus coeruleus (LC) area, the main site of noradrenergic transmission. Thus, this system was one of the first targets of the hypocretinergic system to be analyzed *(13,14)*. Noradrenergic neurons of the LC are active during wakefulness, display low activity during slow-wave sleep, are silent during REM sleep, and are thought to be critical for alternation of REM-NREM sleep *(15)*. Most of the LC neurons express hypocretin-1 receptor but not hypocretin-2 receptor. Local administration of hypocretin-1 in the LC increases wakefulness and suppresses REM sleep in a dose-dependent manner, and this effect can be blocked by antisera that prevent binding of hypocretin to its receptors *(13)*. Application of hypocretin to slices of the LC increased the firing rate of noradrenergic neurons, possibly by decreasing the after hyperpolarization current *(16)*. Interestingly, recent data using retrograde tracing have shown that the suprachiasmatic nucleus of the hypothalamus (SCN) is a target of noradrenergic LC neurons, via the dorsomedial hypothalamus (DMH). In addition, lesion studies confirmed that the DMH is a relay in this circuit *(17)*. This noradrenergic loop connects the circadian output of the suprachiasmatic nucleus to the lateral hypothalamus via the DMH. Also, direct connections between the SCN and hypocretin neurons have been described *(18)*. Thus, hypocretin neurons integrate circadian and metabolic input in the hypothalamus and modulate the firing of noradrenergic neurons in the LC. The LC controls the activity of hypocretin neurons directly, by inhibiting hypocretin firing *(19)*, and indirectly via the DMH.

3.2. Brainstem Cholinergic Nuclei

The major cholinergic input to the thalamus is from the laterodorsal tegmental nucleus (LDT) and the adjacent pedunculopontine tegmental nucleus (PPT). These neurons act on the thalamocortical network to provoke the tonic activation subtending both sensory transmission and cortical activation during arousal *(20)*. Considerable evidence has also indicated that mesopontine cholinergic nuclei also play a role in generating REM sleep, notably by stimulating the medial pontine reticular formation. Thus, cholinergic neurons in the LDT and PPT, by promoting either EEG desynchronization and wakefulness or REM sleep, play a key role in regulating the vigilance state *(21)*. The descending hypocretinergic projection includes the mesopontine cholinergic system *(7)*. Moreover, hypocretin-1 receptor (OX_1R) mRNA has been detected in these mesopontine cholinergic nuclei *(10,22,23)*. Hypocretin peptides excite cholinergic neurons in the LDT *(24,25)*, and injection of hypocretin-1 into the rat LDT increases wakefulness at the expense of NREM sleep *(26)*. It has been hypothesized that the hypocretin system may coordinate activation of the entire ascending reticular activating system (*see* below).

3.3. Intrahypothalamic Network

Even though the effect of the hypocretin peptides on food intake may be weak and transient, there is strong evidence demonstrating that hypocretin neurons are important sensors of metabolic signals. The connectivity between neuropeptide Y (NPY)-positive neurons in the arcuate nucleus and hypocretin neurons has been demonstrated *(27,28)*. These studies also showed that hypocretin neurons contain leptin receptors and that they are contacted by pro-opiomelanocortin (POMC)-containing inhibitory neurons. Furthermore, hypocretin neurons are sensitive to glucose, leptin, ghrelin, and triglycerides *(29–32)*. In an elegant study, Hara et al. *(33)*

showed that genetic ablation of hypocretin neurons in transgenic mice results in obesity and hypophagia, suggesting that the balance between storage and expenditure is impaired in these mice. Together the available data strongly suggest that the main function of the hypocretin peptides is not increasing food intake but generating a coherent output that stabilizes brain states.

In addition to the circuitry that modulates energy balance, hypocretin neurons contact several hypothalamic nuclei involved in sleep and wakefulness, including the ventrolateral preoptic nucleus (VLPO), the DMH, and the tuberomammilary nucleus (TMN). Hypocretin neurons only account for 4% of the lateral hypothalamic input to the VLPO, which is mostly active during NREM sleep *(34)*. The DMH is a key relay nucleus that receives input from the internal clock *(35)*. Both hypocretin peptides excite histaminergic neurons of the TMN, probably acting through hypocretin-2, and knockout mice deficient in histamine receptor 1 are impervious to hypocretin administration, suggesting that at least some of the effects of the hypocretins are caused by release of histamine and activation of postsynaptic H_1 receptors *(36)*.

3.4. The Basal Forebrain

Most neurons in the magnocellular basal forebrain are wakefulness-active, with highest discharge activity during wakefulness and a marked reduction in activity just before and during the entry to NREM sleep. A variety of basal forebrain structures receive a moderate hypocretin innervation. Infusion of hypocretin peptides into the medial septal area significantly increases wakefulness *(37)*. Infusion of hypocretin-1 in slices shows a strong and direct excitatory effect on the cholinergic neurons of the basal forebrain. Interestingly, these effects are mediated through hypocretin-2 receptors, which are lacking in narcoleptic dogs. Thus, the hypocretins excite cholinergic neurons that release acetylcholine in the cerebral cortex and thereby contribute to cortical arousal.

3.5. The VTA/NAcc Reward Circuit

The ventral tegmental area (VTA) contains cell bodies of dopaminergic neurons projecting to the nucleus accumbens, amygdala, hippocampus, and prefrontal cortex. Defined as the mesocorticolimbic dopamine system *(38)*, these neurons are critically implicated in brain mechanisms of reward, reinforcement, and emotional arousal *(39)*. Their activity has been closely correlated to the availability of primary rewards such as food, water, and sexual behavior *(40)*. The mesolimbic dopamine system, which is an established component of the reward system, receives glutamatergic input from cortical structures including the medial and occipital prefrontal cortex and amygdala, γ-aminobutyric acid (GABA) ergic inputs from striatal sources, and cholinergic input from the brainstem *(41)*. Hypocretin activity may mimic lateral hypothalamic self-stimulation, activating the LDT/PPT nuclei and subsequently increase the activity of dopaminergic neurons in the VTA *(41)*. In addition to the hypothetical indirect activation of VTA dopaminergic neurons by hypocretin via the LDT/PPT brainstem nuclei, hypocretin neurons directly excite dopamine fibers in the VTA *(42–44)*, and the VTA dopaminergic system is critically involved in hypocretin-induced hyperlocomotion and stereotypy *(45)*. Lastly, hypocretin-immunoreactive fibers and receptors are present in the nucleus accumbens (NACC) *(7)*, and hypocretin peptides modify the response to glutamate and GABA in this nucleus *(46)*. In this regard, it is interesting to recall the emotional components of cataplexy attacks, suggesting that the loss of hypocretin peptides could destabilize the mesolimbic dopamine system and correspondingly enhance motivational components of arousal.

3.6. The HPA Axis

Hypocretin peptides interact with autonomic, neuroendocrine, and neuroregulatory systems *(14,47)* and have recently been shown to be mediators of the stress response *(48)*. Thus, the hypocretinergic system has been associated with increased sympathetic tone *(49)*. Immunocytochemical studies have shown long descending hypocretin-containing axonal projections from the lateral hypothalamus to the spinal cord *(50)*. Innervation of the intermediolateral column and lamina 10 suggests that hypocretin may participate in the sympathetic and parasympathetic components of the autonomic nervous system. Indeed, injection of an agonist for OX_1R increases heart rate, blood pressure, cerebral blood flow, and renal sympathetic activity in awake animals *(49,51)*, as well as gastric secretion *(52)*.

Hypocretin neurons are modulated by adrenergic input *(53)*. Moreover, centrally administered hypocretin activates the hypothalamic-pituitary-adrenal (HPA) axis in rats *(45,54)*, induces plasma adrenocorticotropin hormone (ACTH) and corticosterone *(48,54,55)* and *c-fos* mRNA in the parvocellular division of the PVN (Fig. 1). In addition, glucocorticoids modulate hypothalamic hypocretin mRNA expression *(56)*, suggesting that this system could constitute a sensitive key relay for mediating stress behavior. Interestingly, hypocretin receptors have been detected in adrenal gland: hypocretin-1 receptor is expressed in the cortex of the normal human adrenal gland (glomerulosa, fasciculate, and reticular zones), and hypocretin-2 receptor is located in the medulla (epinephrine and norepinephrine cells) *(57,58)*. Also, addition of hypocretin to adrenocortical cultures stimulates norepinephrin release *(59)*. However, the origin of the ligand that would bind to hypocretin receptors in the periphery is unclear.

Further supporting the role of hypocretins in activation of the HPA axis, Ida et al. *(48)* have shown that icv administration of the α-helical corticotrophin-releasing factor (CRF) antagonist blocks hypocretin-induced grooming behavior. Also España et al. *(60)* have recently shown that mild stress increases *c-fos* immunoreactivity in hypocretin-positive neurons in the perifornical area. Interestingly, the effect of hypocretin on the stress response appears to be specific and finely regulated, since in vitro, hypocretin-1 inhibits CRF-induced ACTH release via a pertussis toxin-sensitive mechanism but does not affect baseline levels of ACTH, or release of luteinizing hormone (LH), prolactin (PRL), or follicle-stimulating hormone (FSH) from the pituitary *(61)*. These data, and our own preliminary evidence showing that hypocretin neurons are activated by CRF (in press), suggest that the hypocretinergic system is an important component of the neural circuitry modulating the stress response.

4. THE HYPOCRETINS SET THE AROUSAL THRESHOLD

Narcolepsy is a common cause of chronic sleepiness distinguished by intrusions into wakefulness of physiological aspects of REM sleep such as cataplexy and hallucinations *(62)* and can be defined as a dysfunction of the mechanisms that control the boundaries between states of vigilance.

How does the hypocretinergic system exert such control in the transitions between states of vigilance?

Among the neurons of the perifornical lateral hypothalamus, between 40 and 53% increase their firing rates during both wakefulness and REM sleep, but decrease their activities during slow-wave sleep *(63,64)*. An additional 38% of the neurons in this area are activated only during the awake phase. Hypocretin neurons express *c-fos* during the waking period *(65–67;* nighttime in rats), and *c-fos* expression is increased by sleep deprivation and methamphetamine

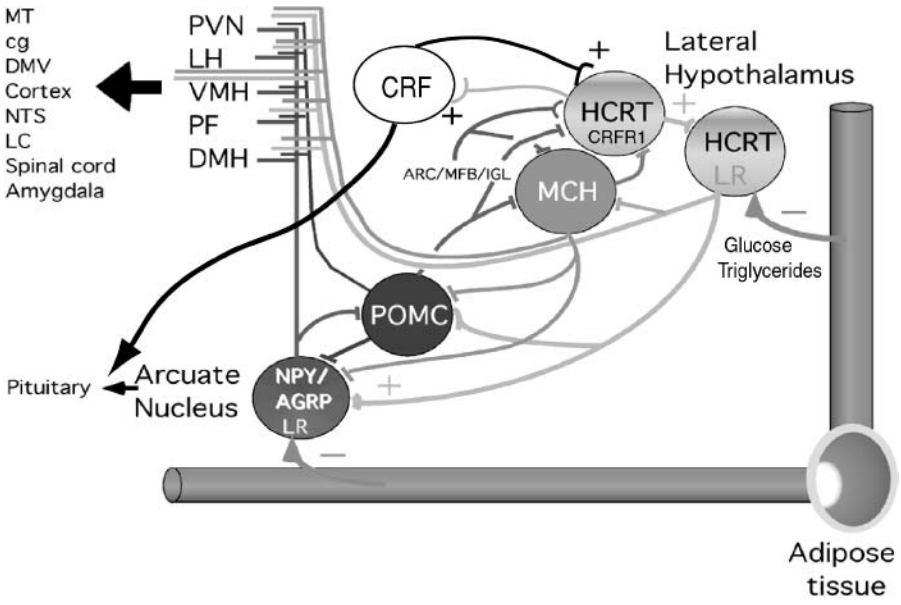

Fig. 1. Representation of the intrahypothalamic network and schematic interactions between hypocretins (HCRT) and the HPA axis. Hypocretin neurons lie in an area known to be critical for metabolic control. They receive input from NPY- and POMC-positive cells and are directly modulated by leptin, glucose, and triglycerides. The hypocretinergic system also receives input from CRF neurons in the PVN and amygdale, and it may contribute to the arousal effect associated with the stress response. Abbrevations: ARC, arcuate nucleus of the hypothalamus; MT medial thalamus; NTS, nucleus tractus solitarius; LC; locus coeruleus; PVN, paraventricular hypothalamus, central nucleus of amygdale; VMH, ventromedial hypothalamus; DMH, dorsomedial hypothalamus; PF, perifornical area; LH, lateral hypothalamus; CRF, corticotropin-releasing factor; NPY, neuropeptide Y; POMC, pro-opiomelanocrotine; AGRP, agouti-related protein; MCH, melanin-concentrating hormone; LR, leptin receptor, CRFR1, corticotropin-releasing factor receptor 1; cg, cingulum; MFB, Medial forebrain bundle; IGL, intergeniculate leaflet.

(66,68). Recordings in the hypothalamic slice have revealed that hypocretin neurons are in an intrinsic depolarized state *(69)*, which is consistent with the maintenance of a tonic excitatory influence on arousal and sympathetic systems during wakefulness. The stimulant modafinil, which is commonly used to treat the drowsiness associated with narcolepsy, greatly elevates *c-fos* expression in hypocretin neurons *(70)*. Hypocretin levels oscillate along the circadian cycle, being highest during waking, and peptide concentrations increase as a consequence of forced sleep deprivation *(71–73)*, suggesting that the hypocretins and the activity of the hypocretin neurons serve as pressures that oppose sleep. The hypocretins do not serve as a fundamental mechanism of sleep or sleep homeostasis, since hypocretin-deficient mice have normal amounts of wakefulness and NREM sleep *(74)*. The increase during forced wakefulness may indicate that the hypocretin system has executive wake-promoting activity, even when there is a need for sleep, or the increase could be a response to the elevated stress that results from such treatments.

The hypocretin neurons project to various brainstem structures of the ascending reticular activating system, which express one or both of the hypocretin receptors and have been implicated in regulating arousal (Fig. 2). The noradrenergic neurons of the LC the serotonergic neurons of the dorsal raphe, and the histaminergic neurons of the TMN are all so-called

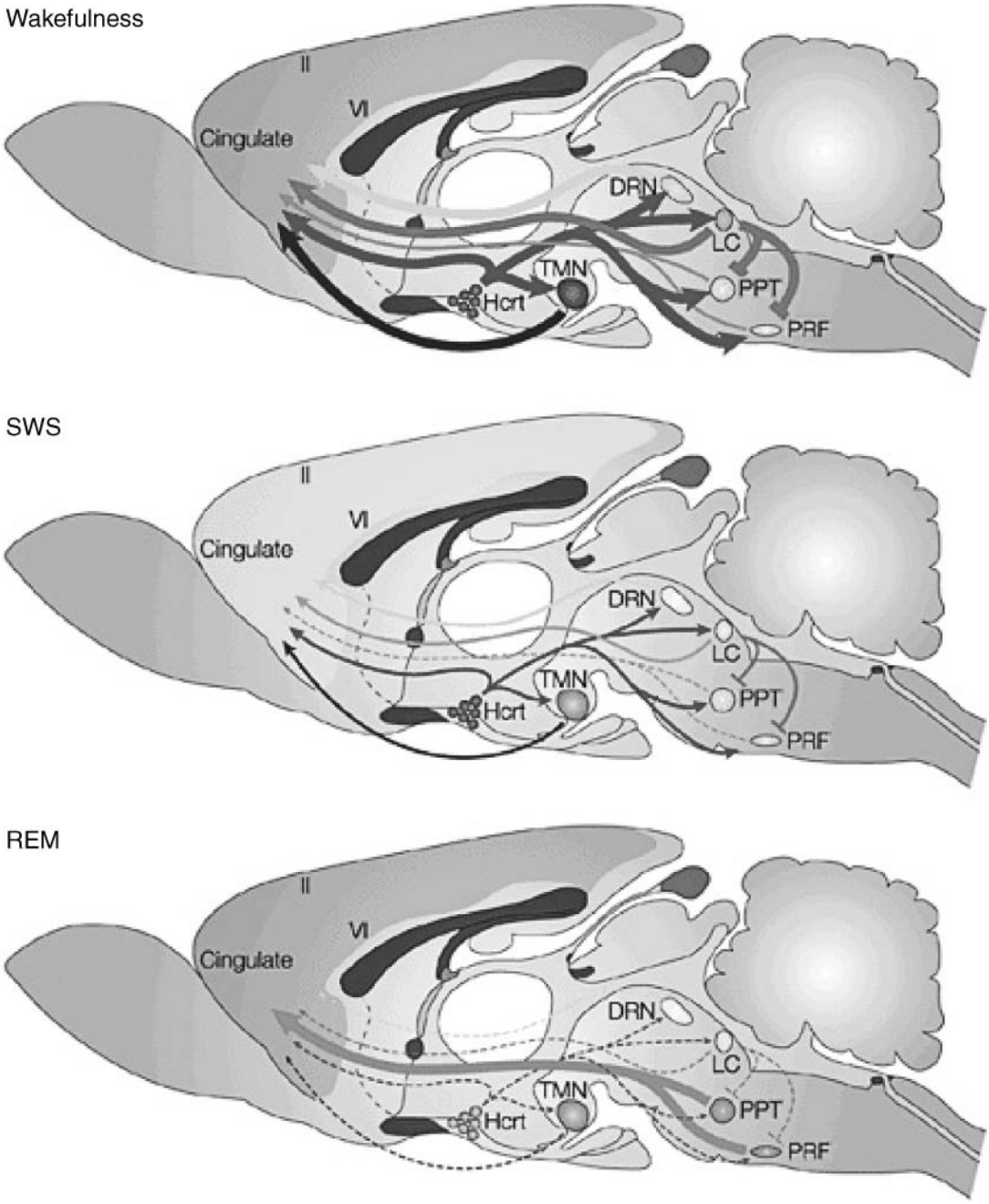

Fig. 2. The hypocretins (Hcrt) set the arousal threshold. Hypocretin neurons integrate information from divergent inputs, including circadian, metabolic, and limbic systems and provide a coherent output that results in the stability of arousal. During waking, hypocretin cells are active and provide direct excitation to REM-off centers and arousal nuclei, including the tuberomammillary nucleus (TMN) and directly to the cerebral cortex. During slow-wave sleep, hypocretin neurons are silent and are permissive to GABAergic activity of sleep-on nuclei such as the ventrolateral preoptic area. During REM sleep, the absence of hypocretin activity releases the inhibition of REM-on cells (From ref. *3*.) DRN, dorsal raphe nucleus; LC, Locus coeruleus; PPT, pedunculopontine tegmental nucleus; PRF, pontine reticular formation; SWS, slow-wave sleep.

REM-off cells: each group fires rapidly during wakefulness, slowly during slow-wave sleep, and hardly at all during REM sleep *(21,75–77)*. The activity state of these groups of monoaminergic neurons is one of the features that distinguish wakefulness from REM sleep.

The noradrenergic LC neurons fire constantly during wakefulness. In addition to their projections to the forebrain, these neurons send inhibitory projections to cholinergic REM-on (fire during wakefulness and more rapidly during REM, but do not fire during slow-wave sleep) generator neurons in the PPT-LDT, which project to the pontine reticular formation (PRF) *(77,78)*. As described above, hypocretin axons form synapses on these LC neurons, which express hypocretin-1 postsynaptically *(13,16)*. Hypocretin-1 excites serotonergic and histaminergic neurons in vitro *(79,80)*. Hypocretin-1 may also promote direct cortical arousal by exciting thalamocortical synapses *(81)*.

Thus, one mode of hypocretin function in arousal is the excitation of brainstem REM-off neurons during wakefulness, probably by signaling through both hypocretin-1 and -2 receptors. The excitation of these groups of monoaminergic cells contributes directly to forebrain arousal.

Local injection of hypocretin-1 into the LDT of freely moving cats increases wakefulness and decreases the number of REM episodes but does not influence episode length, suggesting that the hypocretin system influences the gate (or switch) to REM by reducing the firing rates of the brainstem REM-on cells but does not itself operate during REM. This and the fact that deficiencies in the hypocretin system lead to increases in REM make it more likely that action at REM-on structures by hypocretin occurs only during waking periods. The role of hypocretin in regulating the onset of REM sleep is a complex one in that the REM-on structures receive both indirect hypocretin-initiated inhibitory signals from REM-off cells and direct excitatory projections from hypocretin neurons, and therefore this conflicting information must be integrated into a coherent response.

As the hypocretin neurons are components of the hypothalamic circuitry that determines most homeostatic set points, alterations in the activity of these neurons can have far-reaching influence on other set points, developing into allostasis. The hypocretinergic system can be turned on by components of the stress response and may mediate the increases in sympathetic tone that are associated with alertness. Hypocretin neurons may also be an important component of the circuitry that is engaged in the hyperaroused state associated with stress and drug addiction.

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Hypocretin/Orexin Tonus and Vigilance Control

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1. INTRODUCTION

The importance of the hypocretin/orexin system in vigilance control has rapidly emerged from the discovery of narcolepsy genes in canines and mice and from the findings of ligand deficiency in human narcolepsy *(1–4)*. (An earlier anatomical study suggested this involvement *[5]*.) Narcolepsy, a chronic sleep disorder characterized by excessive daytime sleepiness, cataplexy, and dissociated manifestations of REM sleep *(6)*, is now known to be caused by the loss of hypocretin neurotransmission. The loss could be caused either by a malfunction in hypocretin ligand production or by a loss of function of one of the two hypocretin receptors (i.e., hypocretin receptor 2/orexin 2 receptor) *(1)*.

Narcolepsy first appeared in the medical literature at the end of the 19th century *(7)*. Since subjects affected with narcolepsy exhibit unique sleep-related symptoms (i.e., narcolepsy tetrad or pentad), many researchers interested in sleep regulation have focused intensively on this disorder to explore the pathophysiological mechanisms involved *(6)*. In later years, especially after the discovery of a tight association of narcolepsy-cataplexy with human leukocyte antigen (HLA) DR2/DQ6, human narcolepsy (at least for a core group) has been regarded as a single disease entity *(8)*. Narcolepsy has therefore been a disease model for studying the basic mechanisms of sleep regulation with the hope that by revealing the pathophysiological mechanisms of narcolepsy there will be a better understanding of the physiology of vigilance control *(6)*.

Recent experimental evidence suggests that the hypocretin system is one of the most important regulatory systems in controlling physiological sleep and waking. Its importance lies in the interaction with classical neurotransmitter systems (i.e., monoamines and acetylcholine) as well as with various fundamental hypothalamic functions, such as feeding and/or energy homeostasis *(5,9–14)*. Therefore, the role of the hypocretin system in sleep regulation has become one of the major topics in the sleep research field *(15)*.

Changes in neuronal activity of hypocretin neurons over different circadian times, in different sleep states, and during various manipulations have not been directly measured. However, several researchers have reported fluctuations of hypocretin tonus in relation to vigilance change by measuring the hypocretin gene transcript *(16,17)* in the lateral hypothalamus and hypocretin proteins in the various brain structures *(16)*, in extracellular fluid *(18)*, or in cerebrospinal fluid (CSF) *(19,20)*. In addition, quantifications of Fos immunoreactivity (IR) in hypocretin neurons were carried out *(4,21)*, as well as detailed characterizations of sleep abnormalities in animals that lack hypocretin ligand production *(4,22,23)*. Based on these published

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results, we discuss the roles of the hypocretin system in vigilance control. Technical considerations as well as limitations of the interpretation of these results are also discussed.

2. CIRCADIAN/HOMEOSTATIC REGULATION OF SLEEP

It is often assumed that the pressure to sleep (sleep propensity) is lowest shortly after awakening, increases during the day, peaks at bedtime, and declines during sleep. However, if one stays awake all night, sleepiness, and therefore sleep propensity, increases until a specific circadian time point, usually the individual's customary morning awakening time, at which time he/she will feel less sleepy again for a while. This second signal, of circadian origin, interacts with the sleep debt to maintain alertness evenly across the day. Several models have tried to integrate these two factors. The most established model is Borbély's two-process model *(24)*, whereby one process, process S, measures the homeostatic sleep pressure (Fig. 1). Process S is thought to be dependent on the amount of prior wakefulness and is reflected by the amount of electroencephalographic (EEG) slow-wave activity (0.5–4.5 Hz). Process S increases during extended wakefulness, thereby increasing "sleepiness" with sleep deprivation. Sleep propensity can only be relieved by sleep. The second process, process C, varies as a sinusoidal function across the day, with its intensity being unrelated to the amount of prior wakefulness. Process C is postulated to promote wakefulness during the individual's customary period of activity (daytime in humans) and promote sleep during the customary period of sleep. The circadian activity of process C is also reflected by circadian fluctuations in physiological parameters such as core body temperature, plasma melatonin, and cortisol levels. Whereas the neurobiological substrates mediating process S are still unknown, lesion studies have indicated that process C is generated primarily by the circadian clock in the suprachiasmatic nucleus (SCN) *(25)*, a small cluster of neurons just above the optic chiasm in the anterior hypothalamus. The identification of genetic mutations in *Drosophila* and mice has resulted in the description of a detailed intracellular translation-transcription feedback loop that occurs in single SCN cells and generates circadian rhythmicity to the rest of the organism *(26)*.

Although the general concept of the two-process model for sleep propensity is widely accepted, this model has a number of limitations. Alertness is, for example, not optimal immediately after waking up, a concept called sleep inertia *(27)*. It has also been suggested that process C does not equally promote sleep and wakefulness as a simple symmetrical sinusoid function (see Fig. 1). Lavie et al. *(28)* introduced the notion that it is almost impossible to fall asleep early in the evening independently of any sleep debt, suggesting the existence of a "forbidden zone" followed by a sleep gate where falling asleep is easy. Edgar et al. *(25)* pointed out that after SCN lesions in day-active squirrel monkeys, total sleep time increased, suggesting that the circadian clock primarily promotes wakefulness in mammals, with extended periods of consolidated wakefulness (e.g., some primates and humans). They suggested the existence of an SCN-dependent wake-promoting signal in the evening that opposes the increasing sleep debt that has accumulated after waking up in the morning. Djik and Czeisler *(29)* have also proposed similar models, with both alertness and sleep promoting SCN-dependent signals in humans.

The two-process model does not reveal regulatory mechanisms for many rapid sleep cycle changes, such as alternation of non-REM sleep and REM sleep, and their cyclicity (i.e., ultradian rhythm) (see Fig. 1). Occurrence of REM sleep is controlled by ultradian rhythm, and it occurs within 90–110-min intervals in humans, and 30-min intervals in cats and dogs *(30)*. Earlier brain transection studies suggested that the generation of REM sleep cyclicity originates in the pons *(31)*. However, neuronal afferent systems of this structure as well as many other factors (such as temperature and/or humeral factors) are also likely to affect the cyclicity of REM sleep and other REM sleep characteristics, such as REM sleep amount and stability *(32)*.

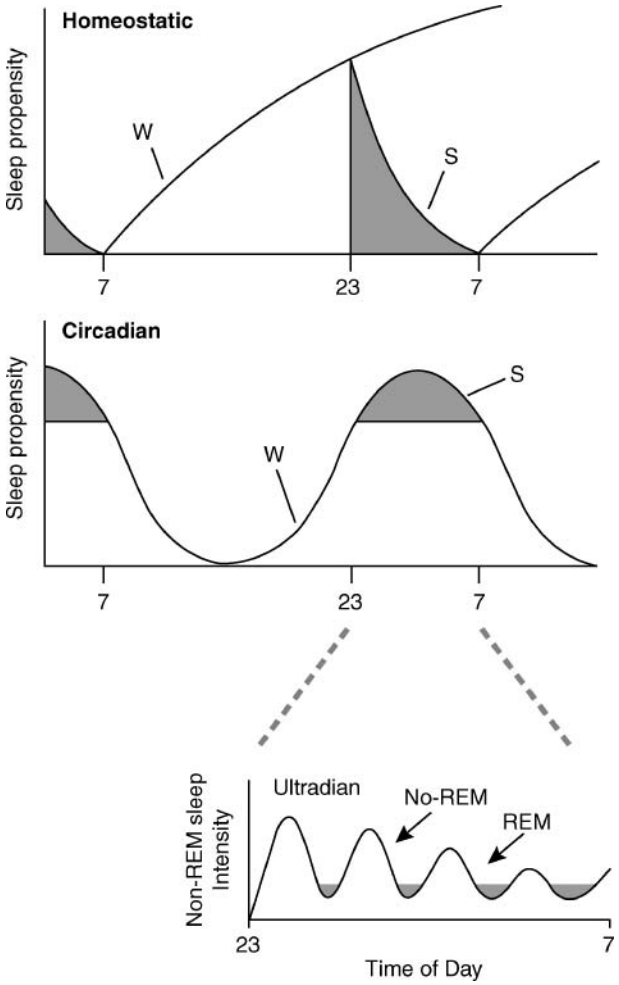

Fig. 1. The three main processes involved in the regulation of sleep. Homeostasis maintains the duration and intensity of sleep, the circadian rhythm determines the timing of the propensity to sleep, and ultradian mechanisms underlie the non-REM-REM sleep cycle. As the sleep episode progresses, the intensity of non-REM sleep declines, and the duration of successive REM sleep intervals increases. W, awake states; S, sleep. (Adapted from ref. *88*.)

3. GENERAL CONCERNS ABOUT MEASURING HYPOCRETIN TONUS IN RELATION TO VIGILANCE CONTROL

The most direct way to address the question of how hypocretin tonus changes with vigilance state may be to measure the neuronal activity of hypocretin neurons across different vigilance states and under the various manipulations that alter the vigilance states. In this regard, two groups recorded the activity of neurons located at the perifornical lateral hypothalamic regions (where hypocretin neurons are enriched). The results demonstrated that several different types of neurons are found in this region, including wake (18–38%), wake/REM (30–53%), slow-wave sleep (SWS; up to 7.5%), and REM (9.4–35%) active neurons *(33,34)*. However, currently there is no method to identify the hypocretin neuron positively during in vivo single-unit recording and neither study confirmed that some of the recorded neurons indeed contained hypocretin. Recently, juxtacellular labeling (with double immunocytochemical staining of

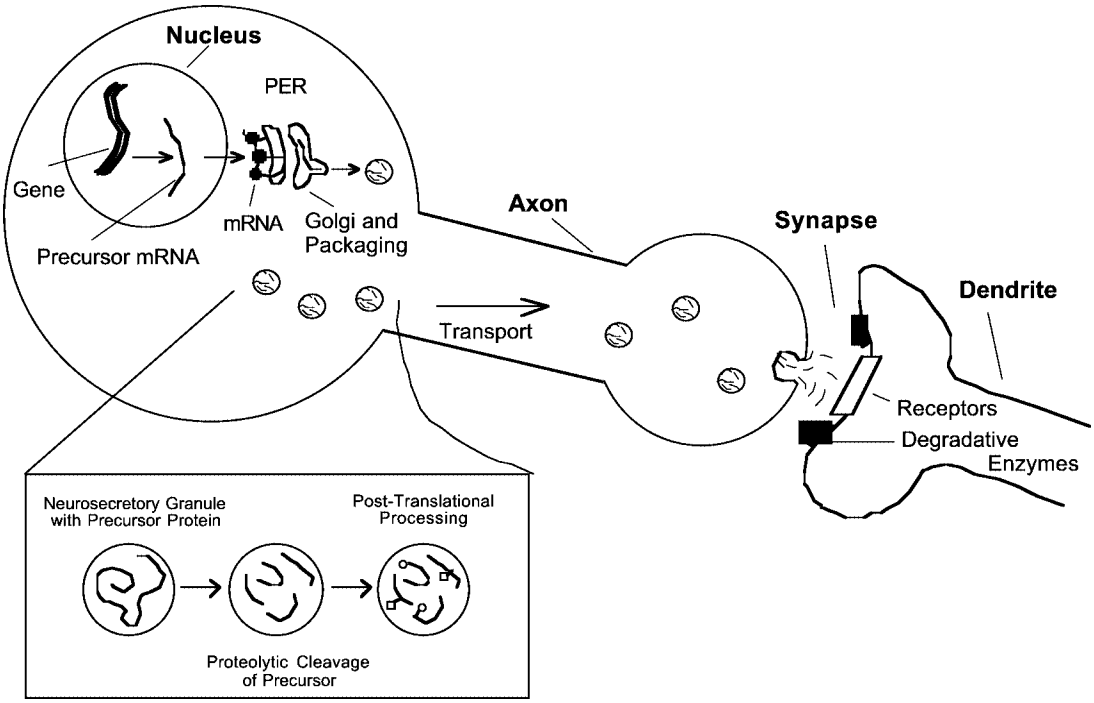

Fig. 2. Schematic representation of a peptidic neuron and synthetic processes of neuropeptides (Adapted from ref. *89*.)

recording neurons) has been successfully used to determine the neurotransmission of recorded neurons in vivo *(35)*, but this method may still have low efficacy for detecting hypocretin neurons because hypocretin neurons are sparsely located among other neuronal populations. Thus, it is still not known whether hypocretin neurons exhibit homogeneous and distinctive firing patterns in relation to vigilance changes and how hypocretin neurons change their firing with circadian, homeostatic, and ultradian vigilance control processes.

Double-labeled immunohistochemistry of Fos (Fos-IR) in the hypocretin neurons may be an alternative to monitoring the activity profile of hypocretin neurons *(4,21)*, but this method does not have enough temporal resolution to study relatively brief phenomena such as non-REM and REM sleep changes in rodents. Furthermore, increased Fos-IR is not always caused by conditions that increase neuronal firing rates or neurotransmitter release, and thus caution needs to be used when interpreting the results *(36)*.

Like other neuropeptides, hypocretin peptides are thought to be released to the synaptic cleft by action potentials. Released peptides are inactivated, and no presynaptic uptake system is reported for neuropetides *(37)*; thus the terminal release of peptides is likely to lead to an increase in synthesis of hypocretin peptides. Several groups have measured preprohypocretin mRNA and peptide levels over 24 h and by several manipulations that affect sleep homeostasis and/or circadian regulation *(16,17)*. However, there are great limitations for these approaches, since the process for the peptide synthesis is relatively slow. The initial synthetic processes of neuropeptides is involved in the synthesis of preprohormone signal peptide cleavage (in the endoplasmic reticulum), and modification (in the Golgi apparatus; Fig. 2). Modified peptides are then transported to the nerve terminals, while posttranslational processing (packing, proteolytic cleavage, and modification) continues during transportation; the peptides are finally

stored in secretory vesicles. Once neuropeptides are released into the synaptic cleft by action potentials, they are rapidly inactivated without re-uptake *(37)*. Thus, changes in mRNA are not likely to vividly reflect terminal peptide release. Similarly, it is possible that peptide levels measured in brain homogenates do not correlate with hypocretin release activity. Neuropeptide levels at the synaptic terminal may instead be low when terminal release is enhanced.

4. SLEEP-RELATED AND DIURNAL FLUCTUATIONS OF HYPOCRETIN mRNA AND PEPTIDE LEVELS IN THE BRAIN PARENCHYMA

Taheri et al. *(16)* reported fluctuations in preprohypocretin mRNA and hypocretin-1 levels in rats over 24 h. Preprohypocretin mRNA and hypocretin-1 levels in the hypothalamus are at their lowest in the beginning of the active phase, gradually increase toward the end of the active phase, and slowly decrease during the resting phase. In contrast, hypocretin-1 peptide in the pons exhibited a mirror image; levels are at their highest in the beginning of the active phase and the lowest at the end of the active phase. These results may suggest that hypocretin release and synthesis are active when animals are awake and possibly modulate the activities of pontine structures, including the noradrenergic locus coeruleus (LC) and the reticular formation for the maintenance of wakefulness *(16)*. Terao et al. *(17)* reported that short-term sleep deprivation in rats had no influence on preprohypocretin mRNA levels in the hypothalamus. However, these results are in contrast to the results later obtained by measuring hypocretin peptide in the CSF and extracellular fluid, which demonstrated increases in hypocretin release during sleep deprivation (*see* Subheading 5. below). Thus changes in preprohypocretin mRNA appeared to be less sensitive to short-tem sleep changes/manipulations.

5. HYPOCRETIN MEASURES IN THE CSF IN RATS WITH VARIOUS MANIPULATIONS. AFFECTING CIRCADIAN AND HOMEOSTATIC REGULATION OF SLEEP

In parallel with the earlier human CSF studies, we have also learned that hypocretin-1 can be reliably measured in a small amount of CSF in all other vertebrates tested (dogs, rats, rabbits, and monkeys) *(19,20,38)*. The nature and origin of hypocretins in the CSF are not fully understood. However, since hypocretin axon terminals protrude into the lumen of the third ventricle, some hypocretins in the CSF may be directly secreted into the CSF and may have functional roles *(39)* such as a volume transmission *(40)*. In contrast to hypocretin-1 levels, hypocretin-2 levels are very low or undetectable in the CSF *(38,41)*, although hypocretin-1 and -2 peptide levels are comparable in the brain parenchyma *(3)*. This discrepancy is probably owing to the difference in stability of hypocretin-1 (with two intrachain disulfide bonds) and hypocretin-2 (a linear peptide). We have reported that hypocretin-1 is very stable in the CSF and that high IR-peptide levels and high biological activities were observed for several hours after exogenously administration of hypocretin-1 in the cerebroventricle of rats *(42)*. Moreover, we found that hypocretin-1 can also be measured in the extracellular fluid collected with a microdialysis probe in rats *(18)*. We therefore focused on measuring changes in hypocretin-1 levels in the CSF and in the microdialysis perfusates in rats over 24 h and with various behavioral manipulations *(18,19)*. These two measures are complementary, but the CSF tap method is simpler; it does not require head stage construction and tubing and is easily applicable to various manipulations whereas measurements taken in the microdialysis perfusates directly reflect the terminal release of hypocretin peptides.

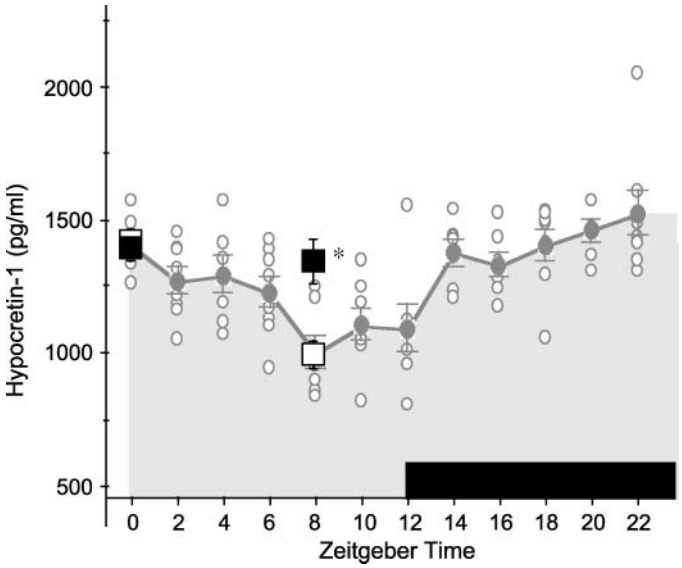

Fig. 3. Fluctuation of CSF hypocretin levels in rat over 24 h and responses to 72-h food deprivation. Open circles represent each individual hypocretin-1 value, and solid circles indicate the mean value with SEM. Mean values with SEM of CSF hypocretin-1 levels at baseline (open squares) and after 72 h of food deprivation (solid squares) at Zeitgaber time (ZT) 0 and 8 h are indicated and are overlaid on the data for the diurnal fluctuation of CSF hypocretin-1 levels. The asterisk indicates a significant increase in the CSF hypocretin-1 level at ZT 8 h from that at baseline in response to 72-h food deprivation ($p < 0.01$). The horizontal black bar indicates the dark phase.

Using a repeatable CSF tap method, CSF measurements of hypocretin-1 were taken by many investigators to monitor hypocretin tonus in several species over 24 h. Measurements were taken during short-term sleep deprivation, long-term food deprivation, forced swimming, and REM sleep deprivation in SCN-lesioned animals and aged animals *(19,20,43–46)*. Sampling intervals of 2–4 h were generally used for most of the studies, and there was a time difference (about 2 h) in peak hypocretin levels in the CSF compared with that in extracellular hypocretin-1 levels *(18,19)*. Since CSF clearance is relatively slow (CSF is replaced about three times a day), the magnitude of changes in hypocretin release may also be attenuated in the CSF.

Regardless of these limitations, we observed a clear diurnal fluctuation of hypocretin-1 levels in the rat cisternal CSF *(19)*. Hypocretin-1 levels are lowest at the beginning of the active phase and become high toward the end of the active phase, declining in the resting phase (Fig. 3). We also observed that 72-h food deprivation, but not 24-h food deprivation, significantly elevated CSF hypocretin-1 levels (at zeitgaber time [ZT] 8) in these animals (Fig. 3). Interestingly, however, this manipulation does not increase the CSF hypocretin-1 levels more than the maximum levels observed during diurnal fluctuation, and no further increase was observed when CSF samples were collected at ZT 0.

Total sleep deprivation as well as forced swimming increased cisternal hypocretin-1 *(44)*. The increase in CSF hypocretin levels during sleep deprivation may be partially owing to the increase in locomotor activity and/or rise of brain temperature during this procedure, since locomotor activation and rise in brain temperature were generally observed during sleep deprivation in these animals *(18)*. In the aged rats, amplitude of diurnal fluctuations of hypocretin-1 was dampened, and an attenuated response to the sleep deprivation compared with young rats

was observed in aged animals *(45)*. Effects of REM sleep deprivation (by a flower pot method) on CSF hypocretin-1 levels was also tested in rats and was high during REM sleep deprivation and low during the recovery phase when the animal spent the most time in REM sleep *(43)*.

The SCN drives the rhythms of sleep and wakefulness through projections to nearby hypothalamic regions. Although a direct projection from the SCN to the lateral hypothalamic area is minor, dense indirect projections to the lateral hypothalamic area (LHA), through the subparaventricular zone (SPZ) *(47,48)* and dorsomedial nucleus of the hypothalamus (DMH) structures are reported *(49,50)*. These projections are probably involved in circadian rhythm of sleep-wake, behavior, autonomic stress responses, and feeding *(51)*. Interestingly, two research groups recently reported that an SCN lesion eliminates the diurnal fluctuation in hypocretin-1 in rats under light-light (LL) and/or dark-dark (DD) conditions *(46,52)*. One of the studies reported that there was a significant correlation between hypocretin-1 and activity prior to the tap in SCN-lesioned rats under constant conditions. The best correlation was between −3 h and −1 h prior to each tap, consistent with CSF fluid dynamics. Interestingly, SCN lesions did not affect hypocretin responsiveness to sleep deprivation *(52)*, indicating that homeostatic control of hypocretin by sleep is still intact in the animals that lost the circadian clock.

6. CSF HYPOCRETIN MEASURES IN DIURNAL ANIMALS

Hypocretin measurements in the cisternal CSF were also performed in diurnal animals such as dogs *(53)* and squirrell monkeys *(20)*, and the results consistently demonstrated that CSF hypocretin levels are high during the active phase and low during the resting phase, regardless of light exposure. As seen in rodents, sleep deprivation increased CSF hypocretin levels in dogs and monkeys. Increased CSF hypocretin levels during sleep deprivation may also be associated with increased locomotor activation in dogs *(53)*, but, interestingly, locomotor activation was not generally observed during sleep deprivation in monkeys. Zeizter et al. *(54)* further assessed the influence of locomotor effects on hypocretin tonus in this animal species. These experiments in monkeys (with a consolidated wake pattern) are critical to assess genuine locomotor effects on hypocretin tonus, since manipulations of locomotion in rodents are likely to be intertwined with changes in wakefulness. Interestingly, the authors found that restricting locomotion to 17% of usual activity had no significant effect on the normal diurnal rise in CSF hypocretin-1 in these animals *(54)*. Taking these results together with the finding that increased locomotion did not significantly increase CSF hypocretin-1 concentrations (but did appear to have a positive modulatory effect on CSF hypocretin-1 levels), these authors concluded that high levels of locomotion may provide a small positive feedback onto the hypocretin system in this wake-consolidating animal. However, these researchers state that locomotion is not necessary for CSF hypocretin-1 to increase across the daytime.

Fluctuations of hypocretin-1 over 24 h were also investigated in humans. Lumbar CSF samples were taken through an intrathecal catheter of bed-restricted subjects, and the results indicated that hypocretin-1 levels are high at night and low in the morning hours *(55)*. However, this paradoxical finding may reflect the additional time delay between the cisternal and lumbar CSF, and thus CSF in the lumbar region may have a decreased value for the functional assessment of hypocretin tonus.

7. SLEEP-RELATED FLUCTUATION OF Fos PROTEIN EXPRESSION IN THE HYPOCRETIN NEURONS

Several authors quantified Fos protein expression in hypocretin neurons under various conditions by using Fos-IR. The results of these experiments are generally consistent: Fos-IR expression in hypocretin neurons is high (about 50% of hypocretin neurons are Fos-positive)

during the active phase, and lower during the resting phase *(21)*. Ambient light had no effect on the activity of hypocretin neurons, but sleep deprivation and methamphetamine and modafinil administrations all increased Fos-IR in the hypocretin neuron *(4,21,56)*. The amount of Fos expression in hypocretin neurons correlated with the amount of wakefulness as well as with body temperature *(21)* and the amount of locomotor activity *(57)*. España et al. *(58)* also reported that hypocretin neurons and their receptive neurons display increased Fos-IR in response to novelty stress. Furthermore, in cat experiments, Torterolo et al. *(57)* reported that Fos expression correlated mainly with increased locomotor activity. All these observations indicate that hypocretin neurons are active during wakefulness, especially in association with stress, locomotion, and other high-arousal conditions

8. HYPOCRETIN-1 RELEASE IN EXTRACELLULAR SPACE IN RATS OVER 24 H AND WITH SLEEP-RELATED MANIPULATIONS

Together with CSF hypocretin measures, we also initiated hypocretin measures in microdialysis perfusates in freely moving rats with simultaneous sleep recordings *(18)*. Like other neurotransmitters, hypocretin concentration in the microdialysis perfusate is dependent on the microdialysis membrane length and flow rate. The pore size of the dialysis membrane is another important factor to be considered in peptide sampling (*see* next paragraph). We found that hypocretin is easily adsorbed into the microdialysis membrane and tubes, and this significantly reduced the final hypocretin-1 concentration in the collected perfusate. Thus, all connecting and sampling tubes should be siliconized prior to use to avoid adsorption of hypocretins. However, in vitro recoveries of hypocretin-1 are still very low at 0.8% (by nonlabeled standard hypocretin-1 solutions) and 2.4% (by ^{125}I hypocretin-1), in our microdialysis setting (C-I-8 microdialysis probes from EiCOM, Kyoto, Japan; membrane length, 3 mm; o.d., 0.22 mm; cutoff value, 50 KDa: flow rate, 0.4 μL/min). These recoveries are much lower (1/10–1/20) than those for monoamines or acetylcholine at the same setting. The baseline hypocretin-1 level obtained in the LHA was about 200–300 pg/mL. Thus, calculated basal extracellular hypocretin-1 levels are 4–8 n*M* and are roughly in the same range as the in vitro affinity for hypocretin receptors *(59)*. These concentration and flow rate ranges enabled us to set the maximum time resolution for 1–2 h. We also confirmed that perfusion of a γ-aminobutyric acid $(GABA)_A$ antagonist, bicuculline (100 μ*M*), significantly increased hypocretin-1 levels in the LHA, suggesting that the hypocretin levels measured in microdialysis perfusates reflect the terminal release of hypocretin-1 *(18)*.

Probes with a large pore membrane, such as one with a 1000-kDa cutoff, may improve the recovery of hypocretin-1. However, negative pressure must be applied from the outlet (i.e, the push–pull method) to use a large pore membrane, since a fluid leak into the brain is likely without negative pressure. We found that this is extremely difficult working with in vivo freely moving animals. Indeed, we could not obtain a consistent flow rate with the push-pull method owing to the difficulty of keeping the lines closed, and thus we decided to use a regular microdialysis membrane and system.

By trial and error, we were able to detect hypocretin-1 levels reliably in a 1–2 h sampling bin, and we found that extracellular hypocretin-1 levels in the lateral hypothalamus and medial thalamus significantly fluctuate across 24 h (Fig. 4). Hypocretin-1 release was lowest at the end of the light phase. After the light was turned off, the animals immediately became active and remained awake most of the time, whereas hypocretin-1 levels gradually increased over time. Hypocretin-1 levels continued to increase for the duration of the active phase and

reached a maximum at the end of the active phase. When the lights were turned on and the animals started to spend a greater percentage of time in sleep, hypocretin-1 levels decreased gradually and reached a nadir after 6 h *(18)*. The difference between the minimum and maximum level was about 1.6-fold, and there was a significant difference in mean hypocretin-1 levels between the active (dark) phase and the resting (light) phase. Hypocretin-1 levels were positively correlated with the amount of wakefulness and negatively correlated with the amount of slow-wave sleep (SWS) and REM sleep over 24 h (Fig. 4). However, hypocretin-1 levels and sleep measurements were clustered in the dark and light phases, and the amount of wakefulness, SWS, and REM sleep were not significantly correlated with hypocretin-1 levels during either the dark or the light phase (Fig. 4). Brain temperature during the dark phase was the only variable that was significantly correlated with hypocretin-1 level. Acute light shift (the 4-h phase advance) did not modify the hypocretin release pattern. In contrast, 6 h of sleep deprivation starting at the beginning of the rest phase significantly increased hypocretin-1 levels (Fig. 5).

Kiyashcenko et al. *(60)* also studied changes in the terminal release of hypocretin-1 in feline brain across sleep-wake states. These authors used a large-pore 1000-KDa dialysis membrane with the push-pull technique and found that hypocretin-1 levels in the lateral hypothalamus were higher during active waking and REM sleep than during SWS. Although the results showing high hypocretin release during REM sleep are interesting and may be important, they need to be replicated with other experimental settings since our microdialysis experiments in rats indicate that hypocretin release tends to correlate negatively with the amount of REM sleep *(18)*. Similarly, a negative correlation between Fos expression in hypocretin neurons and the amount of preceding REM sleep was reported *(21)*. It was also reported that REM sleep deprivation in rats increased CSF hypocretin-1 levels and that CSF hypocretin-1 was low during REM sleep rebound *(43)*.

9. PHYSIOLOGICAL HYPOCRETIN RELEASE AND ABNORMAL SLEEP CHARACTERISTICS IN HYPOCRETIN-LIGAND DEFICIT RATS

Previous human studies have suggested that problems with the maintenance of the vigilance stage (i.e., fragmented sleep/wake pattern) together with cataplexy are the primary symptoms in narcolepsy. However, no apparent abnormalities have been found in sleep homeostasis and SCN function in human narcoleptics (i.e., they show compensatory responses to sleep loss and normal entrainment to light/dark cycles) *(61–64)*. Abnormal sleep patterns of prepro-orexin (preprohypocretin) gene knockout (KO) mice and hypocretin neuron-ablated (*orexin/ataxin-3 transgenic*) mice were also characterized; these mice exhibit highly fragmented vigilance states, occasional direct transition to REM sleep from wakefulness, and behavioral arrest similar to cataplexy *(4,22)*. Thus, loss of hypocretin signaling is likely to contribute instability to vigilance states. The possible mechanism for this is well explained in a recent review by Saper et al. *(65)* and Chapter 15 of this volume. Despite the absence of hypocretin release, diurnal non-REM sleep distribution seems to be intact in these animals, whereas distribution of REM sleep is significantly impaired. (The animals have a large amount of REM sleep during the active phase.)

More recently, hypocretin neuron-ablated *orexin/ataxin-3* transgenic rats were produced, and their sleep characteristics have been reported by Beuckmann et al. *(23)*. These animals also exhibit fragmented vigilance states, decreased latency to REM sleep, direct transitions from wakefulness to REM sleep, and brief episodes of muscle atonia and postural collapse resembling cataplexy. Twenty-four-hour sleep/wake patterns of these narcoleptic rats were

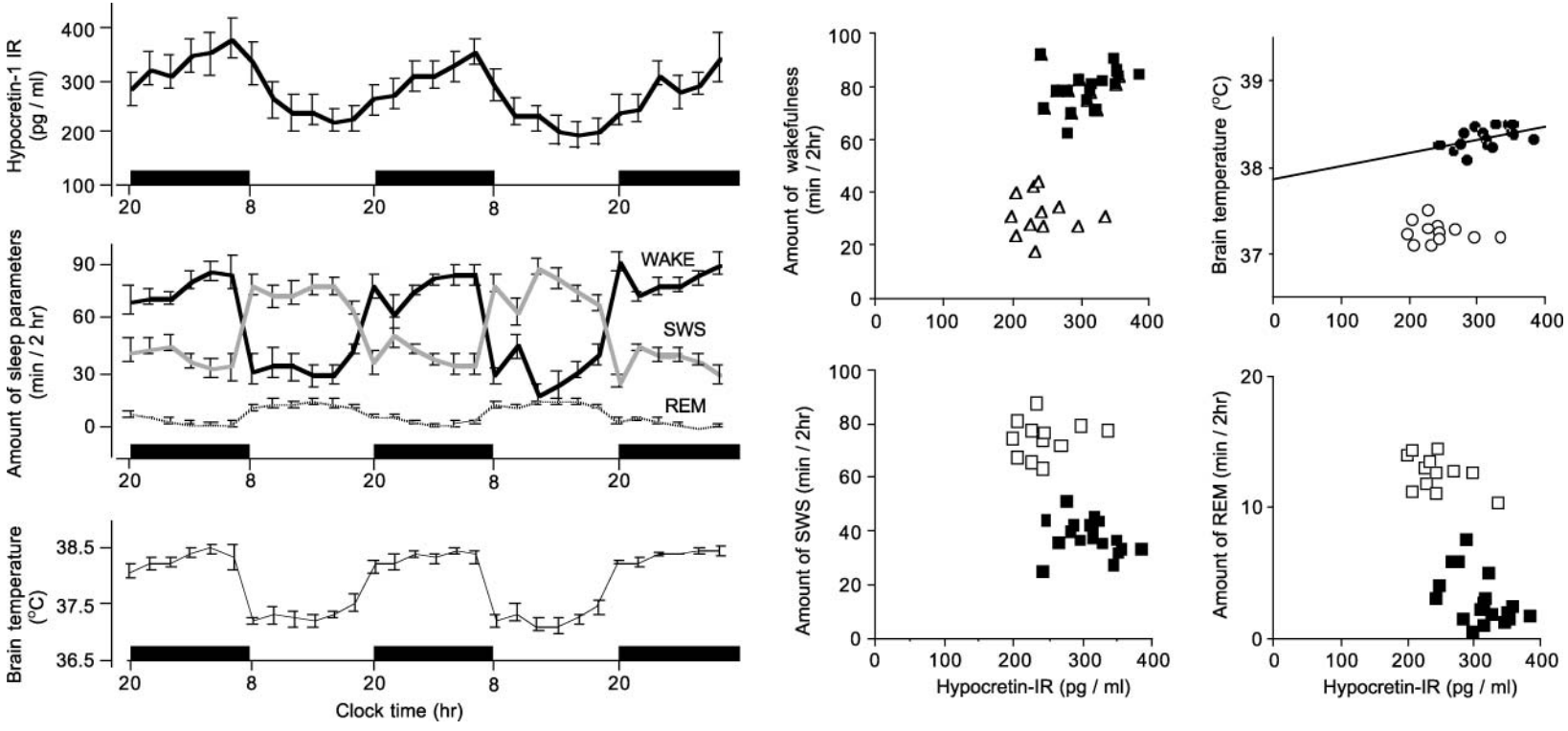

Fig. 4. Fluctuation of extracellular hypocretin-1 levels in the LHA under 12-h light/12-h dark conditions in relation to the sleep–wake cycle and brain temperature. Correlations among wakefulness, slow-wave sleep (SWS), REM sleep, and hypocretin-1 levels were investigated. The mean (± SEM) of the data for eight animals is displayed. Dark phases are indicated by black bars. **(A)** Mean sleep and brain temperature data of eight animals were correlated with hypocretin-1 levels measured at 2-h intervals. **(B)** Hypocretin-1 levels clustered in the dark (filled symbols) and light phases (open symbols). Stepwise analysis revealed that brain temperature level in the dark phase (filled circles) is the only variable that significantly correlated with hypocretin-1 ($p < 0.05$), and hypocretin-1 was not significantly correlated with wakefulness, SWS, and REM sleep. (Adapted from ref. *18*.)

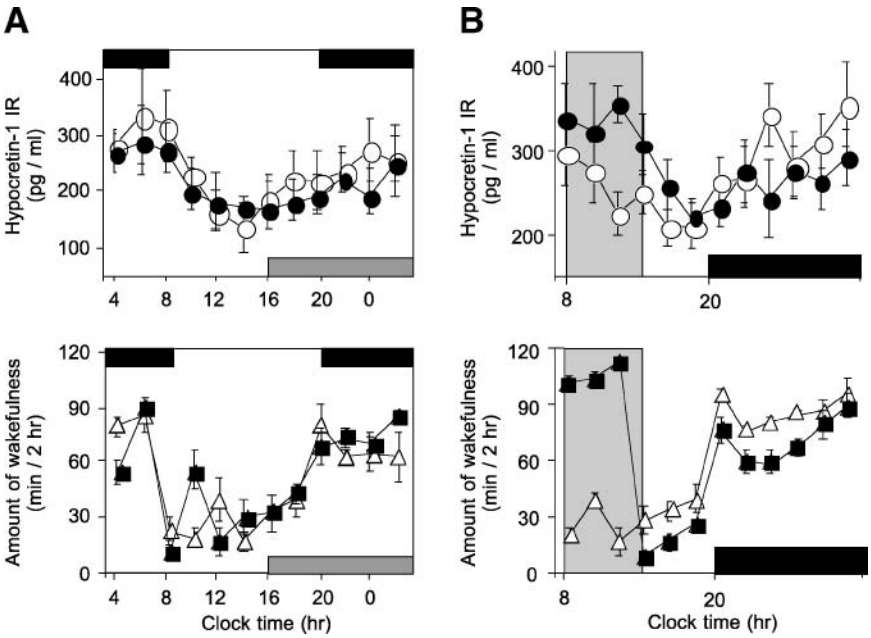

Fig. 5. Effect of 4-h light shift and 6-h sleep deprivation on hypocretin-1 levels in the LHA and amount of wakefulness. **(A)** Lights were turned on at 04.00 h, 4 h earlier than the regular light/dark schedule. Hypocretin-1 levels (filled circles), as well as amount of wakefulness (filled triangles), were overlaid on the traces from the day before (open symbols). Filled bars on the top of the figure represent baseline dark phases, and filled bars on the bottom of the figure represent dark phases after phase shift. Although 4-h phase advance caused a transient disturbance of sleep/wake activities, the levels of hypocretin-1 did not show significant changes compared with the baseline level. **(B)** Sleep deprivation was initiated at the beginning of the light phase on day 4 (shaded area) (see Fig. 4) and resulted in an 85% increase in wakefulness (filled triangles). During SD, hypocretin-1 levels (filled circles) were maintained at maximal levels and were significantly different from the levels at the respective baseline time points (open symbols; time 3 manipulations effect, $p < 0.0001$). (Adapted from ref. *18*.)

also analyzed and exhibited clear diurnal distribution patterns of non-REM sleep (Fig. 6), suggesting that an intact hypocretin system is not required for the diurnal distribution of non-REM sleep (if the SCN function is intact). However, total wakefulness time was reduced during the dark phase in narcoleptic rats; they have more non-REM sleep at the end of the active phase (Fig. 6). This finding is very interesting since the results of our microdialysis experiment in rats demonstrate that hypocretin-1 levels build up toward the end of active phase (Figs. 4 and 6). The gradual buildup during the active phase and the exponential decline during the rest phase strikingly resemble the time-course of NREM sleep delta power in the rat *(18,66)*. Thus, the hypocretin system may be one of the important factors in counteracting increasing sleep propensity at the end of the active phase in these animals. In contrast, decline of hypocretin tonus after sleep onset may help to free the sleep propensity that accumulated during wakefulness and result in consolidated sleep *(67)*.

Similar to the findings seen in hypocretin-deficient narcoleptic mice *(22)*, the diurnal distribution of REM sleep in hypocretin neuron-ablated rats was significantly impaired *(23)*. It thus appears that distribution patterns of REM sleep are highly dependent on the availability of the hypocretin system and on the changes in tonus. Hypocretin deficiency may therefore result in disinhibition of REM sleep, especially during the active phase; therefore narcoleptic

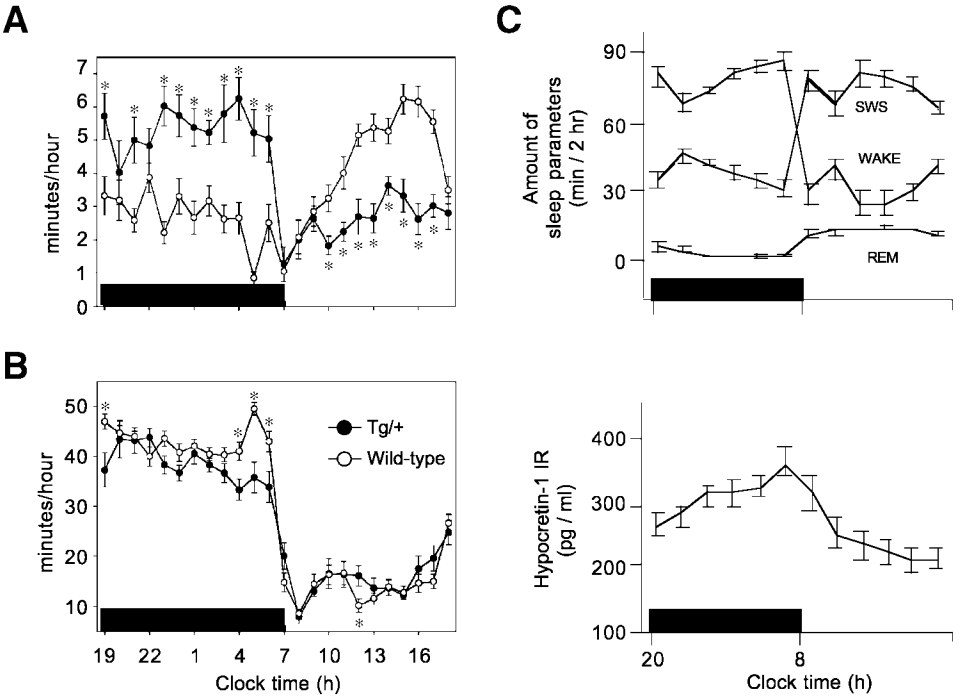

Fig. 6. Time spent each hour (in minutes; mean ± SEM) in REM sleep **(A)** and wakefulness **(B)** for wild-type rats and their *orexin/ataxin-3* hemizygous transgenic litter mates **(C)** in relation to fluctuation of hypocretin-1 levels in the extracellular space in normal rats. Significant differences between the genotypes (*t*-test; $p < 0.05$) are marked by asterisks. The dark phase is denoted by the horizontal bar. Although *orexin/ataxin-3* hemizygous transgenic animals exhibit a clear diurnal distribution patterns of non-REM sleep, total wakefulness time was reduced during the dark phase in these narcoleptic rats. The results of the microdialysis experiment in rats conducted by Yoshida et al. *(18)* demonstrate that hypocretin-1 levels build up toward the end of the active phase, and thus lack of the buildup of hypocretin may contribute to this difference. The diurnal distribution of REM sleep in hypocretin neuron-ablated rats was significantly impaired, and it appears that distribution patterns of REM sleep are highly dependent on the availability of the hypocretin system. SWS, slow-wave sleep. (Adapted from refs. *18* and *23*.)

subjects may have various REM sleep-related abnormalities during the daytime (such as frequent REM sleep episodes during daytime naps) as well as sleep onset and offset.

10. THE ROLE OF HYPOCRETIN IN VIGILANCE CONTROLS AND THEIR INTERACTION WITH OTHER REGULATORY SYSTEMS

The results of monitoring hypocretin tonus over 24 h consistently showed that hypocretin-1 release is high during the active phase, when animals spend most of the time awake, and low during the resting/sleeping phases *(16,18–20,46)*. Several in vivo manipulations that enhance hypocretin release were identified; forced wakefulness by behavioral manipulation and some wake-promoting compounds elevated hypocretin-1 levels (Table 1). Stress reactions, locomotor activation, and high brain temperature are often associated with forced wakefulness and high arousal conditions, and these may also be involved in activation of the hypocretin system. The hypocretin system, the most unique from other classical neurotransmitters, is also activated by fasting or low glucose utilization in the brain *(14)*.

Table 1
Known Behaviors/Manipulations Reported to Increase/Decrease Hypocretin Tonus

Increase
Physiological wakefulness
Forced wakefulness (sleep deprivation)
Exercise
High temperature
Wake-promoting compounds (modafinil, amphetamine)
Fasting/low-glucose utilization
Stress reactions
Decrease
Physiological sleep
Sleep (non-REM and REM sleep) rebound

Experimental evidence has suggested that the manipulations listed in the table are associated with high or decreased hypocretin tonus. It is not known whether these factors directly affect hypocretin tonus or are secondary to other effects, and some of these changes are also tightly connected with each other. Neurotransmitters activating hypocretin tonus are also known, including glutamates and acetylcholine. Ghrelin also activates hyocretin neuronal activity. In contrast, norephinephirnie and serotonin, as well as leptin, inhibit hypocretin activity.

These observations suggest that hypocretin neurons are activated during the wake phase by various alerting stimuli to maintain wakefulness and are inactive during the sleep phase. The activities of the monoaminergic neurons in the posterior hypothamaus (histaminergic neurons in the tuberomamimillary neurons [TMN]) and brainstem (noradrenergic LC neurons and serotonergic neurons in the dorsal raphe [DR]) are also strongly associated with behavioral states. They fire tonically during waking, and less during non-REM sleep and are silent during REM sleep *(68–70)*. This regulation might be mediated at least in part by hypocretin neuronal activity, since hypocretin neurons project to and potently excite these neurons, inducing alertness *(9,10,71)*. Hypocretins also have a strong excitatory effect on cholinergic neurons of the basal forebrain (mostly wake/REM active) that plays an important role in behavioral and electrocortical arousal *(72)*, and thus this mechanism is also important for maintaining wakefulness.

One of the important findings discussed above is that the diurnal distribution of REM sleep is highly dependent on the hypocretin system. Hypocretin neurons project directly to the cholinergic pedunculopontine (PPT) and laterodorsal tegmental (LDT) nuclei *(5)*. Injections of hypocretin-1 into the LDT of cats resulted in an increase in wakefulness and a decrease in REM sleep *(73)*. However, LDT/PPT cholinergic neurons include heterogeneous groups regarding activity during REM sleep: REM-on neurons that are preferentially active during REM sleep *(74–76)* and help to generate REM sleep and produce atonia through descending projections *(77)*. The lesions of the LDT/PPT region abolish REM sleep *(78,79)*. The wake/REM active neurons also exist in LDT/PPT neurons. Although local administration of hypocretin generally excites most brainstem cholinergic neurons, the physiological role of hypocretin input to these cholinergic neurons in vivo whole system is not known. Hypocretin may selectively excite, inhibit (through inhibitory interneuron), or modulate the occurrence of REM sleep. Recent anatomical and electrophysiological experiments revealed that these aminergic/cholinergic neurons send inhibitory input to the ventrolateral preoptic area (VLPO) sleep-active neurons, thereby maintaining wakefulness *(80)*. In the absence of hypocretin, these arousal regions may have reduced or nonsynchronized activity, resulting

in an inappropriately low threshold for transition into non-REM sleep (sleep fragmentation). Hypocretin neurons might also be necessary for maintenance of non-REM sleep.

Recent electrophysiological studies using hypothalamic slices of transgenic mice expressing green fluorescent protein (GFP) in hypocretin neurons have also identified several activators and inhibitors of hypocretin neurons. It was shown that agonists of ionotropic glutamate receptors (AMPA and NMDA) excite hypocretin neurons, whereas glutamate antagonists reduce their activity *(13,81)*. Several researchers have hypothesized that amines should excite hypocretin neurons, forming positive feedback loops that would maintain wakefulness *(65)*. However, Yamanaka et al. *(13)* reported that both nonadrenaline and serotonin hyperpolarize GFP-expressing hypocretin neurons, suggesting more complex feedback loops for the monaminergic hypocretinergic interactions.

Some of the mechanisms for the activation of hypocretin neurons during fasting are also becoming elucidated by in vitro electrophysiological studies. The results of these studies revealed that peripheral humoral factors related to energy metabolism modulate the activity of hypocretin neurons; the activity of isolated hypocretin neurons is inhibited by glucose and leptin and stimulated by ghrelin *(14)*.

Proper maintenance of arousal during food seeking is essential for an animal's survival. Therefore, these two vital physiological processes, feeding and vigilance control, should be tightly linked. When faced with a negative energy balance owing to reduced food availability, mammals respond behaviorally with phases of increased wakefulness and locomotor activity that support food seeking *(82–86)*. Hypocretin neurons in the LHA are anatomically well located to connect energy homeostasis and wakefulness. In this regard, Yamanaka et al. *(14)* recently reported that hypocretin neuron-ablated narcoleptic mice fail to respond to fasting with increased wakefulness and activity. Thus it appears that the hypocretin system plays important roles in the molecular and physiological basis of this evolutionarily conserved phenomenon.

11. CONCLUSIONS

A loss of hypocretin neurotransmission causes sleep disorder narcolepsy in humans and animals. The hypocretin system is a recently discovered hypothalamic neuropeptidic system involved in vigilance control and various fundamental hypothalamic functions (*see* review in ref. *87*).

Recent experiments revealed that hypocretin-1 release is high during the active phase when animals spend most of their time awake and low during the resting/sleeping phases. Several in vivo manipulations that enhance hypocretin release were identified, and forced wakefulness by behavioral manipulation and some wake-promoting compounds elevated hypocretin levels. Stress reactions, locomotor activation, and high brain temperatures are often associated with forced wakefulness, and these may also be involved in activation of the hypocretin system. Most uniquely, hypocretin systems are also activated by fasting or decreased glucose utilization in the brain.

These observations suggest that hypocretin neurons are active during the wake phase, maintaining wakefulness by various stimuli, whereas they are inactive during the sleep phase. Anatomical and functional findings suggest that this regulation is likely to be mediated through activation of other wake-active monoaminergic and cholinergic neurons as well as inhibition of the hypothalamic sleep-promoting system

The phenotype of hypocretin-deficient narcolepsy is characterized as fragmentation of sleep and wake (instability of vigilance state) and dissociated manifestations of wake and REM sleep. Narcoleptic subjects could not maintain wakefulness and did not respond to the

alerting stimuli as normal people do. Animal experiments demonstrated that diurnal distribution of REM sleep is also highly dependent on the hypocretin tonus. Thus, the hypocretin system is likely to be involved in (1) stabilizing the vigilance state, (2) mediating various alerting stimulus (including peripheral humoral factors related to energy metabolism) to wakefulness, and (3) REM sleep inhibition/modulation.

Recent electrophysiological studies have begun to reveal the in vitro cellular mechanisms modulating hypocretin tonus. A complex feedback loop with the monoaminergic and cholinergic systems is likely to be involved in this regulation; however, the precise afferent system/mechanism mediating these effects is not yet known. The hypocretin system is one of the most critical systems for regulating vigilance, linking fundamental hypothalamic functions required for survival, and it is essential to gain further knowledge of this system in physiological and pathophysiological conditions.

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14

Hypocretin System and Aminergic and Cholinergic Systems in the Control of Vigilance

Krister S. Eriksson, PhD, and Helmut L. Haas, MD

1. INTRODUCTION

The hypothalamus plays a central role in the maintenance of homeostasis by integrating central and peripheral signals and controlling behaviors like sleep and food intake. Neural pathways connecting different hypothalamic and brainstem nuclei, peripherally released hormones, receptors, and outputs from the hypothalamus constitute the anatomical substrate for this homeostatic regulation. It has long been known that the posterior hypothalamus acts as a waking center and that damage to this region causes profound increases in the time spent sleeping, whereas the anterior hypothalamus contains regions that promote sleep, and lesions in this region cause insomnia *(1,2)*.

The hypocretin/oxerin neurons of the posterior hypothalamus innervate the whole brain and spinal cord and send especially dense projections to the aminergic cell groups, i.e., the tuberomammillary nucleus (TMN), raphe nucleus, locus coeruleus (LC), and ventral tegmental area (VTA; *3,4*). In this chapter we review the recent findings regarding the roles of these neurons, and their apparent interaction, in the regulation of waking. As some data indicate that the histaminergic (HAergic) neurons of the TMN are an especially important target for hypocretin signaling *(5)*, and since this nucleus has received less attention in the literature compared with the other aminergic systems, we emphasize the literature on the histaminergic system.

2. HYPOCRETIN

Hypocretin neurons are diffusely distributed in the lateral hypothalamus and they lack distinctive morphological features, which has hampered electrophysiological recordings from these neurons. Recently transgenic mice expressing green fluorescent protein in the hypocretin neurons have been used to characterize these neurons. Hypocretin neurons exhibit variable firing patterns; some are silent, some fire single-action potentials, and others fire bursts of action potentials. During depolarization, they are able to fire at very high frequency (up to 200 Hz) with very little frequency adaptation *(6,7)*. There appear to be no properties that distinguish hypocretin neurons from neighboring neurons, so recording from them will remain problematic.

Published reports of the action of hypocretin in aminergic and cholinergic neurons have uniformly described them as excitatory. Thus, the peptides have been shown to have excitatory actions or to increase the intracellular Ca^{2+} concentration in, e.g., the TMN and the arcuate, dorsal raphe, LC, lateral and medial hypothalamic, and laterodorsal tegmental (LDT) nuclei; they can also increase transmitter release (Fig. 1) via presynaptic actions *(3,8–13)*.

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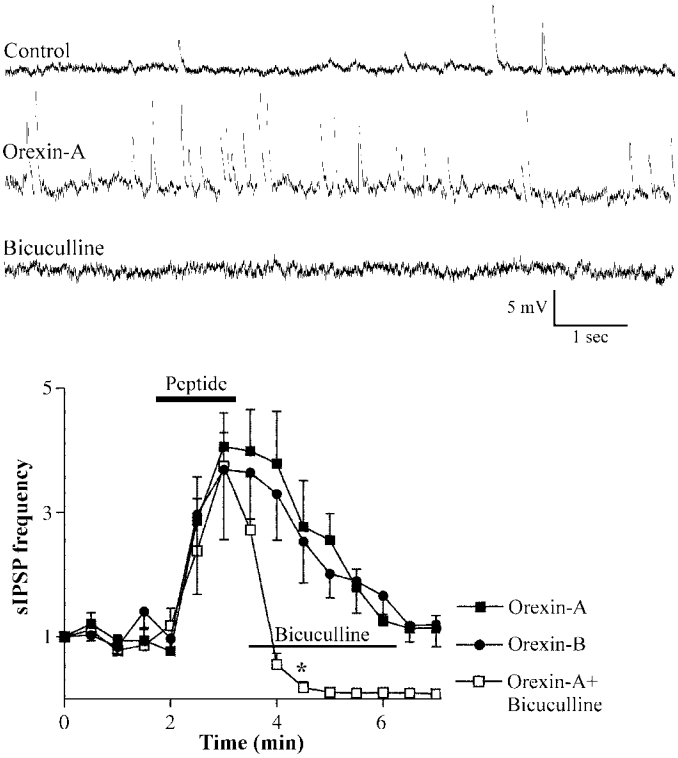

Fig. 1. Intracellular recordings from TMN neurons in brain slices. Both hypocretin/orexin peptides increase the frequency of inhibitory postsynaptic potentials (IPSPs) in TMN neurons. The $GABA_A$-receptor antagonist bicuculline completely suppresses the hypocretin-induced IPSPs, but an increased membrane noise is unaffected by bicuculline. As can be seen in the graph, both peptides increase IPSP frequency with equal potency and bicuculline reduces the number of IPSPs which exceed the detection threshold to values below control conditions. (Reprinted from ref. *44* with permission from Blackwell Publishing).

Li and colleagues *(6)* studied the responses to neurotransmitters of these neurons and found that noradrenaline (NA) and serotonin (5-HT) hyperpolarized the hypocretin neurons via postsynaptic actions. Thus, the LC and raphe neurons exert a negative feedback on the hypocretin neurons. Yamanaka and coworkers *(7)* found that carbachol depolarized hypocretin neurons, which suggests that the cholinergic neurons in the pedunculopontine tegmental (PPT) and LDT nuclei form a positive feedback circuitry with the hypocretin neurons. As these neurons are active during rapid eye movement (REM) sleep, it is possible that they activate the hypocretin neurons during this state. However, Li and colleagues *(6)* did not see any effect when they applied acetylcholine. In spite of the described HA-immunoreactive axons that contact hypocretin neurons *(10)*, neither of the groups detected any effect of HA on these neurons. This discrepancy may be caused by variations in receptor expression and innervation between subgroups of the hypocretin neurons. Both hypocretin peptides depolarize the hypocretin neurons. This is not a postsynaptic effect, however, as hypocretin excites local glutamatergic neurons that regulate the activity of hypocretin neurons *(6)*, thereby forming a positive feedback circuit. In the light of this, it is interesting that the hypocretin neurons are sensitive to excitotoxic insults. Incubating hypothalamic slices with glutamatergic agonists led to selective loss of hypocretin neurons, whereas surrounding neurons were largely spared *(14)*.

Such a positive feedback loop could render the hypocretin neurons especially sensitive to conditions that lead to excessive local glutamate release, e.g., anoxia. The hypocretin neurons are also inhibited by γ-aminobutyric acid $(GABA)_A$-receptor agonists.

Hypocretin neurons have also been implicated in the regulation of feeding, and a recent paper supports this notion, as such neurons respond to regulatory feeding signals. Thus, these neurons are inhibited by leptin and by increased glucose levels, whereas they are excited by ghrelin *(15)*.

3. HISTAMINE

The HAergic neurons of the TMN in the posterior hypothalamus (Fig. 2) send out varicose axons that innervate most parts of the central nervous system (CNS; *16*). Electrophysiological studies of TMN neurons show that their regular pacemaker-like firing pattern is controlled by an ensemble of membrane currents that maintain a stable firing rate (2–5 Hz) even in synaptic isolation *(17)*. The activity of the TMN neurons is strongly associated with behavioral state, and they fire tonically in a regular pattern during waking, little during light slow-wave sleep (SWS), and not at all during deep SWS and REM sleep *(18)*.

The physiological functions in which the HAergic system have been implicated include the regulation of waking and feeding behaviors *(19)*. Several pharmacological studies have shown that HA influences the ability to sustain waking and suppress sleep, especially SWS. Thus, treatment of rats with an inhibitor of HA synthesis leads to significantly increased sleep during the dark phase, while leaving the total 24-h amount of sleep unaffected *(20)*. In the cat, inhibition of HA synthesis increases sleep, whereas prolonged waking is induced if the HA levels are increased with an inhibitor of HA catabolism *(21)*. The release and production of HA is controlled by autoinhibiton via H_3 receptors located on the TMN neurons *(22)*, which makes it possible to influence the levels of HA with agonists and antagonists of H_3 receptors. Thus, oral application of an H_3 agonist to the cat leads to a dramatic increase in cortical slow activity and increase in SWS, whereas an H_3 antagonist induces cortical desynchronization and waking *(23)*. In addition to the cortex, the cholinergic basal forebrain appears to be an important target for HA's waking action. Injections of HA in this region greatly reduce SWS without affecting the amount of REM sleep *(24)*.

It is well known that, in humans, the classical antihistamines, which are H_1-receptor antagonists, used to treat asthma and allergy cause sedation and drowsiness, and one would therefore expect that HA's effect on waking would be mediated by this receptor. Mepyramine, an H_1-receptor antagonist, causes an increase in cortical slow waves, a decreased latency to SWS, and a prolongation of SWS that is associated with decreases in both waking and REM sleep in the cat *(25)*. Activation of the H_1 receptor with selective agonists, on the other hand, markedly increases wakefulness and decreases all phases of sleep in rats *(26,27)*. It has recently been shown that knockout (KO) mice lacking either the HA-synthesizing enzyme histidine decarboxylase or the H_1 receptor are unable to stay awake in response to environmental changes. In the beginning of the lights-off period, wild-type mice spend a comparatively large amount of time awake, and if they are transferred to another cage, they may stay awake for several hours. These waking responses are greatly diminished in the KO mice *(28)*. Furthermore, H_1-KO mice have a disturbed circadian rhythm of locomotor activity, with increased locomotion during the light phase and decreased locomotion during the dark phase *(29)*.

Both anatomical and physiological evidence exists for an interaction between the hypocretin and TMN neurons. The TMN neurons are densely innervated by hypocretin-containing axons that form synapses onto their somata and proximal dendrites *(4,30)*. The hypocretin

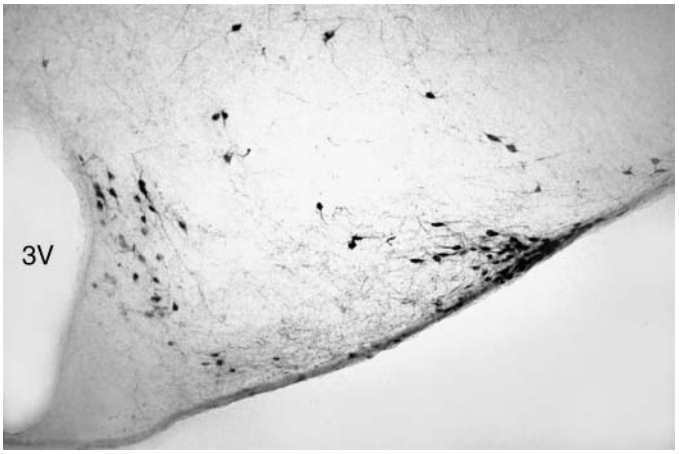

Fig. 2. The tuberomammillary nucleus of mouse posterior hypothalamus. The neurons are immunocytochemically stained for histamine. The lateral (ventral) part of the nucleus, which contains most of the histaminergic neurons is seen at the right, and the medial (dorsal) part of the nucleus is located next to the third ventricle (3V).

neurons also receive innervation by HA-IR axons that appear to terminate on their somata, and there is some overlap between the anatomical distributions of these two neural populations *(10)*. It has been shown in vitro that both hypocretin-1 and -2 strongly depolarize the TMN neurons and increase their firing rate (Fig. 3) via a postsynaptic mechanism involving the hypocretin-2 receptor *(10,31,32)*, and injection of hypocretin-1 into the TMN nucleus markedly increases HA release *(5,33)*. Hypocretin's effect on wakefulness seems to be largely mediated by the HAergic system and activation of the H_1 receptor. In rats, injection of hypocretin-1 directly into the TMN during the inactive light phase increases waking severalfold, and this resulting increase in waking is largely reversed by the H_1 antagonist pyrilamine. In mice, icv injection of hypocretin-1 during the light period increases waking to levels seen during the dark period, whereas its effect is completely absent in H_1-receptor KO mice *(5,32)*. In narcoleptic dogs, the HA levels are significantly decreased in regions that are important for HA-mediated cortical arousal (cortex and thalamus), whereas other amines are less affected *(34)*.

The TMN neurons are probably inhibited during sleep by the prominent GABAergic and galaninergic inputs they receive from the sleep-active neurons in the ventrolateral preoptic area (VLPO), and both GABA and galanin inhibit the TMN neurons *(35–38)*. Injections of the $GABA_A$-receptor agonist muscimol into the posterior hypothalamus cause hypersomnolence *(39)*, and TMN neurons are the target for the sedative component of general anesthesia induced by agents acting on $GABA_A$ receptors, e.g., pentobarbital *(40)*. VLPO inhibition of TMN neurons appears to be important for transitions between waking and sleep; these transitions are disturbed in narcolepsy. The orexin neurons also produce dynorphin *(41)*, and dynorphin-containing axons densely innervate the TMN *(42)*, but dynorphin has no postsynaptic effect on the TMN neurons *(43)*. We found, however, that dynorphin suppresses GABAergic inputs to TMN neurons, thereby reinforcing hypocretin's excitatory postsynaptic action, with an additional presynaptic disinhibition of these neurons (Fig. 4). Thus, dynorphin acts in concert with hypocretin to increase the excitability of the TMN neurons *(44)*.

Adrenergic neurons of the brainstem are involved in the control of arousal and behavioral states (for references, *see* ref. *45*), and there is ample evidence for a functional interaction of the

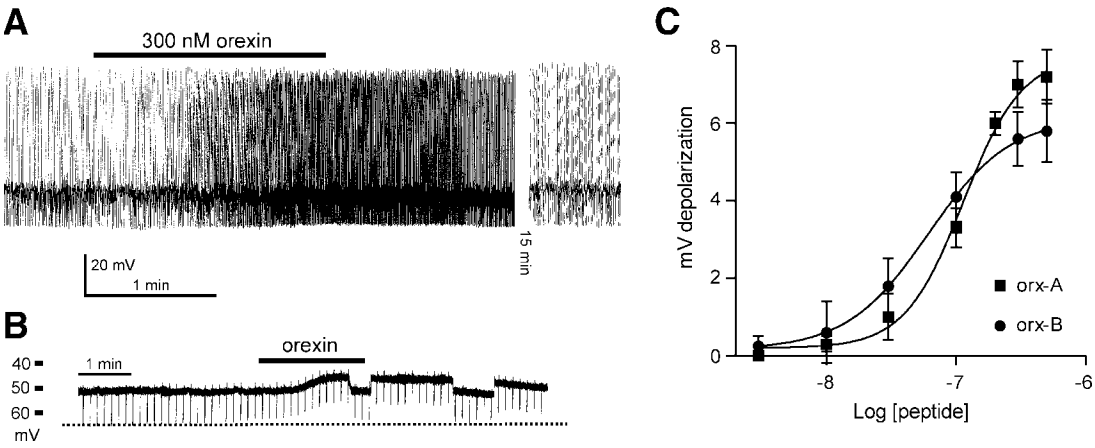

Fig. 3. Intracellular recordings from TMN neurons in hypothalamic slices. The bar indicates the presence of hypocretin in the recording chamber. **(A)** Orexin-A 300 n*M* increases the firing rate of the neuron; this effect was reversed after a washout period. **(B)** A neuron recorded in the presence of tetrodotoxin, which prevents firing and causes synaptic isolation. Orexin-A strongly depolarizes the neuron, indicating a postsynaptic action. Hyperpolarizing current pulses were used to study changes in the input resistance of the neuron. When the membrane potential is manually retuned to the resting value, a small decrease in the input resistance is seen. **(C)** Dose dependence of the depolarization. The data were obtained in the presence of tetrodotoxin and demonstrate the maximal postsynaptic effect of different doses of hypocretin-1 (orx-A) and hypocretin-2 (orx-B). (Reprinted from ref. *10*. Copyright 2001 by the Society for Neuroscience.)

adrenergic and HAergic systems *(46,47)*. The release of HA is inhibited by NA in the cortex as well as in the hypothalamus, and this action of NA appears to be mediated by α_2-receptors located on HA terminals *(48,49)*. The TMN itself receives strong projections from noradrenergic cell groups of the brainstem and LC *(50)*. NA has no direct postsynaptic effects on TMN neuron activity, whereas GABAergic inputs are inhibited presynaptically by adrenergic α_2-receptors. Inputs from noradrenergic neurons of the brainstem will thus reduce the GABAergic inhibition, resulting in disinhibition of TMN neurons *(51)*.

All parts of the TMN receive serotonergic inputs from the raphe nuclei *(50),* and the TMN neurons of monkey and rat express the 5-HT_{2C} receptor *(52)*. We found that 5-HT strongly excites TMN neurons by acting at the 5-HT_{2C} receptor (Fig. 5), and an in vivo microdialysis study has shown that 5-HT increases HA release *(53,54)*. Another study, however, suggests that histamine release is under tonic inhibition by 5-HT via 5-HT_{2A} receptors *(55)*.

4. SEROTONIN

The neurons of the midbrain raphe nuclei release 5-HT and give rise to ascending projections to the forebrain and cortex *(56)*. Their firing rate is highest during waking and lower during SWS, and they are silent during REM sleep *(57,58)*. Their firing is driven by a tonic NA input that excites them by acting on adrenergic α_1-receptors *(59,60)*.

Compounds that increase the release of 5-HT produce a quiet waking state and satiety, decrease eating, and may prevent sexual behavior. Such compounds can also be used in humans as anxiolytic and antidepressant drugs *(61)*. Lesioning of the cat dorsal raphe nucleus leads to prolonged insomnia and agitation *(62)*. This effect is not seen in rats, however *(63)*, and 5-HT_2 receptor antagonists support sleep *(64)*. Recent findings show that inhibition of

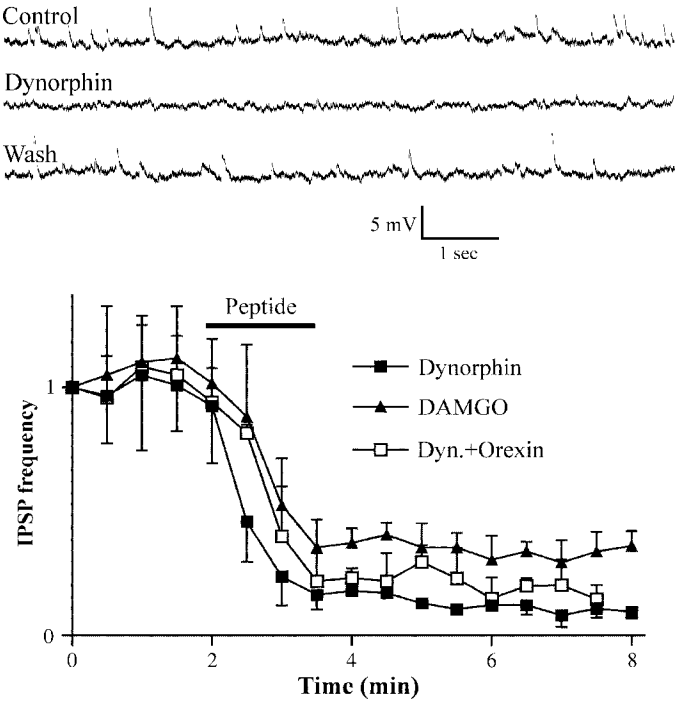

Fig. 4. Activation of opioid receptors suppresses inhibitory postsynaptic potentials (IPSPs) in TMN neurons. The trace shows the effect of the κ-receptor agonist dynorphin and the reversal of the dynorphin effect after a washout period. The μ-receptor agonist DAMGO also suppresses IPSPs. The graph shows the effects of dynorphin and DAMGO on IPSP frequency. Coapplication of dynorphin and orexin-A suppressed the IPSPs to a level that was not significantly different from that obtained with dynorphin alone. (Reprinted from ref. *44* with permission from Blackwell Publishing.)

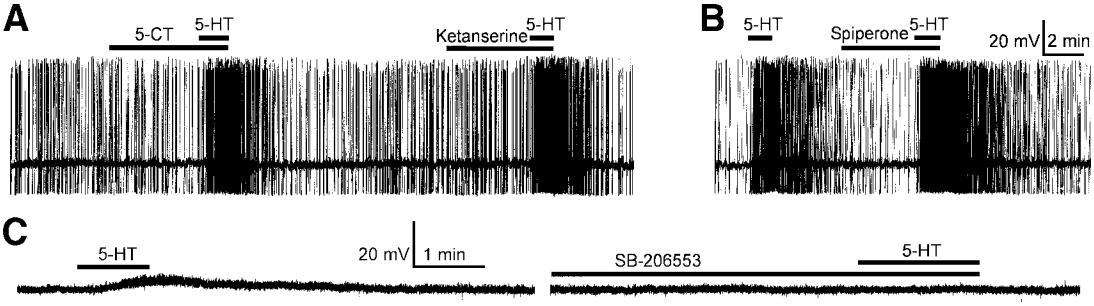

Fig. 5. The TMN neurons are excited by 5-HT. Some of the experiments done to identify the 5-HT receptor involved are shown. (**A**) The $5\text{-HT}_1/5\text{-HT}_7$ receptor agonist 5-carboxamidotryptamine (5-CT) does not affect the firing rate of this TMN neuron; 5-HT strongly increases it. After a washout period, the 5-HT_{2A} antagonist ketanserin does not affect the response to 5-HT. (**B**) Pretreatment with the 5-HT_{2A} antagonist spiperone does not affect the response to 5-HT. (**C**) A tetrodotoxin-treated TMN neuron is depolarized by 5-HT. After a washout period, during which the selective $5\text{-HT}_{2C/2B}$ antagonist SB-206553 is added to the superfusate, 5-HT has no effect, suggesting that the involved receptor is the 5-HT_{2c} subtype. (Reprinted from ref. *53* with permission from Elsevier.)

raphe nucleus activity induces waking, and the role of 5-HT in sleep generation remains unclear *(65)*.

Hypocretin excites the 5-HT neurons of the dorsal raphe nucleus *(66)*. In addition to exciting the 5-HT neurons, hypocretins also activate GABAergic neurons in the dorsal raphe, increasing inhibitory inputs to 5-HT neurons and thus providing a negative feedback during high levels of hypocretin activity *(67)*. The 5-HT neurons in the dorsal raphe nucleus are also excited by HA *(8)*, Thus, a positive feedback loop appears to exist between the serotonergic and histaminergic systems.

5. DOPAMINE

The dopaminergic neurons of the ventral mesencephalon are located in the VTA and substantia nigra (SN). They project through the medial forebrain bundle to the dorsal striatum (nigrostriatal projection) and to the basal forebrain, ventral striatum, and cortex (meso-limbocortical projection). The dopaminergic neurons either fire in a single-spike pattern or in bursts. Their firing rate does not appear to change over the sleep-wake cycle, but burst firing is induced by aroused and rewarding conditions, such as feeding *(68–70)*.

Although the dopamine (DA) system stimulates arousal and attention and supports locomotor and exploratory reward-seeking behaviors during waking, the role of DA in sleep-wake regulation is less studied. Drugs that increase the release of DA, such as cocaine and amphetamine, cause arousal and increased waking *(71)*, but these drugs are not selective for DA and also cause increased extracellular levels of 5-HT and NA. Modafinil is a relatively new drug that induces wakefulness, and there is evidence that this compound also increases extracellular DA levels by blocking DA reuptake, although there are sufficient pharmacological differences between modafinil and amphetamine to suggest some other mechanism of action, e.g., α_1-adrenoceptor agonism *(72–74)*.

The VTA is densely innervated by hypocretin-containing fibers, whereas the SN, mainly the caudal part, receives a rather low innervation *(4)*. We found that hypocretin excited GABAergic neurons in both the SN and the VTA *(75,76)*, and the firing rate of these neurons is highest during active waking and REM sleep *(69)*, which could reflect their excitation by hypocretin during these stages. Dopaminergic neurons of the SN were unresponsive to hypocretin, whereas DAergic neurons of the VTA either were unresponsive, increased their firing rate, or switched from regular firing to burst firing *(75,76)*. Recently a group of wake-active DAergic neurons in the periaqueductal gray have been described *(77),* and it is possible that these neurons together with the DAergic neurons in the VTA are more important for regulating wakefulness than those of the SN.

The SN and VTA are also innervated by HAergic axons *(78),* and HA inhibits DA release. The mechanism of this inhibition is excitation of GABAergic interneurons in the SN and VTA, which in turn inhibit the DAergic neurons via $GABA_A$ receptors *(79,80)*.

6. NORADRENALINE

The noradrenergic cells of the pontine LC form dense clusters adjacent to the fourth ventricle and give rise to a diffuse innervation of the whole CNS *(81)*. They fire tonically at their highest rate during active waking and less during SWS and go virtually silent during REM sleep *(82)*. The LC neurons are electrotonically coupled via gap junctions, and the whole nucleus can fire synchronously. During waking, their highest firing rates are associated with behavioral signs of arousal and attentiveness, including stress. During periods of sustained

attention, the LC neurons may switch to a burst firing pattern *(83,84)*. Several studies suggest that the LC system increases the animal's ability to process relevant stimuli while suppressing responses to irrelevant stimuli (for references, *see* ref. *85*).

Whereas NA clearly contributes to cortical activation and high enough LC firing rates appear to prevent sleep *(82,86)*, lesioning of the LC does not have long-lasting effects upon waking. Thus, the LC's main function may be to stimulate and enhance cortical activation and arousal, especially during periods of stress, rather than being necessary for the induction of these states during waking *(61,87)*.

The LC receives the densest innervation from the hypocretin neurons *(4)* and hypocretin excites the firing of LC neurons in vitro and increases arousal and locomotion in the rat when given icv *(11)*. Injections of hypocretin into the LC also greatly suppress REM sleep and increase waking at the expense of deep SWS *(88)*. The LC neurons cease firing during cataplectic episodes in narcoleptic dogs, supporting the idea that LC activity contributes to the maintenance of muscle tone in waking *(89)*.

The HAergic and NAergic neurons exhibit similar reciprocal excitatory actions. It has been shown that LC neurons are excited by HA *(90),* and we have shown above that NA increases the excitability of the TMN neurons. Interactions between the HA and the NA systems modulating release of several endocrine substances have also been reported *(46)*.

7. ACETYLCHOLINE

The cholinergic pontomesencephalic neurons are located in the LDT and PPT nuclei. They project dorsally to the thalamus and ventrally to the hypothalamus and ventral forebrain. The firing patterns of these neurons fall into two distinct categories: neurons that fire at rates above 10 Hz during waking, slow down during SWS, and fire at their highest rate during REM sleep and neurons whose firing rate is less than 2 Hz during waking and display an increase in SWS and even more in REM sleep *(91,92)*. This activity pattern indicates the importance of the pontine cholinergic neurons in the generation of neocortical desynchronization occurring during waking and REM sleep. These neurons also inhibit reticulospinal and spinal systems, to produce the muscle atonia normally seen in REM sleep *(93)*. Lesions that include the LTD and PPT do not affect cortical activation during waking but lead to a loss of REM sleep, suggesting that cholinergic neurons may be critically involved in the generation of cortical activation and muscle atonia during REM sleep *(94)*.

Serotonergic inputs to REM sleep-active cholinergic neurons in the PPT and LDT nuclei suppress REM sleep, and 5-HT or 5-HT_{1A}-receptor agonists inhibit these neurons in vitro *(95–97)*. It has also been shown that injections of 5-HT into the LDT area inhibit REM sleep, whereas inhibition of raphe neurons, and thus 5-HT release, increases REM sleep *(98,99)*.

8. INHIBITION OF THE AMINERGIC NUCLEI BY THE VENTROLATERAL PREOPTIC AREA

The HAergic neurons appear to gate the transition between waking and sleep; they fire regularly during waking, slow their firing during light SWS, and are silent during deep SWS and REM sleep *(18)*. Transitions between SWS and REM sleep are regulated by reciprocal monoaminergic (5-HT, NA)/cholinergic interactions in the brainstem. The monoaminergic neurons of the brainstem are most active during waking, decrease their activity during NREM sleep, and go almost silent during REM sleep. In contrast, brainstem cholinergic activity is high during waking and REM sleep and promotes the active state during this sleep state *(100)*.

The core region of the VLPO sends an especially dense projection to the TMN and may selectively promote SWS by inhibiting the TMN neurons, whereas the more diffuse parts of the VLPO project to the raphe and LC and may turn these neurons off, to liberate the cholinergic neurons from the aminergic inhibition and thereby promote REM sleep *(101)*. The VLPO is densely innervated by HAergic, 5-HTergic, and NAergic axons *(102),* and at least 5-HT and NA inhibit the firing of VLPO neurons *(103)*. It has been proposed that this arrangement could form a bistable "sleep switch" *(104)*, whereby the sleep-active VLPO inhibits the aminergic nuclei during sleep and the aminergic systems inhibit the VLPO during waking. Such an arrangement would ensure fast transitions between sleep and waking. Hypocretin probably acts as a stabilizer of the waking state by driving the aminergic nuclei during waking, thus preventing the sudden transitions from waking to sleep that are seen in narcolepsy, in which this stabilization is absent.

9. CONCLUSIONS

The hypocretin and aminergic and cholinergic systems, as well as the VLPO, are mutually interconnected and are thus able to modulate each other's activity. Although the different transmitter systems appear to have their unique roles, it is likely that they are partially redundant in the regulation of vigilance and that they may, to some extent, compensate for the loss of one of the systems. As the regulation of wakefulness and cortical activation is crucial for the survival of animals, it is not surprising that this function is distributed over several overlapping systems. As an example, it was recently shown that the HAergic system appears to be upregulated as a response to the lack of DAergic signaling in Parkinson's disease *(105)*.

Most of the prescribed anticataplectic drugs, including tricyclic antidepressants such as imipramine and chlomipramine, increase the availability of NA by inhibiting its uptake or degradation. Serotonin and DA reuptake inhibitors and (at least in dogs) H_3-receptor antagonists that increase the release of HA are also effective. This emphasizes the functional convergence and interactions among aminergic, cholinergic, and hypocretinergic signaling and food, mood, sleep, and motor functions (for references, *see* refs. *34* and *106*).

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15
Orexin and Hypothalamic Control of Sleep and Waking

Rodrigo A. España, PhD, and Thomas E. Scammell, MD

1. INTRODUCTION

The hypothalamus regulates emotions, eating, drinking, body temperature, circadian rhythms, autonomic tone, and pituitary function. It also controls sleep and wakefulness; however, until recently, little was known about the specific neurons and pathways that control behavioral state. With the discovery of orexin/hypocretin, a flood of important discoveries has provided key insights into the mechanisms that stabilize sleep and wakefulness, as well as the pathways that underlie the circadian timing of sleep. This chapter reviews the hypothalamic systems regulating sleep/wake behavior and highlights how the orexin neurons play an essential role in stabilizing wakefulness and integrating arousal with other hypothalamic functions.

2. OVERVIEW OF BRAINSTEM AND OTHER STATE-REGULATORY SYSTEMS

To understand the role of the hypothalamus in behavioral state control, it is helpful to first review some of the brainstem and midbrain systems (Fig. 1) that control sleep and wakefulness (for review, *see* ref. *1*). The reticular formation is a loose collection of neurons extending from the caudal medulla up through the core of the midbrain *(2)*. Through projections to the thalamus, the reticular formation induces a single-spike mode of firing in thalamic and cortical neurons that is necessary for cortical activation and wakefulness. The importance of this projection is clear in patients with lesions of the rostral reticular formation, who often have severe, persistent coma or hypersomnolence.

The brainstem also contains state-regulating neurons that produce norepinephrine (NE), serotonin (5-HT), and dopamine (DA). NE is synthesized in several brainstem nuclei, but the best understood of these regions is the locus coeruleus (LC). The LC projects extensively to the cortex and hippocampus, as well as to subcortical areas such as the thalamus and hypothalamus *(3,4)*. 5-HT is produced by the median and dorsal raphe (DR) nuclei as well as other regions *(5,6)*. The raphe nuclei project throughout the pons, midbrain, hypothalamus, striatum, hippocampus, and frontal cortex *(7)*. Neurons producing DA are abundant in the substantia nigra and the ventral tegmental area as well as in the posterior hypothalamus and several brainstem nuclei. These DA neurons innervate specific central nervous system (CNS) regions including the frontal cortex, striatum, limbic areas, and many parts of the thalamus *(8,9)*.

These aminergic systems are essential for the regulation of behavioral state. For example, LC and DR neuronal activity and neurotransmitter release is highest during waking, less so

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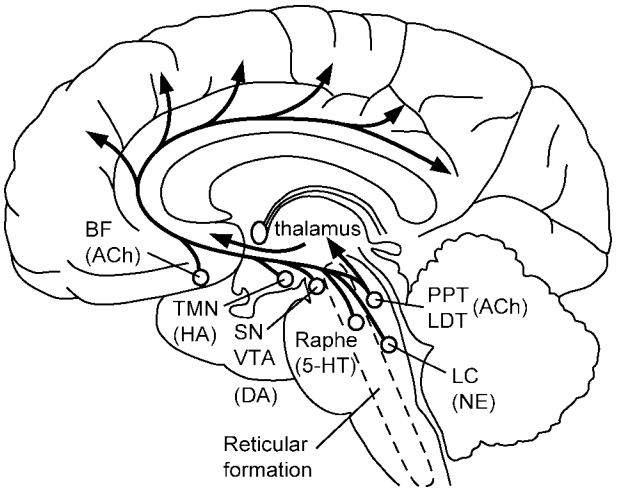

Fig. 1. Ascending arousal systems in the brainstem and posterior hypothalamus project throughout the forebrain. Cholinergic neurons in the pedunculopontine and laterodorsal tegmental areas (PPT/LDT) activate many forebrain targets, including the thalamus. Neurons in the locus coeruleus (LC), dorsal raphe (DR), tuberomammillary nucleus (TMN), substantia nigra and ventral tegmental area (SN/VTA), and basal forebrain (BF) excite many cortical and subcortical targets. The reticular formation projects to the thalamus, hypothalamus, and basal forebrain. ACh, acetyleholine; DA, dopamine; HA, histamine; NE, norepinephrine (Adapted from ref. *1*.)

during NREM sleep, and lowest during REM sleep *(10–17)*. Although the firing rates of DA neurons are less clearly linked to behavioral state, extracellular levels of DA are elevated during periods of waking and are at their lowest during sleep *(18–20)*. Manipulations that increase NE, 5-HT, or DA signaling elicit waking and reduce NREM and REM sleep *(21–29)*. In contrast, NE and DA antagonists decrease waking *(30–35)*. These observations suggest that NE, 5-HT, and DA are important for the regulation of wakefulness.

Cholinergic neurons in the laterodorsal and pedunculopontine tegmental nuclei (LDT and PPT) of the dorsal pons densely innervate the thalamus, lateral hypothalamus, and basal forebrain *(36–40)*. A separate group of cholinergic neurons in the basal forebrain projects heavily to the cortex, hippocampus, and amygdala *(38)*. Neurons in both of these cholinergic regions are active during waking and REM sleep but are relatively inactive during NREM sleep *(41,42)*. Consistent with these observations, acetyleholine (Ach) agonists produce desynchronized cortical activity and increase waking *(43–47)*, whereas ACh antagonists or lesions of the basal forebrain produce electroencephalographic (EEG) slowing *(48–51)*. These findings demonstrate that cholinergic neurons are necessary for full cortical activation.

The LDT/PPT also contain neurons that help generate REM sleep. These cholinergic neurons are preferentially active during REM sleep *(52–55)*, and lesions of the LDT/PPT region abolish REM sleep *(56–58)*. Like their wake-active counterparts, these REM-active neurons release ACh in the thalamus during REM sleep, thus activating the thalamus and producing cortical desynchrony. In addition, these neurons produce atonia through a series of descending projections. REM sleep is increased by drugs that enhance ACh signaling and is reduced by cholinergic antagonists *(44,59)*. These observations indicate that specific cholinergic neurons help drive the cortical activation and atonia of REM sleep.

The cholinergic and aminergic systems all promote wakefulness, but most likely, each arousal system is particularly active under specific circumstances. For example, through limbic

and striatal projections, DA may promote waking when an individual is motivated or physically active. In fact, enhanced DA signaling increases locomotor and behavioral activity, whereas reduced DA signaling produces the opposite effects *(29,60)*. Neurons in the LC are especially active during stress or when an animal encounters a novel stimulus *(13,14)*. After a mild stressor, mice lacking NE fall asleep more rapidly than controls *(61)*, suggesting that the NE system may be necessary for arousing to novel or important stimuli. Because wakefulness is the coordinated expression of many behaviors and physiologic changes, normal wakefulness most likely requires coordinated activity in all the arousal systems.

3. HYPOTHALAMIC WAKE-PROMOTING SYSTEMS

In the first half of the 20th century, clinical and experimental observations suggested that the hypothalamus plays a critical role in the regulation of arousal and sleep/wake behavior (for historical review, *see* ref. *62)*. Following up on Von Economo's *(116)* reports of intense sleepiness in patients with encephalitis of the posterior hypothalamus, Nauta and others demonstrated that mechanical and electrolytic lesions of the posterior hypothalamus produced marked and persistent somnolence in rats *(63,64)*. Additionally, Nauta observed that destruction of the anterior hypothalamus, in the region of the preoptic area, produced unrelenting insomnia. Based on these observations, Nauta agreed with Von Economo that the posterior hypothalamus probably contained a "waking center" and the anterior hypothalamus contained a "sleep center." More recently, many studies have expanded on these early observations and verified that the hypothalamus contains specific wake- and sleep-regulating neurons.

The lateral and posterior hypothalamus contain wake-active, wake-promoting neurons. Most neurons in these regions fire more rapidly during waking than during NREM sleep *(65–68)*. In anesthetized animals, electrical or chemical stimulation of the posterior hypothalamus increases EEG activation *(69–71)*. Most importantly, chemical lesions of these regions decrease waking *(72),* and inhibition of this region with the γ-aminobutyric acid (GABA) agonist muscimol increases NREM sleep *(73)*. Combined, these observations indicate that wake-promoting neurons in the posterior hypothalamus are critical for the production of wakefulness.

One of these wake-promoting systems is the histamine (HA)-producing neurons of the tuberomammillary nucleus (TMN). These posterior hypothalamic neurons are the only neuronal source of histamine, and they project extensively throughout the cortex, basal forebrain, hypothalamus, and brainstem *(74,75)*. As with other aminergic neurons, TMN neuronal activity is highest during waking, lower during NREM sleep, and lowest during REM sleep *(66,76)*. Drugs that enhance HA signaling increase waking, and extracellular levels of HA are high during spontaneous waking, especially at the beginning of active period *(77–79)*. Much of this arousing influence may be mediated by H_1 receptors, as drugs that block histamine H_1 receptors increase NREM and REM sleep in both animal studies and clinical practice *(77,78,80,81)*. On the other hand, H_3 agonists produce sleep, most likely by stimulating autoinhibitory H_3 receptors on HA and other aminergic neurons *(82)*. The importance of this system is apparent in mice unable to synthesize HA, which have less wakefulness at the beginning of the active period and do not maintain wakefulness in a mildly stressful environment *(83)*. Together, these observations indicate that histaminergic neurons promote waking and probably enhance arousal when animals are presented with challenging conditions.

4. OREXIN AND THE CONTROL OF BEHAVIORAL STATE

The orexin neurons play a unique role in the regulation of wakefulness and sleep. Orexin-A and -B (hypocretin-1 and -2) are synthesized solely by neurons in the lateral hypothalamus

(LH) and adjacent hypothalamic regions and bind to the orexin-1 and orexin-2 receptors *(84–88)*. Originally thought to promote feeding *(84,89)*, extensive evidence now suggests that the orexin system modulates arousal and arousal-related processes. For example, orexin neurons heavily innervate the LC, DR, TMN, PPT, LDT, and ventral tegmental area (84,86,90,91). When injected into the ventricles, LC, or within select basal forebrain regions, orexins increase waking and suppress NREM and REM sleep *(92–97)*. These wake-promoting actions are accompanied by marked increases in locomotor activity as well as other behaviors *(96,98–100)*.

Considerable evidence demonstrates that deficient orexin signaling is associated with narcolepsy. About 90% of narcoleptics with cataplexy have no detectable orexin in their cerebrospinal fluid *(101)*, and the brains of narcoleptics show a marked reduction in the number of orexin-producing neurons *(102,103)*. In mice, rats, and dogs, a lack of orexin/hypocretin or dysfunction of the orexin receptors produces narcolepsy-like symptoms, with poor maintenance of wakefulness and sudden episodes of cataplexy *(87,104–107)*.

The behavior of mice lacking orexin may be best described as behavioral state instability, with frequent transitions between sleep and wakefulness *(108)*. These mice lack consolidated waking and NREM sleep and display episodes of cataplexy resembling REM sleep atonia during wakefulness *(87)*. This poor consolidation of wakefulness is not caused by a circadian defect because even in constant darkness, the pattern of sleep/wake rhythms is normal in orexin knockout mice *(108)*. Unlike the NE and HA knockout mice *(61,83)*, orexin knockout mice remain awake just as long as normal mice when presented with a mild stress. In addition, orexin-deficient mice have normal accumulation and expression of homeostatic sleep drive. Thus, the fragmented wakefulness and sleep of orexin knockout mice appears to be caused by inappropriately low thresholds to transition between behavioral states.

The activity profile of the orexin neurons is not well understood, but examination of Fos-immunoreactivity (IR) in the orexin neurons has provided several useful insights. Fos is a transcription factor that can be rapidly induced by synaptic activity and elevated intracellular concentrations of cAMP or calcium *(109,110)*. Although often used as a marker of increased neuronal activity, Fos-IR has several limitations. For example, Fos may persist in neurons for several hours after a stimulus *(111)*, and thus, Fos production does not have the temporal resolution for studying relatively brief phenomena such as REM sleep in rodents. In addition, increased Fos-IR is not always caused by conditions that increase neuronal firing rates or neurotransmitter release *(100,110,112)*. Lastly, one cannot determine whether Fos expression in a particular neuron causes a behavior or is simply a consequence of that behavior. Thus, although Fos is a useful marker of neuronal activity, it should be interpreted cautiously.

Using Fos-IR as a marker of neuronal activation, we examined the activity of orexin neurons across the sleep-wake cycle of rats *(113)*. During the night, in animals that were mainly awake, about half of the orexin neurons contained Fos-IR. Fos expression in these cells was much lower during the day when the rats were mainly asleep. Ambient light had no effect on the activity of the orexin neurons, but sleep deprivation, methamphetamine, and modafinil all increased Fos-IR in the orexin neurons *(87,113,114)*. Across all these condition, orexin neuron Fos expression correlated with the amount of wakefulness, body temperature, and the amount of locomotor activity. These studies demonstrate that the orexin neurons are active during wakefulness and suggest that other factors such as locomotor activity may also be influential.

In further experiments, España et al. *(100)* examined the degree to which orexin-synthesizing neurons and orexin-receptive neurons display increased Fos-IR across the sleep-wake cycle

and in response to novelty stress. Compared with rats that were sleeping or spontaneously awake during the day, rats spontaneously awake during the night had more Fos-containing orexin neurons. Interestingly, when introduced into a moderately stressful novel environment during the day, animals displayed higher levels of Fos within orexin-synthesizing and orexin-1-receptor-expressing- neurons in the LC and basal forebrain compared with spontaneous waking at night. Similar to the Estabrooke studies, increases in Fos were correlated with time spent awake; however, increases in Fos were significantly more correlated with the amount of locomotor and behavioral activity preceding sacrifice. This observation is consistent with experiments in cats showing increased Fos expression mainly with increased locomotor activity *(115)*. Combined, these observations indicate that the orexin neurons are active during wakefulness, especially in association with stress, locomotion, and other high-arousal conditions.

5. HYPOTHALAMIC SLEEP-PROMOTING SYSTEMS

Von Economo *(116)* and Nauta *(63)* showed that large clinical or experimental lesions of the anterior hypothalamus produce insomnia. Subsequent studies determined that injury to the preoptic area (the most rostral part of the anterior hypothalamus) produced marked reductions in sleep *(117–119)*, whereas electrical or chemical stimulation of this region increased NREM sleep *(120–123)*. Many of these sleep-active neurons are concentrated in the ventrolateral preoptic area (VLPO) and extend up into the median preoptic nucleus *(124–130)*. Fos expression in VLPO neurons is strongly correlated with the amount of sleep during the 1–2 h before sacrifice *(129,131,132)*. Electrophysiological recordings in freely moving animals demonstrated that VLPO neurons fire rapidly during NREM and REM sleep and are virtually inactive during waking *(133)*. During sleep deprivation, VLPO neurons fire infrequently, but during the subsequent deep recovery sleep, they fire rapidly, indicating that the activity of these cells does not code for "sleep drive" but rather reflects the intensity of sleep. Consistent with the idea that these are essential sleep-producing neurons, lesions of the VLPO and adjacent regions substantially reduce the amounts of NREM and REM sleep *(119,134,135)*. Most VLPO neurons contain the inhibitory neurotransmitters GABA and galanin *(132,136)*, and they directly innervate wake-promoting brain regions such as the TMN, LC, DR, LDT/PPT, LA (Fig. 2) *(136)*. Through these inhibitory projections, VLPO neurons probably promote sleep by coordinating the inhibition of these arousal regions.

The activity of these sleep-producing preoptic neurons may be regulated by endogenous somnogens such as adenosine and prostaglandin D_2. Prolonged waking increases the concentration of adenosine in the basal forebrain, and adenosine levels fall rapidly during the subsequent recovery sleep *(137)*. Central administration of adenosine agonists or prostaglandin D_2 increases sleep and EEG delta power *(138,139)*. These somnogens also increase Fos within VLPO neurons, suggesting that they may produce sleep by activating the VLPO *(140,141)*. Adenosine may promote sleep by disinhibiting the VLPO and inhibiting wake-active neurons *(142,143)*, but the mechanism through which prostaglandin D_2 acts remains unclear. Sleep is influenced by many factors, and activation of preoptic neurons by endogenous somnogens may play an essential role in driving sleep.

6. DYNAMIC CONTROL OF BEHAVIORAL STATE: THE FLIP-FLOP SWITCH

When animals switch between behavioral states, they spend little time in intermediate states. This is clearly adaptive for survival, as an animal that performed daily tasks in a state of muddled drowsiness, neither fully awake nor asleep, would be in danger from predators

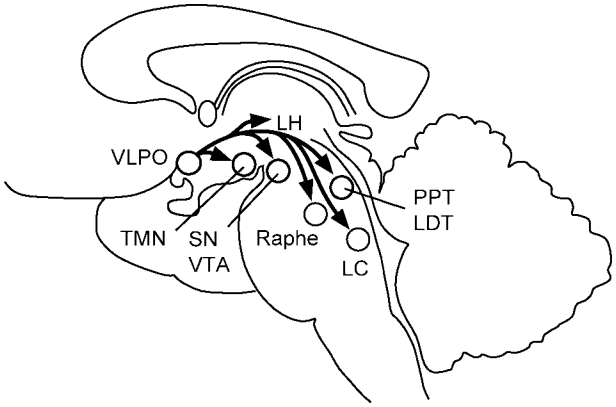

Fig. 2. The ventrolateral preoptic area (VLPO) contains sleep-promoting neurons. Neurons of the VLPO produce GABA and galanin and inhibit all the arousal systems during NREM sleep. Many of these cells are active during REM sleep as well. All the arousal systems, with the notable exception of pedunculopontine and laterodorsal tegmental areas (PPT/LDT), also project to the VLPO. LC, locus coeruleus; LH, lateral hypothalamus; SN, substantia nigra; TMN, tuberomammillary nucleus; VTA, ventral tegmental area. (Adapted from ref. *1*.)

and an inability to carry out essential tasks. The generation of discrete behavioral states and the ability to switch rapidly between states may be governed by reciprocal interactions between sleep- and wake-regulating regions *(144)*.

The sleep-producing neurons of the VLPO receive substantial aminergic inputs *(145)* and are inhibited by NE, ACh, and 5-HT *(146)*. During wakefulness, high monoaminergic activity probably inhibits the VLPO, thus reducing inhibition of the arousal regions that further enhances their activity (Fig. 3). Conversely, during sleep, VLPO neurons are active and probably inhibit the arousal regions, thus disinhibiting and reinforcing their own firing. This mutually inhibitory relationship may create a bistable feedback loop that avoids intermediate states and inappropriate transitions between states when input signals to the VLPO or the arousal regions transiently fluctuate. However, large-scale changes, such as rising homeostatic pressure for sleep, should gradually shift the balance, eventually triggering a reversal of firing patterns. This neural switch therefore changes behavioral state infrequently but rapidly, just like the flip-flop circuits familiar to electrical engineers.

A loss of wake- or sleep-regulating neurons should destabilize this switch, resulting in poor control of behavioral state. Accordingly, rats with lesions of the VLPO display half the normal amount of NREM sleep, very brief sleep episodes, and they enter sleep much more frequently than normal *(134)*. Most likely, their sleep is easily disrupted because they are unable to inhibit the arousal regions in a coordinated fashion. Their fragmented sleep may result in persistently high homeostatic sleep drive, as indicated by their many attempts to sleep.

7. OREXIN STABILIZES THE FLIP-FLOP SWITCH

The physiologic consequences of deficient orexin signaling demonstrate orexin's important role in stabilizing the flip-flop circuit. People with narcolepsy have frequent, although usually brief, naps and tend to awaken several times during the night. They also quickly transition into REM sleep and have REM sleep-like phenomena such as hallucinations and

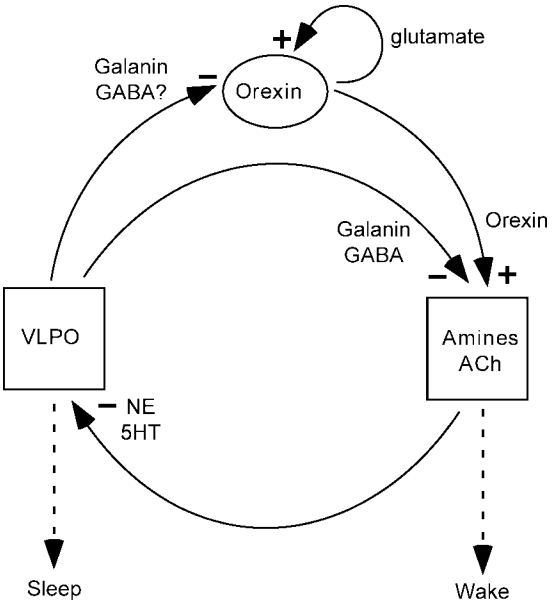

Fig. 3. Reciprocally inhibitory pathways may help stabilize sleep and wakefulness. Aminergic and cholinergic (Ach) neurons promote wakefulness. Ventrolateral preoptic area (VLPO) neurons help generate sleep by inhibiting these arousal regions using γ-aminobutyric acid (GABA) and galanin. During wakefulness, norepinephrine (NE) and serotonin (5HT) inhibit the VLPO neurons. This inhibition of the VLPO should foster more sustained activity in the arousal regions. During sleep, the VLPO neurons are not inhibited by amines, thus allowing sustained production of sleep. This mutual inhibition may help generate full wakefulness and sleep, with quick transitions between states. The orexin neurons help stabilize this circuit. Through an unusual positive feedback loop, orexin increases the activity of orexin neurons by increasing glutamate signaling. This sustained activity in the orexin neurons during wakefulness probably increases activity in the arousal regions, thus stabilizing wakefulness. In the absence of orexin, this flip-flop circuit becomes less stable.

paralysis (cataplexy) during wakefulness. Mice lacking orexin have similar deficits, including cataplexy and an inability to sustain wakefulness and sleep *(108,147)*.

Orexin probably influences both sides of the flip-flop circuit. Through direct, excitatory projections, orexin increases the firing rate of neurons in the LC, DRN, TMN, and ventral tegmental area *(92,148,149)*. Additionally, injection of orexin into the preoptic area, near the VLPO, increases wakefulness and decreases both REM and NREM sleep *(94,96)*. Most likely, this is not a direct influence on the VLPO neurons because they lack orexin receptors *(150)* and have no direct physiologic response to orexin *(151)*. Instead, release of orexin in the VLPO region may act presynaptically, enhancing the release of amines that then inhibit the VLPO. By increasing the activity of several arousal regions and their inhibitory projections to the VLPO, orexin should help keep the flip-flop switch in the "wake" position. In the absence of orexin, arousal region activity might be reduced, less consistent, or less coordinated, resulting in less inhibition of the VLPO and inappropriate transitions into sleep. In other words, without the activating influence of orexin, the flip-flop circuit becomes unstable and more vulnerable to small changes in homeostatic sleep drive or other factors.

In the clinic, narcolepsy is the most striking example of behavioral state instability, but this phenomena may not be unique to orexin deficiency. Neural network models and lesions of

the VLPO both suggest that injury to any part of the flip-flop circuit can produce instability, although each specific lesion may alter sleep architecture in a distinct fashion *(134);* (Regulation of sleep-wake timing: circadian rhythms and bistability of sleep-wake states, T.C. Chou, PhD thesis, Harvard University, 2003).

8. HYPOTHALAMIC TIMING OF SLEEP AND WAKEFULNESS

Circadian rhythms strongly govern the timing of REM sleep, the onset of wakefulness, and behavioral alertness *(152)*. These endogenously generated, daily rhythms are controlled by the suprachiasmatic nucleus (SCN), a small cluster of neurons just above the optic chiasm in the anterior hypothalamus. The SCN acts as a master clock, keeping time with an accuracy of a few minutes each day *(153)* and adjusting bodily rhythms to seasonal variations in day length. Most likely, each SCN neuron is a competent circadian pacemaker, and the coordinated activity of all SCN neurons results in highly accurate 24-h rhythms. The importance of this nucleus is apparent in people and animals with lesions of the SCN, who may sleep and wake at any time of day.

In addition to its role in timing behavior, the SCN may also promote and consolidate wakefulness. In monkeys, lesions of this nucleus reduce the daily amount of wakefulness and produce very fragmented waking *(154)*. These observations form the foundation of the opponent process model in which the SCN is hypothesized to have an alerting function that opposes rising homeostatic sleep drive across the day (154). In rodents, this wake-promoting role is less apparent, although lesions of SCN outflow regions do produce small reductions in wakefulness *(155)*.

The SCN drives the rhythms of sleep and wakefulness through projections to nearby hypothalamic regions. The SCN sends only minor, direct projections to brain nuclei implicated in state control *(145,156)*, but it heavily innervates several hypothalamic regions, especially an area just dorsal to the SCN known as the subparaventricular zone (SPZ) *(157,158)*. Lesions of the ventral SPZ markedly reduce the circadian rhythms of sleep/wake behavior, but the body temperature rhythm is relatively preserved, demonstrating that the pacemaker itself is intact *(159)*. The SPZ projects to the dorsomedial nucleus of the hypothalamus (DMH), a region implicated in autonomic stress responses, feeding, and the circadian release of corticosteroids *(160)*. Anatomically precise lesions of the DMH reduce the circadian rhythms of wakefulness, NREM, and REM sleep, with relatively little effect on the daily amounts of these states or the body temperature rhythm *(155)*. The DMH sends excitatory (glutamate and thyrotropin-releasing hormone) projections to wake-promoting regions such as the orexin neurons and LC *(155,161)*. A separate population of GABA-containing DMH neurons innervate the VLPO. Through these excitatory and inhibitory projections, the DMH may integrate rhythmic circadian signals with those mediating feeding and stress to help promote wakefulness and suppress sleep at the appropriate times of day.

Neurons in the lateral hypothalamus are essential for diurnal variations in sleep and wakefulness. Neurochemical lesions of the lateral hypothalamus in rats eliminate the nocturnal rise in wakefulness, resulting in 40–50% waking at all times *(162)*. Because these lesions markedly reduce the number of orexin neurons, one might hypothesize that orexin is essential for the timing of sleep/wake behavior. However, this now appears unlikely because orexin knockout mice housed in constant darkness have normal circadian variations in wake, NREM, and REM sleep *(108)*. Although this demonstrates that orexin peptides are not necessary for these rhythms, the "orexin neurons" are still present in these mice and contain other neurotransmitters including dynorphin and probably glutamate *(163,164)*. Studying the behavior of mice

lacking the orexin neurons *(165)* may help resolve this question. Alternatively, the loss of the wake rhythm with LH lesions may reflect injury to an as-yet-undiscovered class of wake-promoting cells.

Humoral factors secreted from the SCN also influence the timing of sleep. SCN-lesioned animals lack circadian rhythms, but rhythms can be restored with SCN transplants. This restoration of rhythmicity does not require synaptic connections because the daily rhythm of locomotor activity can be partially restored with SCN transplants encased in a polymer that blocks the outgrowth of neurites yet allows passage of small molecules *(166)*. These secreted timing factors include transforming growth factor-α (TGF-α) *(167)* and prokineticin 2 (PK2) *(168)*. In nocturnal rodents, TGF-α and PK2 expression is high during the day, and infusion of these peptides suppresses locomotor activity. TGF-α and PK2 receptors are expressed in targets of the SCN, including the SPZ and DMH. Chronic infusions of TGF-α block the circadian variation in sleep/wake behavior in a pattern very similar to that seen with lesions of the SPZ. Thus, release of TGF-α and PK2 from the SCN may act on nearby hypothalamic regions to drive the circadian rhythms of locomotor activity and wakefulness.

9. OREXIN NEURONS MAY INTEGRATE SLEEP/WAKE BEHAVIOR WITH OTHER HYPOTHALAMIC FUNCTIONS

For an animal to properly attend to and respond to its environment requires coordination of many physiologic functions. For example, a mouse foraging for food must be alert, hungry, and physically active with relatively high sympathetic tone (blood pressure, heart rate, metabolic rate, and so on). The orexin neurons are well positioned to coordinate these disparate physiologic functions.

Anatomic and physiologic studies highlight the multifunctional nature of the orexin system. The orexin neurons directly respond to neuromodulators implicated in feeding such as ghrelin and leptin *(169,170)* and send projections to appetite-regulating regions including the arcuate, paraventricular, and ventromedial nuclei of the hypothalamus *(86)*. Central injection of orexin during the light period dose-dependently increases feeding *(84,89,171–173)*, whereas blockade of orexin receptors suppresses feeding *(169,174)*. The orexin neurons innervate cells in autonomic regulatory regions such as the LH, ventrolateral medulla, nucleus of the solitary tract, and intermediolateral cell column *(86,175,176)*, and ventricular injection of orexin increases sympathetic activity and modulates body temperature *(177–181)* Through their projections to the arcuate nucleus and median eminence, the orexin neurons also influence neuroendocrine responses *(182)*. Few of these physiologic studies have controlled for the wake-promoting aspects of orexin, but orexin may increase autonomic tone, and the release of pituitary peptides beyond that seen with wakefulness itself.

The integrating role of the orexin neurons is apparent in the behavioral response to food deprivation. In normal mice, food deprivation increases waking and locomotor activity, perhaps to facilitate food-seeking behavior. However, these responses are absent in mice lacking the orexin neurons *(183)*. Food restriction markedly increases locomotor activity *(184,185)*, which promotes and consolidates wakefulness *(186)*. This locomotor response may depend on orexin, as exogenous orexin increases locomotion and wheel running *(92,96,99,100,187)*, and the orexin neurons are most active during periods of high locomotor activity *(100,113,115,188)*. The response to food deprivation may also be mediated by appetite-regulating signals. Although the hormones that mediate this response are not fully defined, food deprivation increases ghrelin *(189)*, and ghrelin excites the orexin neurons *(183,190)*.

Conversely, fasting decreases leptin levels, and leptin inhibits the orexin neurons *(183)*. Thus, a combination of increased locomotor activity, increased ghrelin, and decreased leptin may contribute to increased orexin signaling and wakefulness during food deprivation. Coordination of these signals confers an essential behavioral advantage, allowing a hungry animal to remain awake for longer periods and forage more effectively.

10. CONCLUSIONS

The orexin neurons play a central role in the hypothalamic control of sleep and wakefulness. By exciting arousal regions and indirectly inhibiting the VLPO, the orexin neurons help stabilize the flip-flop switch, avoiding inopportune transitions into sleep. The orexin neurons may also play an essential role in integrating hypothalamic signals related to stress and appetite into appropriate sleep/wake behavior. Despite advances in our current understanding of hypothalamic function, many questions remain regarding the hypothalamus and state control: Is homeostatic sleep drive caused by changes in hypothalamic neurochemistry or physiology? What brain regions are necessary for orexin's wake-promoting role? Does the LH contain other wake-promoting neurons besides the orexin neurons? Addressing these and related questions should lead to a much better understanding of the role of the hypothalamus in the control of sleep and wakefulness.

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16
Hypocretin/Orexin and Motor Function

Jerome M. Siegel, PhD

1. INTRODUCTION

As the literature on hypocretin/orexin has grown, so has the list of functions attributed to this system. Recent papers have routinely listed sleep and arousal control, blood pressure regulation, feeding, motor control, and others as functions for the hypocretin system *(1–4)*. The question I address here is whether such an inclusive listing is an appropriate acknowledgement of the complexity and subtlety of hypocretin's function or whether a simpler relationship may underlie the multiple correlations that have been seen.

Some neuronal cell groups have well-understood functions. Although motor neurons can be fairly described as being correlated with many different behaviors (e.g., food consumption, arousal, blood pressure elevation, and so on), the most accurate formulation is that they cause muscular contraction and that this is utilized in multiple functions. The same sort of argument can be applied to neurons in sensory pathways. However "interneurons" in integrative regions such as the hypocretin neurons of the hypothalamus cannot be so easily understood because their anatomy alone does not explain their function, and the relevant physiological and behavioral parameters are often unknown. In the case of the hypocretin system, the dynamics of its behavioral physiology are just beginning to be understood.

What we now know about the behavioral physiology of hypocretin neurons suggests that they function to support certain kinds of motivated motor activity *(5)*. According to this hypothesis, they facilitate muscle contraction and simultaneously activate brain arousal systems so as to allow appropriate perceptual processing of signals generated by movement. Many of the correlations with other behaviors can be best understood as a consequence of this relationship.

2. HYPOCRETIN RELEASE

We taken advantage of the ease of extracting cerebrospinal fluid (CSF) from the canine cisterna magna to perform a series of studies on hypocretin release. Hypocretin neurons are located in both the lateral and medial hypothalamus. The lateral hypothalamus is well known to have a role in feeding as well as other motivated behaviors. Because early studies administering large amounts of hypocretin to rats icv had suggested that this peptide might mediate feeding behavior, we studied the pattern of release of this peptide under food deprivation and feeding conditions *(6)*. Figure 1 shows the result. The level of feeding inducing peptides such as neuropeptide Y increases with food deprivation. However, hypocretin levels are not elevated across a 48-h period of food deprivation in the normal dog, instead showing a

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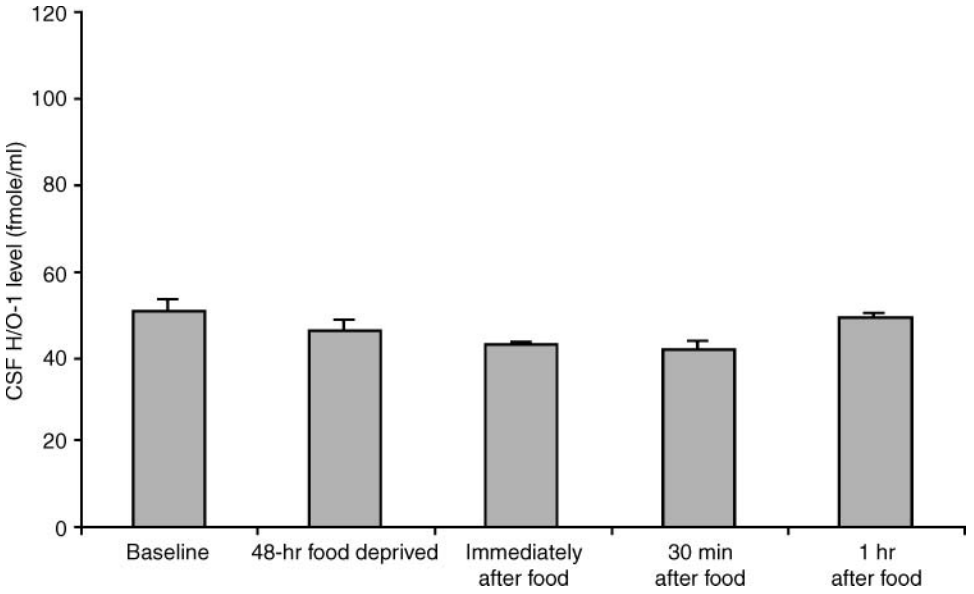

Fig. 1. Food deprivation and refeeding do not substantially alter hypocretin levels in the dog. CSF, cerebrospinal fluid. (From ref. *6*.)

nonsignificant decrease. We also sampled hypocretin at various intervals after feeding. We found that this also did not produce a significant change in hypocretin level.

Because our work and the work of others had implicated hypocretin loss in the pathology of human narcolepsy (*7–9*), we studied the effect of sleep deprivation under the same CSF sampling and assay conditions as our food deprivation and refeeding studies (*6*). We found that 24 h of sleep deprivation produced a significant and substantial increase in hypocretin (Fig. 2). However, when we studied the correlation between the amount of hypocretin elevation and sleep loss within our group of animals, we saw no relationship. However, we found that the amount of motor activity during sleep deprivation was strongly correlated with hypocretin elevation. This led us to hypothesize that motor activation or behaviors associated with motor activation, rather than sleep loss, might be the determinant of hypocretin level. Therefore we exercised these dogs for 30 min to 2 h and compared hypocretin levels after exercise with those in the same animals kept awake for the same period. Figure 3 illustrates that this manipulation produced a marked elevation of hypocretin level. This work demonstrates that exercise is sufficient to elevate hypocretin level even in the absence of differences in sleep.

We next studied the same phenomenon in normal cats. Figure 4 shows that we saw an elevation of hypocretin level with exercise (*10*). We were able to conduct the first microdialysis assay of hypocretin across the sleep cycle. We found that levels were maximal in active waking, reduced in non-REM sleep, and increased again in REM sleep (Fig. 5). This finding fits with the motor activation explanation of hypocretin release, since REM sleep is a state of internal motor activation, blocked at the periphery by motoneuron atonia.

3. DIRECT VERSUS INDIRECT ACTIONS OF HYPOCRETIN

Hypocretin release can activate the motor system through several routes. Torterolo and colleagues (*11*) have shown that hypocretin neurons directly contact motoneurons. This may

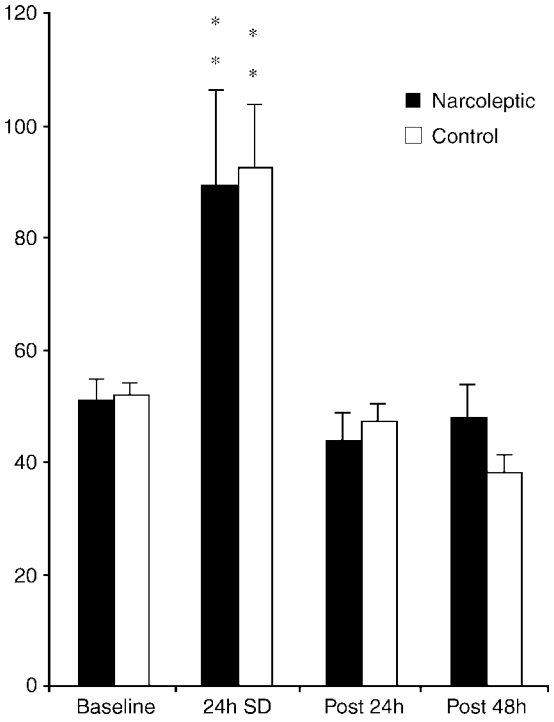

Fig. 2. Sleep deprivation produces a substantial increase in hypocretin levels in both normal and hypocretin receptor-2 mutant dogs. (From ref. *6*.)

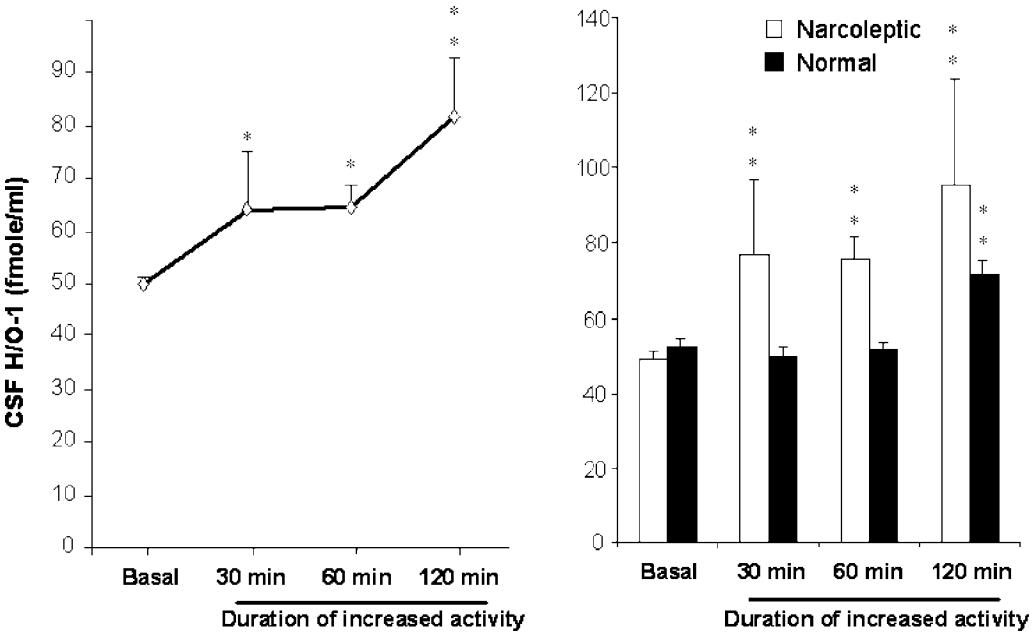

Fig. 3. Increasing duration of motor activity during exercise in a yard produces increasing levels of hypocretin (H/O) relative to the levels seen in the same dogs during equal periods of waking without vigorous movement. CSF, cerebrospinal fluid. (From ref. *6*.)

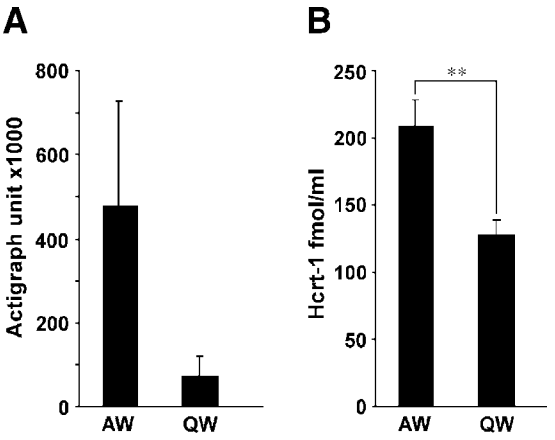

Fig. 4. Motor activity in the normal cat as quantified by actigraphy produces a marked increase in hypocretin (Hcrt) levels relative to equal durations of continuous waking, as in the dog. AW, active waking; QW, quiet waking. (From ref. *23*.)

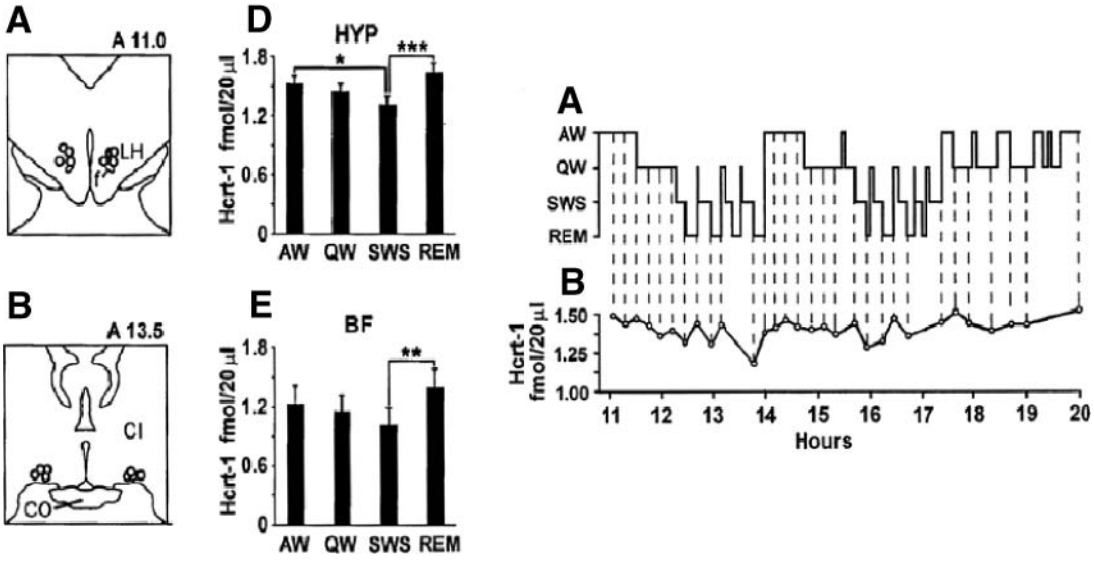

Fig. 5. Release of hypocretin-1 (Hcrt-1) across the sleep cycle. Hypocretin release is maximal in active waking (AW), decreases in quiet waking (QW), and further decreases in non-REM sleep. Release increases to waking levels in REM sleep, a state of intense activation of brain motor systems and hyperpolarization of motoneurons. (From ref. *23*.)

contribute to motor activity in waking, although motor activation is blocked in REM sleep by the simultaneous release of inhibitory neurotransmitters such as γ-aminobutyric acid (GABA) and glycine (*12*) as well as the decreased release of norepinephrine and serotonin (*13*). The action of hypocretin at the motoneuronal level is mediated by the release of glutamate. Figure 6 shows that intravenous administration of hypocretin causes calcium-dependent (vesicular) release of glutamate in hypocretin-innervated regions. Blockade of glutamate receptors prevents the motoneuronal activation produced by hypocretin (*14*) (Fig. 7).

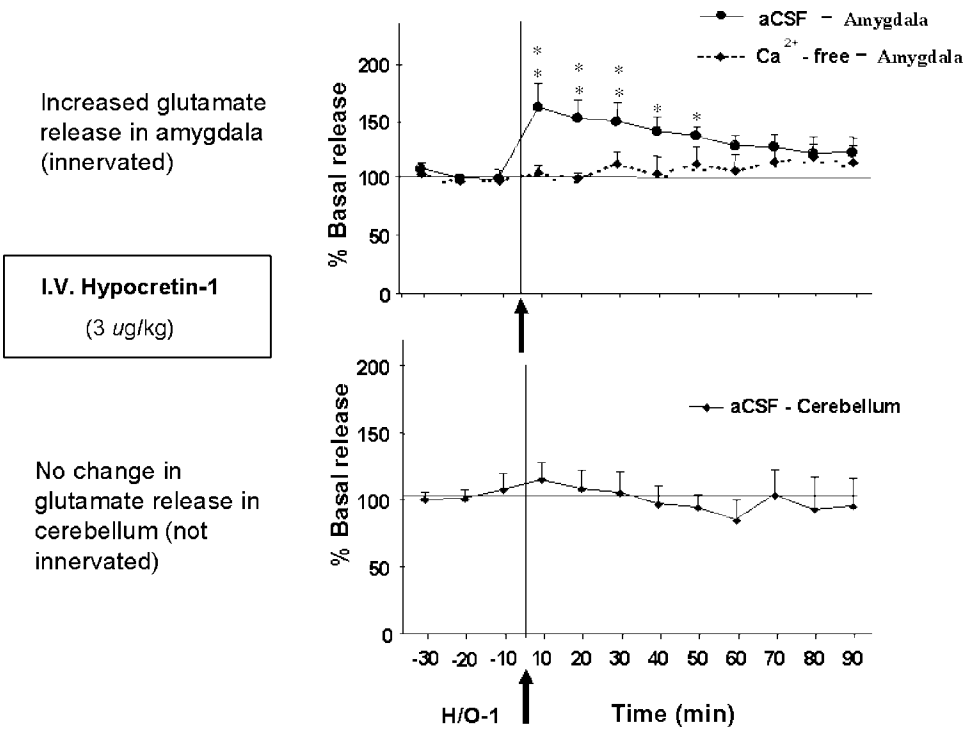

Fig. 6. Intravenous administration of hypocretin-1 to rats causes a calcium-dependent release of glutamate in the amygdala, hypocretin-innervated region, but not in the cerebellum, a region not innervated by hypocretin. a CSF, artificial cerebrospinal fluid. (From ref. *22*.)

Indirect routes of hypocretin activation of motor neurons include a major link through the monoaminergic systems. Hypocretin neurons have their most potent extrahypothalamic projection onto the noradrenergic neurons of the locus coeruleus. Excitation of the locus coeruleus *(15)* (Fig. 8) and other noradrenergic neurons *(16)* produces increased release of norepinephrine onto motoneurons *(13)*. Similar effects are seen with activation of serotonergic neurons *(13,16,17)*. This route is blocked in REM sleep by GABA release *(10)*. Figure 9 shows a simplified outline of the hypocretin pathways facilitating muscle tone.

4. DISCUSSION

Clearly the motor facilitation produced by hypocretin neurons is not tonic. Narcoleptics are not continuously weak, rather, their weakness is apparent only during the sudden onset of strong emotions, such as those accompanying laughter or anger. Normal individuals can also experience a less severe form of weakness at these times, with people "doubling over" with laughter or needing to sit down when strong emotions are triggered. One can postulate that in normals these episodes of weakness are kept in check by actions of the hypocretin system. However, in narcoleptics the loss of hypocretin neurons leaves this weakness unopposed, causing cataplexy. Loss of the hypocretin-mediated facilitation of forebrain arousal systems results in interruptions of waking arousal, producing the characteristic sleepiness of narcolepsy.

The motor relation of hypocretin cells can most parsimoniously explain the involvement of hypocretin in food intake. Stimulation of the lateral hypothalamus has long been

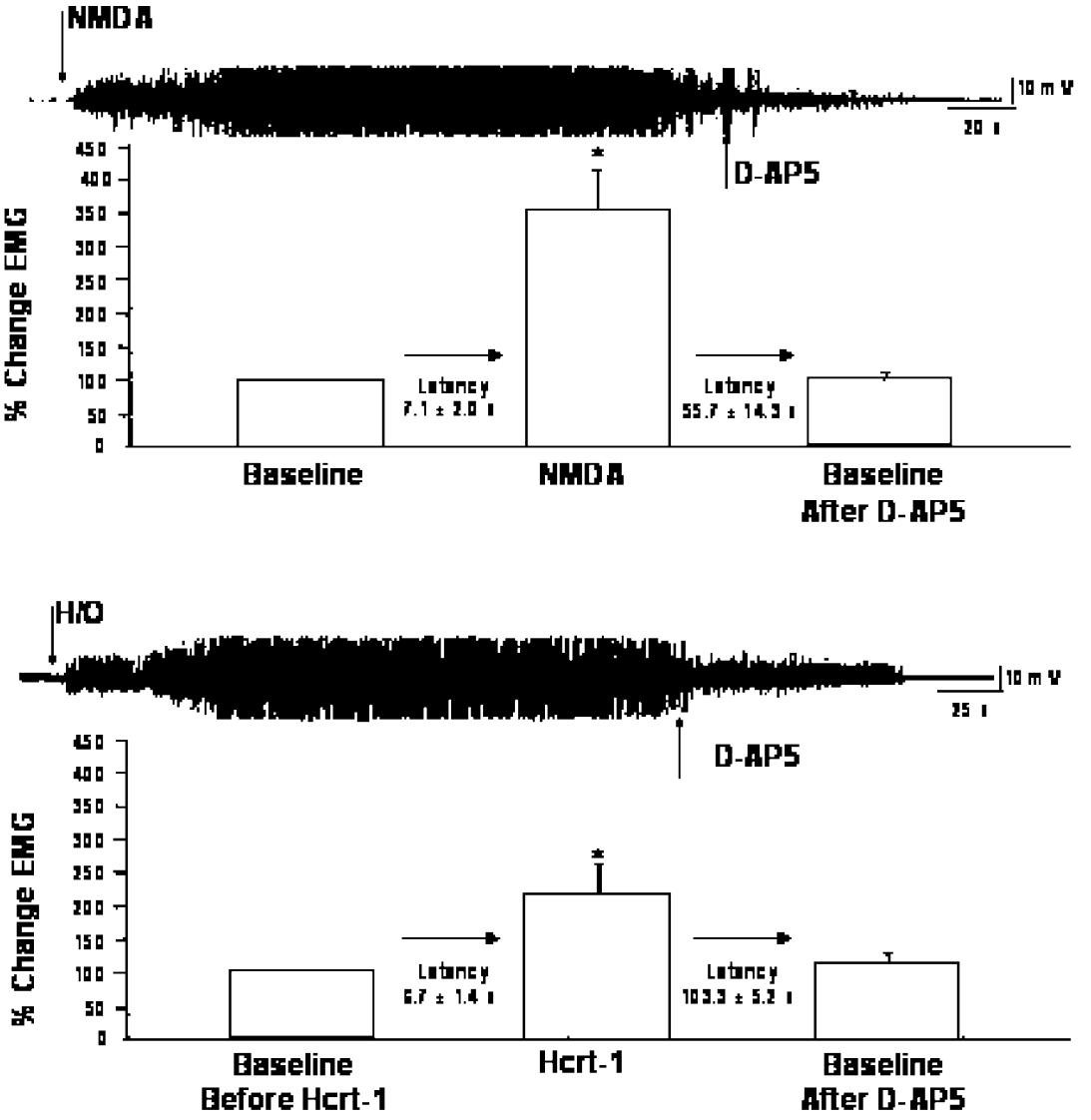

Fig. 7. The increased muscle tone produced by hypocretin-1 (Hcrt-1) microinjection into masseter motoneuron pool is blocked by DAP-5, a non-NMDA glutamate antagonist, indicating that the hypocretin effect is mediated by glutamatergic mechanisms. (From ref. *14*.)

known to produce activation and food consumption if food is presented. However, food consumption was not found to be a consistent response, since drinking would occur if water was presented, gnawing if a wood block was presented and sexual behavior if a receptive female was presented. The constant was motivated motor activation *(18,19)*. Studies of the effects of hypocretin administration on feeding have also produced variable responses, with some increase in eating in certain studies, increased grooming and head shake responses in some studies, and no overall change in others *(20)*. Hypocretin knockout rats do not eat less, and ataxin knockout animals actually gain weight relative to controls (reviewed in ref. *6*). Narcoleptics are not underweight despite the loss of hypocretin,

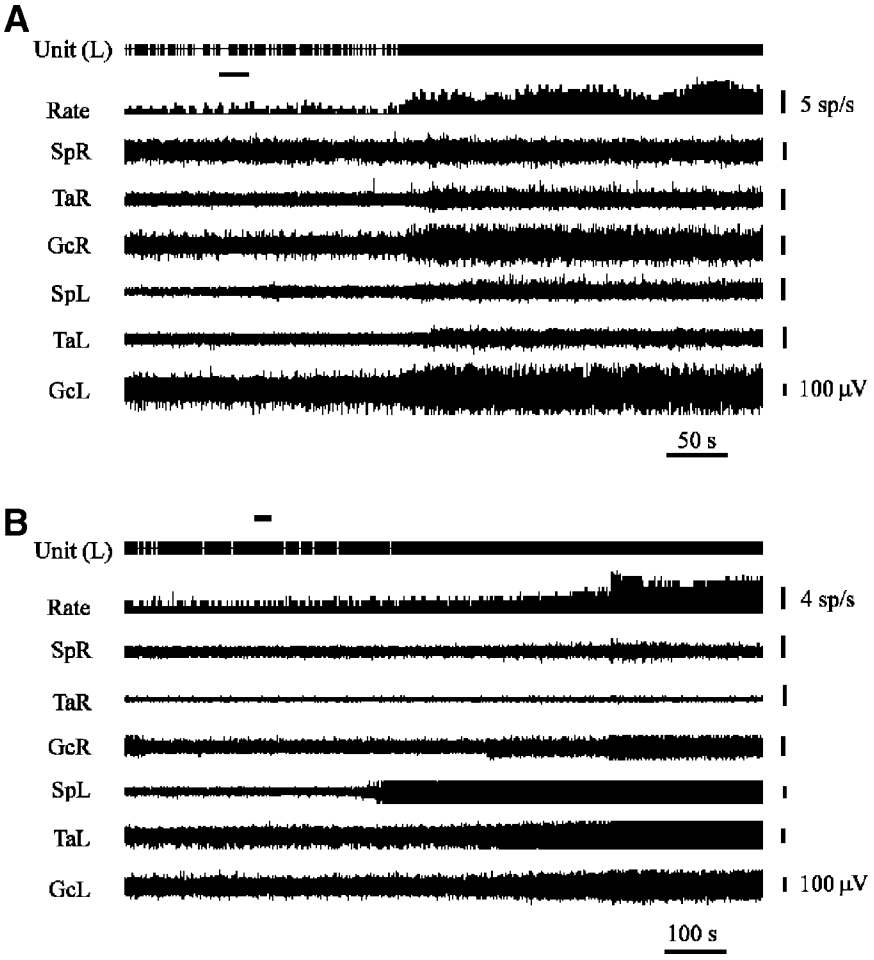

Fig. 8. Microinjection of hypocretin into the locus coeruleus increases muscle tone via adrenergic input to motoneurons SpR, and SpL (splenius, right and left); TaR, and TaL (tibialis anterior, right and left); and GcR, and GcL (gastrocnemius, right and left). (From ref. *23*.)

but rather have a tendency toward obesity (*21*). All these findings can be explained by hypocretin mediation of motor activation, with reduced motor activity producing weight gain even in the absence of increased food intake. Correlations among blood pressure, arousal, and circadian rhythms of hypocretin can be explained by the modulation of motor activity over the 24-h cycle. Although we have tested some aspects of this hypothesis, others remain to be studied.

5. CONCLUSIONS

Hypocretin has a major role in the activation of motor systems. As we have proposed before, the ascending projections of the hypocretin system, particularly those to the histamine and ascending cholinergic system, may be seen as coordinating motor activation with sensory processing and the activation of thalamocortical motor systems. The loss of the hypocretin system causes not only episodic loss of muscle tone (cataplexy) and reduction in the total

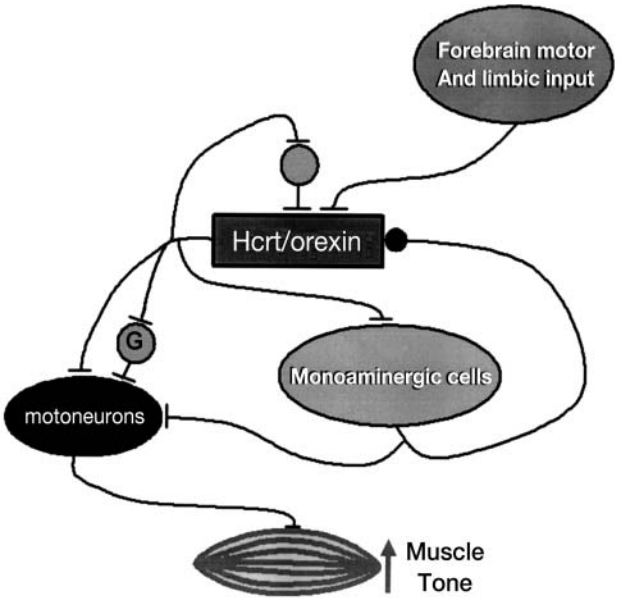

Fig. 9. Hypocretin (Hcrt/orexin) can increase muscle tone by direct action on motoneurons, but more potently by indirect action mediated by glutamate-and monoamine-containing cells. Simplified model of connections between hypocretin neurons and motoneurons. G indicates glutamate cells.

amount of motor activity, but also an impairment of the ability to maintain waking as a result of disfacilitation of these ascending systems.

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V

Pathophysiology: Narcolepsy and Orexin/Hypocretin Deficiency

A. Orexin/Hypocretin Deficiency and Narcolepsy

17
Overview of Human Narcolepsy

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1. HISTORICAL PERSPECTIVE

Narcolepsy research began at least 125 yr ago and has since been steadily advancing (Table 1). In the late 1800s, physicians first began to notice an unusual constellation of symptoms. In 1877, Westphal *(1)* was the first to describe an association between hypersomnia and episodic muscle weakness that was felt to be more than simply an "epileptoid" phenomenon. This is usually considered to be the initial documentation of narcolepsy, although a few years before, in 1862, Caffé *(2)* documented a severely sleepy patient with hallucinations. Fischer *(3)* described a similar case in 1878. In 1880, Gelineau *(4)* used the term *narcolepsy*, derived from the Greek *narke* (numbness, stupor) and *lepsis* (an attack, seizure), to describe patients with primary hypersomnia. Loewenfeld *(5)* emphasized the significance of cataplexy, and during the epidemic of encephalitis lethargica from 1917 to 1927, Von Economo *(6)* discussed the posterior hypothalamus as an important region for the promotion of wakefulness. In 1915, Redlich *(7)* noted the frequency of dreams during sleep attacks. In 1934, Daniels *(8)* wrote an important review of the current knowledge pertaining to narcolepsy, which helped to focus interest on the topic. By 1957, the clinical tetrad of hypersomnia, cataplexy, hypnagogic hallucinations, and sleep paralysis was described by Yoss and Daly *(9)*. In 1960, Vogel *(10)* discovered that patients with narcolepsy had early onset of REM sleep on electroencephalography. The symptom of disturbed nocturnal sleep was recognized at the First International Symposium on Narcolepsy in 1975 *(11)*.

In 1973, Knecht et al. *(12)* reported a case of canine narcolepsy in a dachshund, which stimulated research in the genetics and pathophysiology of narcolepsy in both dogs and humans. Later, a case was described in a poodle by Mitler et al. *(13)*. In 1975, narcoleptic Dobermans were bred, leading to the first successful genetic transmission in canines *(14,15)*. The story of canine narcolepsy is discussed in more detail in Chapter 18. Shortly thereafter, Dr. Honda began a search for a human genetic marker, eventually leading to the discovery of the increased frequency of the human leukocyte antigen HLA-DR2 allele *(16,17)*. This was later refined to the HLA-DQB1*0602 marker, which applied more generally over all ethnic groups *(18,19)*. The fact that HLA genes are important components of the immune system led researchers to contemplate an autoimmune hypothesis for narcolepsy.

After a long and complicated search, the cause for inherited canine narcolepsy was determined to be a mutation in the canine hypocretin (orexin) receptor 2 gene (Hcrtr2) *(20)*. Shortly after this discovery, a preprohypocretin knockout mouse was found to have symptoms consistent with narcolepsy *(21)*. Given the discoveries involving defective hypocretin in animals with

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Table 1
Important Events in Narcolepsy Research

Year	*Event*
1877	Westphal first to describes for the first time a convincing case with both sleepiness and episodes of muscle paralysis triggered by emotions syndrome in the literature *(1)*
1880	Gelineau coins the term "narcolepsy" *(4)*
1902	Loewenfeld emphasizes the importance of cataplexy *(5)*
1917	Von Economo recognizes the posterior hypothalamus as critical for maintenance of wakefulness through his studies of encephalitis lethargica *(6)*
1935	First use of amphetamines in the treatment of narcolepsy *(77)*
1960	Sleep onset REM periods described in narcoleptic patients *(10)*
1960	First report on use of a tricyclic antidepressant in the treatment of cataplexy *(78)*
1973	Canine narcolepsy discovered *(12,13)*
1983	HLA DR2 association found *(16,17)*
1992	Narcolepsy found to be more closely associated with HLA DQB1*0602 *(18,19)*
1999	Narcolepsy in mice and dogs caused by hypocretin mutations *(20,21)*
2000	Human narcolepsy associated with hypocretin deficiency *(22–24)*

narcolepsy, human patients with narcolepsy were evaluated. In 2000, patients with narcolepsy and cataplexy were found to have deficient or absent cerebrospinal fluid hypocretin-1 levels *(22)*. Nine months later, neuropathologic studies showed an absence of hypothalamic hypocretin neurons but no clear damage in surrounding neurons *(23,24)*. Hypocretin genes (receptors and ligand gene) were also screened for mutations in a large number of patients. Only one narcolepsy case with very early onset was found to have a causative mutation in the preprhypocretin gene *(23)*. Thus, given our current state of research, one possible etiology for narcolepsy is an autoimmune attack on the hypothalamus, leading to nonfunctioning hypocretin neurons. Currently, it is unclear what this attack would be and how hypocretin deficiency leads to the symptoms of narcolepsy.

2. PREVALENCE AND GENETICS

The prevalence of narcolepsy appears to vary across ethnic groups. In the general population, prevalence ranges from approximately 0.02 to 0.06% *(25–29)*. In Japan, however, two studies have estimated prevalences as high as 0.16% and 0.59% *(30,31)*. In Israel the prevalence may be as low as 0.002% *(32)*. A recent study of 18,980 randomly selected subjects in five European countries, using the diagnostic criteria from the International Classification of Sleep Disorders (ICSD), found a prevalence of 0.047% *(33)*. Note, that the above statistics pertain to narcolepsy *with* cataplexy. The prevalence of narcolepsy without cataplexy is much less clear. A recent study of all patients with narcolepsy in the medical records of the Rochester Epidemiology Project found a prevalence of 0.021% for patients with narcolepsy without cataplexy *(34)*.

Although originally thought to be a familial disease, more recent studies indicate that the risk of a first-degree relative developing narcolepsy with cataplexy is only 1–2% *(25)*. This is still 10–40-fold higher than in the general population. Even though heritability is low, there has been a great focus on the genetic component of narcolepsy. As mentioned above, in 1983 an association of narcolepsy-cataplexy with HLA DR2 was reported in Japan *(16,35)* and was quickly confirmed in Caucasian patients *(36–41)*, with 85–98% of patients being positive for

this antigen. It was later noted that DR2 was a poor marker in the black population, but HLA DQB1*0602 was more tightly associated across all ethnic groups *(18)*. The association with DQB1*0602 is much stronger in patients who have narcolepsy with cataplexy *(42,43)*.

Given the HLA association, an autoimmune hypothesis has been pursued but has thus far been difficult to verify *(44,45)*. Since attempts at confirming the autoimmune hypothesis have been unfruitful, further attention has been paid to the HLA genetics. Research now indicates that the both the DQB1*0602 and DQA1*0102 alleles may be necessary for maximal susceptibility *(46)*. There also appears to be an increased risk in DQB1*0602/DQB1*0301 heterozygotes and a decreased risk in DQB1*0602/DQB1*0601 and DQB1*0602/DQB1*0501 heterozygotes *(47)*. Finally, it appears that homozygosity for DQB1*0602 confers a two to four times greater risk for narcolepsy compared with heterozygosity *(48)*.

3. CLINICAL SYNDROME

Narcolepsy is primarily characterized by a disabling excessive daytime sleepiness (EDS). The EDS is thought to be secondary to the inability to regulate sleep-wake states properly, a condition also leading to disrupted nighttime sleep. The other primary symptoms are, as mentioned above, cataplexy, sleep paralysis, hypnagogic/hypnopompic hallucinations, automatic behaviors, and disrupted nighttime sleep. These symptoms are all thought to be secondary to improperly regulated sleep states and disrupted nighttime sleep. Cataplexy and EDS are the most important symptoms for diagnosis. Most of the other symptoms can occur in other disorders of EDS or even in normal individuals. Despite being called the "tetrad," most patients only have a few of these symptoms, and it is unusual to have all four symptoms together in one patient *(49)*.

In general, EDS in narcolepsy is considered to be more severe than in other sleep disorders, such as obstructive sleep apnea (OSA). It is present both as a chronic continuous daily sleepiness and as intermittent sleep attacks and is often the first symptom of developing narcolepsy. The chronic component of EDS leads to difficulty concentrating, decreased vigilance, and automatic behaviors. The term "sleep attack" is somewhat of a misnomer in that the episodes are not so much "attacks" as unavoidable naps. They are not as abrupt as the word "attack" implies but rather an irresistible desire to sleep that usually occurs at inopportune times throughout the day. The EDS of narcolepsy can be quite disabling, leading to difficulties in concentration and poor performance at work. Usually the daytime sleepiness can be temporarily relieved by a brief nap, but it returns quickly. This can distinguish narcoleptics from patients with idiopathic hypersomnia, who usually take long, unrefreshing naps. Despite frequent napping, patients with narcolepsy appear to have normal total amounts of sleep over the 24-h period *(50,51)*.

Automatic behaviors are activities that occur without full awareness or memory. A clinical description of these events involves a number of features. Their duration can last anywhere from minutes to a few hours. Typically, patients will notice that they are drowsy immediately preceding the behavior. This is followed by a period of amnesia. An observer might notice that the patient continues his or her previous activity in a semiautomatic way. If the activity was simple or routine, the patient may be able to continue without a problem, but if the activity is complicated, mistakes usually occur. This can lead to serious problems in the workplace. When asked a simple question, the patient may be able to answer, but complicated questions elicit inappropriate or bizarre answers. Patients will continue to write or talk on the phone in a daze, only to discover later that their writing or conversation was nonsensical. Many patients experience automatic behaviors while driving; having complete amnesia for an

entire trip is not uncommon. These episodes are similar to sleep-walking episodes in many ways, except that they occur in the daytime.

There are many examples that may help illustrate the nature of these episodes. One patient reported that she would drive for 30 min to her boyfriend's house but could not remember any details from the drive, except getting into and out of her car. Another patient reported putting away the groceries, only to discover later that she had put the milk in the cupboard and the cereal in the refrigerator. A third patient, who was a pharmacist, reported frequent miscounting of pills when filling prescriptions. Usually there is nearly complete amnesia, but occasionally patients will remember vague bits and pieces of the events.

The term *cataplexy* comes from the Greek word *cataplessa,* which means "to strike down with fear or the like." It is a sudden, reversible, bilateral loss of muscle tone triggered by emotions. It can range in severity from a slight change in facial tone to complete collapse. At its worst, only the respiratory muscles are spared. There is no loss of consciousness, and the duration is usually brief, lasting seconds to minutes. The event occurs almost simultaneously with the offending trigger; there is little or no lag between the trigger and the weakness. Usually patients have just enough time to break the fall or lean against a wall. Severe events can be frightening because the patient is fully conscious but unable to move, particularly if passersby attempt life-saving maneuvers. The emotional trigger is usually humor or laughter, especially in the case of telling a joke or relaying a funny story *(52)*. It occurs more often when the emotional trigger is active rather than passive. In other words, it is more likely to occur if someone is telling a joke rather than hearing a joke. Other emotions such as anger, surprise, or elation can also be a trigger. The likelihood of cataplexy occurring during a particular trigger increases if a patient is sleepy or if they are withdrawing from an anticataplectic medication. If the patient is examined during an episode, the deep tendon reflexes are absent. Cataplexy is almost pathognomonic for narcolepsy. If a patient has it, then he or she almost certainly has narcolepsy. However, it is important to note that the diagnosis of narcolepsy can be made without the presence of cataplexy.

One of the most difficult issues in evaluating cataplexy is differentiating it from normal weakness. Often, normal individuals feel weakness after laughing very hard or after sexual intercourse. Also, normal people can feel "weak in the knees" with public speaking. These episodes are usually longer lasting and more gradual in onset than true cataplexy. Also, these patients typically do not report more subtle episodes of cataplexy such as loss of facial expression or control of the neck. Patients with narcolepsy can usually differentiate between "normal" weakness and true cataplexy. The difficulty arises when a young patient is developing narcolepsy and has not had experience with cataplexy. Cataplexy is frequently more atypical in its presentation closer to the onset of the disease.

Hypnagogic and hypnopompic hallucinations are dreamlike hallucinatory phenomena that occur on transitions into and out of sleep, respectively. Most are hypnagogic in nature, and true hypnopompic hallucinations are rare. They occur with both nighttime sleep and daytime napping. They are usually visual or auditory in nature, but they can involve other senses as well. They can involve cenesthopathic sensations like light touch on the skin, vestibular sensations like turning in space, or hallucinations involving body parts such as a limb disappearing or transforming. They can range from vague shapes or sounds to complex images involving multiple senses and storylines. Often there is an awareness of another presence in the room, such as an intruder. There is frequently a distressing emotional component to these events, although they can be pleasurable as well. An important defining characteristic is that these sensations occur superimposed on the patient's true surroundings, thus the term hallucinations. Patients

can transition in and out of REM sleep during these events, leading to an intertwining of hallucinations and dreams. They are often so realistic that patients are convinced of their reality. For example, a patient might call the police over multiple nights, convinced that there is an intruder in the house, despite frequent searches and reassurances to the contrary. In some cases this has led to the unfortunate misdiagnosis of psychotic disorders *(53)*.

Sleep paralysis is the inability to move upon falling asleep or awakening from REM sleep *(54)*. It occurs while the patient is conscious and thus can be very disconcerting. Patients are usually completely paralyzed, and some even complain of difficulty taking a full breath. It usually lasts for seconds, although rarely can last for minutes. During this period, the patient is involved in an internal struggle to move the body. It always ends spontaneously. In patients with narcolepsy, it can occur in either nocturnal sleep or at naps. It can be accompanied by vivid hypnagogic hallucinations. This combination of sleep paralysis and hypnagogic hallucinations can be very frightening, and is described as being "scared stiff."

Disrupted nighttime sleep is a common problem, usually manifesting as a sleep maintenance issue, rather than difficulty with sleep onset *(55)*. It can be very disruptive and distressing to the patient. There are often vivid, bizarre dreams at night as well as periodic limb movements and REM behavior disorder. Most awakenings are brief, although patients can also have difficulty getting back to sleep for hours. The overnight sleep study shows severe fragmentation of sleep at night particularly involving REM sleep.

Most of the above-described symptoms are thought to be related to an inability to regulate aspects of REM sleep. Cataplexy is thought to be the paralysis of REM sleep intruding into wakefulness *(56)*. This is also the proposed mechanism of sleep paralysis. Hypnagogic and hypnopompic hallucinations are thought to be the dream phenomena of REM sleep expressed during wake. Disrupted nighttime sleep is thought to be partially due to the poor regulation of REM sleep during nocturnal sleep.

4. DIAGNOSIS

The diagnosis of narcolepsy hinges on a careful history of cataplexy, if present. If a patient has both EDS and cataplexy, nothing else is required for diagnosis *(57)*, although diagnostic testing is highly recommended for purposes of documentation. This is particularly true if aggressive treatment measures are required in the future. Attention must be paid to the nature of the trigger, the description of the episode, and the duration and frequency of events. If the description of cataplexy is unclear or atypical, for purposes of diagnosis it is often prudent to assume that it is not cataplexy until further diagnostic information can be gathered. In patients without cataplexy, it is important to note the presence of other associated symptoms.

If a patient does not have cataplexy, polysomnographic testing is necessary for diagnosis. Two or more sleep-onset REM periods (SOREMPs) and a mean sleep latency less than or equal to 8 min on a multiple sleep latency test (MSLT) are diagnostic for narcolepsy *(58)*. The MSLT is comprised of a series of five 20-min daytime naps taken every 2 h. The amount of time that it takes to fall asleep (sleep latency) as well as the presence of REM sleep during the nap is recorded. The MSLT must be preceded by an overnight polysomnogram (PSG) the night before the MSLT is to be performed. This is to ensure that the patient does not have a significant sleep disorder other than narcolepsy that could explain the daytime sleepiness or SOREMPs. Also, it documents sufficient sleep the night before (>6 h). If a significant concomitant sleep disorder is found, then the problem must be treated before proceeding to MSLT. It is also important that medications that can suppress REM sleep, particularly serotonin selective reuptake inhibitors (SSRIs), be discontinued at least 2 wk prior to the test.

Wake-promoting agents must also be avoided prior to MSLT testing. It is also helpful to have sleep logs and/or actigraphy for the weeks preceding the MSLT.

Although MSLT is required for diagnosis in patients without cataplexy, it is an imperfect test. Up to 33% of patient with narcolepsy-cataplexy can have false-negative results *(59)*. Also, it is difficult in young children, patients who cannot follow commands, or patients who are unable to discontinue their SSRIs. It has been proposed that if the suspicion for narcolepsy is high, but the MSLT is negative or impossible to do, cerebrospinal fluid (CSF) hypocretin-1 measurements may be useful *(60)*. A direct-assay CSF hypocretin-1 level < 110 pg/mL has been found to be highly sensitive and specific for narcolepsy-cataplexy *(61)*.

5. TREATMENT

Novel therapeutic approaches are discussed elsewhere in this text, but a brief introduction to current therapy is warranted. Both behavioral and pharmaceutical methods are necessary for adequate results. There are a number of behavioral methods that are often overlooked in favor of medications. Proper sleep hygiene is always necessary. Short naps should be encouraged, and employers should be educated as to their necessity. Patients should choose vocations that are less sedentary. They should avoid vocations that require driving during the course of employment, and if necessary the physician should notify the proper authorities depending on state requirements.

Three types of compounds are used to treat narcolepsy: wake-promoting agents, anticataplectic agents, and medications that consolidate nighttime sleep (Table 2). All these compounds act symptomatically rather than on the cause of the disease, hypocretin deficiency. Most act by promoting monoaminergic transmission (Table 2). Hypocretin systems are well known to project heavily onto monoaminergic neurons, with generally excitatory effects. The activation of monoaminergic transmission by these drugs is thus probably acting downstream of the primary defect in narcolepsy, hypocretin deficiency.

Amphetamines like dextroamphetamine, methamphetamine, methylphenidate, and pemoline have long been the primary drugs used to promote wakefulness, but a newer drug, modafinil, has been developed and has a lower occurrence of side effects *(62,63)*. Their pharmacologic effect is primarily through a global, nonspecific release of monoamines from presynaptic terminals. More specifically, the wake-promoting effects are thought to be secondary to the promotion of dopamine release and/or via dopamine reuptake inhibition *(64,65)*. The mechanism for modafinil is still controversial, but it is also thought to act through dopamine uptake inhibition *(66)*. Because of the lower incidence of side effects, modafinil is now becoming a first-line treatment for EDS associated with narcolepsy.

Cataplexy has been traditionally treated with antidepressants. Initially, the tricyclic antidepressants were the most commonly prescribed treatment for cataplexy, but more recently the SSRIs have been used because of their lower incidence of side effects *(67)*. All the antidepressants work via complex pharmacologic mechanisms involving monoamine uptake inhibition. The older tricyclics also have cholinergic, histaminergic, and α-adrenergic blocking properties (*68–70*). Studies in narcoleptic canines have shown that adrenergic but not dopaminergic or serotonergic uptake inhibition is necessary for the anticataplectic effects to manifest. Drugs that specifically block noradrenergic reuptake, such as protriptyline, desipramine, viloxazine, and atomoxetine are usually more effective than SSRIs *(69,70,71)*. In contrast, much higher doses of the SSRIs are required to treat cataplexy *(69,70)*.

The third type of medication used to treat narcolepsy is thought to act through the consolidation of nighttime sleep. Sodium oxybate (γ-hydroxybutyrate [GHB]) is the newest agent

Table 2
Commonly Prescribed Treatments and Their Pharmacological Properties

Compound	Pharmacological properties
Stimulants	
Amphetamine (sulfate)	Produces a reverse efflux of monoamines through monoaminergic transporter (reuptake) sites. This increases monamine release (DA > NE >> 5-HT); at higher dose, blocks monoamine vesicular storage (VMAT), monoamine reuptake, and monoamine oxiydase at high doses
Methamphetamine (HCl)	Increased central penetration and fewer peripheral effects compared with amphetamine
Methylphenidate	Blocks more monoamine uptake and has less effect on monoamine release
Pemoline	Dopamine reuptake inhibition
Selegiline (L-deprenyl)	Monoamine oxidase B inhibitor
Modafinil	Mode of action is debated but inhibition of dopamine reuptake is likely to play a role
Anticataplectic compounds	
Venlafaxine	Dual serotonin and adrenergic reuptake blocker (5-HT = NE); no anticholinergic effects; very effective
Atomoxetine	Specific adrenergic reuptake blocker (NE); normally indicated for attention deficit hyperactivity; very effective
Fluoxetine	Specific serotonin uptake blocker (5-HT >> NE = DA); active metabolite norfluoxetine has more adrenergic effects; high therapeutic doses often needed
Protriptyline	Tricyclic antidepressant; monomoaminergic uptake blocker (NE > 5-HT > DA); anticholinergic effects
Imipramine	Tricyclic antidepressant; monoaminergic uptake blocker (NE = 5-HT > DA); anticholinergic effects; desipramine is an active metabolite
Desipramine	Tricyclic antidepressant; monoaminergic uptake blocker (NE >> 5-HT > DA); anticholinergic effects
Chlomipramine	Tricyclic antidepressant; monoaminergic uptake blocker (5-HT > NE >> DA); anticholinergic effects; desmethyl-chlomipramine (NE >> 5-HT > DA) is an active metabolite; very effective
Other	
γ-Hydroxybutyric acid (GHB, sodium oxybate)	May act via $GABA_B$ or via specific GHB receptors; reduces dopamine release

Abbreviations: DA, dopamine; NE, norepinephrine; 5-HT, serotonin; MAO, monoamine oxidase

approved for treating narcolepsy. It is a sedative hypnotic compound that is known to increase slow-wave sleep and to a lesser extent REM sleep *(68)*. Not only does it consolidate nighttime sleep, but it also seems to improve daytime sleepiness and cataplexy *(72–74)*. The exact mechanism of action is not clear, but a number of properties have been observed. Dopamine transmission is affected, with the end result of reducing firing rates and increasing brain levels of dopamine *(68,75)*. Effects on opioid, glutamatergic, and acetylcholine transmission have been observed *(75)*. The sedative hypnotic effects are thought to be related to its known $GABA_B$ agonist activity; alternatively, specific GHB receptors have been identified and may

be relevant to the therapeutic effects of GHB *(75,76)*. Although the mechanism of GHB is unclear, it is felt that the consolidation of nighttime sleep somehow helps to correct the abnormalities associated with narcolepsy.

6. SUMMARY

Researchers have been studying narcolepsy for over 125 yr. During the first 100 yr, much attention was paid to defining the syndrome and developing diagnostic strategies. Although Von Economo emphasized the importance of the posterior hypothalamus in the 1920s, it was not until the last quarter century that a clear understanding of the genetics and pathophysiology of narcolepsy was developed. The fact that narcolepsy is associated with HLA DQB1*0602 as well as hypocretin deficiency suggests an autoimmune cause for narcolepsy. With any luck, in the next quarter century we will be able to use this knowledge to develop more effective treatment strategies than are currently available. The most promising approaches, discussed elsewhere in this monograph, include the use of hypocretin-based replacement therapies or the possibility that immune suppression early in the course of the disease (i.e., in children) could be used to prevent full development of the disorder.

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18
Canine Models of Narcolepsy

Seiji Nishino

1. INTRODUCTION

One of the major aims of the Stanford Sleep Research Center is to find the cause of human narcolepsy, a unique sleep disorder that affects 1 in 2000 of the general population. Since narcolepsy does not directly cause death, patients may ultimately pass away from an accident or illness, making the clinical-neuropathological correlation difficult to obtain from human subjects. Because of the nature and treatment of these accidents and unrelated illnesses patient specimens will generally not be available as uncontaminated neuropathological specimens of narcolepsy. The development of an animal model of narcolepsy was thus an urgent issue in the Stanford Sleep Research Center. In 1973, the Center had the extraordinary good fortune to acquire a female miniature French poodle who exhibited cataplexy and sleep onset rapid eye movement (REM) sleep periods *(1)*. Active recruiting efforts resulted in the acquisition of additional narcoleptic dogs in small breeds, and finally in 1976, four narcoleptic Dobermans (two males and two females, including two litter mates) were obtained *(2)*. With these multiple narcoleptic dogs, the Stanford Canine Narcolepsy Colony was established, and a breeding program was initiated *(2)*.

Subsequently, it was demonstrated that narcolepsy in Dobermans and Labradors was transmitted through a single autosomal recessive gene, *canac-1*, whereas the disease in other breeds was sporadic *(2)*. Twenty-three years later, *canac-1* was identified by positional cloning, revealing that the gene encodes one of the two hypocretin/orexin receptors (i.e., hypocretin receptor-2 [*Hcrtr 2*]) *(3)*. This, together with an independent discovery of a narcolepsy phenotype in mice lacking hypocretin ligand (preprohypocretin gene knockout mice) *(4)*, immediately led to the discovery of the major pathophysiological mechanism of human narcolepsy (i.e., hypocretin ligand deficiency) *(5–7)*. This chapter, summarizes what knowledge we have gained about the basic mechanisms of narcolepsy derived from these narcoleptic dogs over a 30-yr period.

2. THE PRIMARY SYMPTOM OF CANINE NARCOLEPSY

Just as in humans, cataplexy is a hallmark of canine narcolepsy. Affected dogs exhibit very pronounced attacks of cataplexy that are mainly triggered by positive emotional experiences, such as being fed a favorite food or engaging in play. Cataplectic attacks in dogs often begin with buckling of both hind legs, which is often accompanied by a drooping of the neck (see video clips at http://med.stanford.edu/school/Psychiatry/narcolepsy/moviedog.html). The dog

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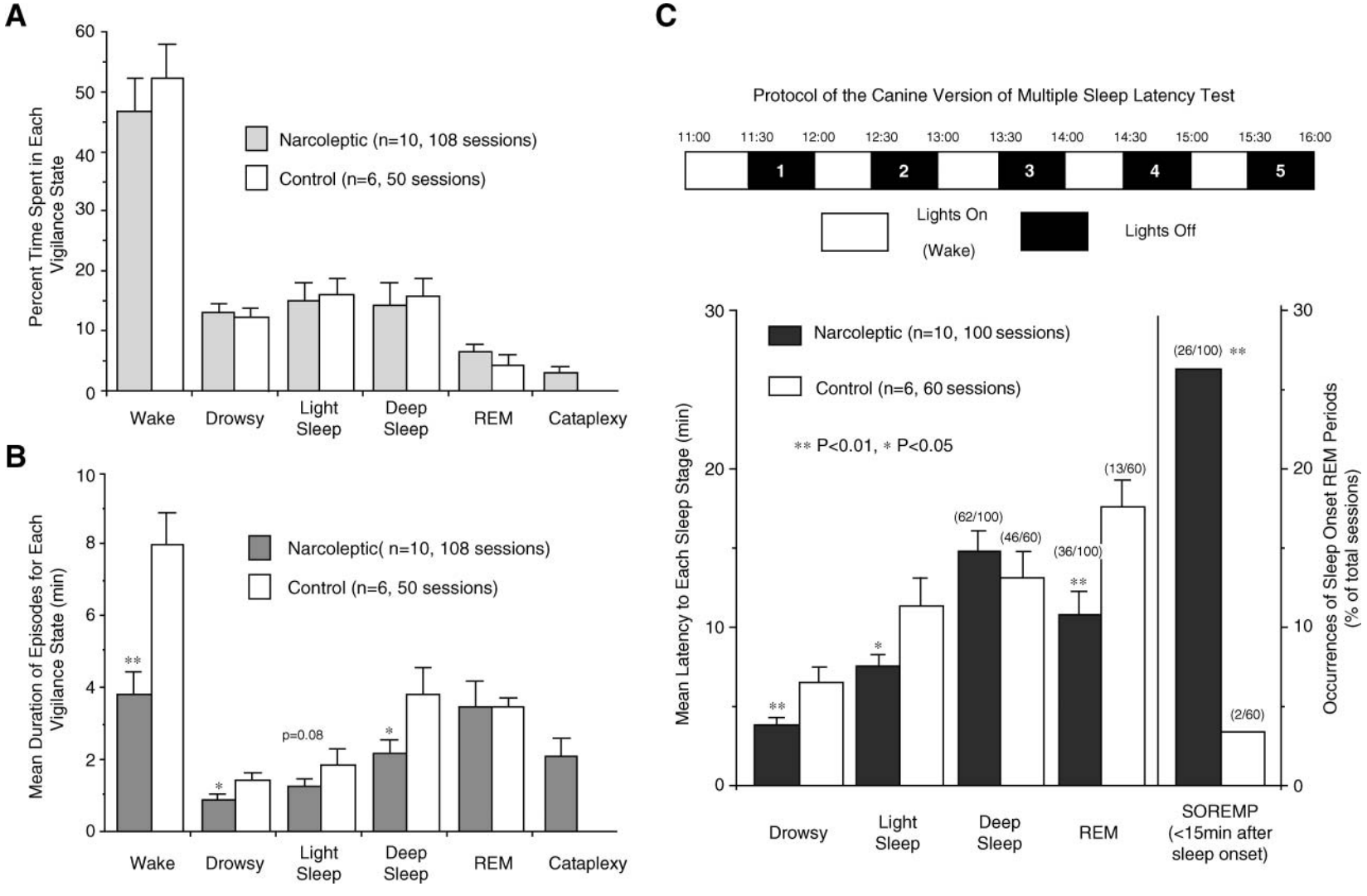
A
Percent Time Spent in Each Vigilance State
Narcoleptic (n=10, 108 sessions)
Control (n=6, 50 sessions)
Wake
Drowsy
Light Sleep
Deep Sleep
REM
Cataplexy
B
Mean Duration of Episodes for Each Vigilance State (min)
Narcoleptic(n=10, 108 sessions)
Control (n=6, 50 sessions)
p=0.08
C
Protocol of the Canine Version of Multiple Sleep Latency Test
11:00
11:30
12:00
12:30
13:00
13:30
14:00
14:30
15:00
15:30
16:00
Lights On
(Wake)
Lights Off
Narcoleptic (n=10, 100 sessions)
Control (n=6, 60 sessions)
** P<0.01, * P<0.05
Mean Latency to Each Sleep Stage (min)
Occurrences of Sleep Onset REM Periods (% of total sessions)
(62/100)
(46/60)
(36/100)
(13/60)
(26/100)
(2/60)
SOREMP (<15min after sleep onset)

may collapse to the floor and remain motionless for a few seconds or several minutes. In contrast to some forms of epilepsy, excess salivation or incontinence were not observed during cataplectic attacks. During long cataplectic attacks, rapid eye movements, muscle twitching, and/or fast repetitive movement of the fore- and hindlimbs may occur. These phenomena are thought to be related to the active phase of REM sleep. The muscle is always flaccid and never stiff during cataplectic attacks, a unique characteristic that is not found in seizure-related attacks. Polygraphic recordings during the attacks also demonstrated that the brain patterns measured by electroencephalogram (EEG) during cataplexy resembled those seen during normal wake or REM sleep periods, and no abnormal spikes or waves were observed *(1,8)*. Dogs usually remain conscious (especially at the beginning of attacks), with eyes open, and are capable of following moving objects with their eyes. If the attack lasts for an appreciable length of time (usually longer than 1–2 min), the dog may transit into sleep (often REM sleep). Dogs are often easily aroused out of an attack either by a loud noise or by being physically touched. Although hypnagogic hallucinations and sleep paralysis may also occur in these dogs, there is no objective way to determine this at the present time.

Like narcoleptic humans, narcoleptic dogs are sleepier (fall asleep much more quickly) during the day. However, this was not noticeable, because even normal dogs take multiple naps during the daytime.

A series of polygraphic studies clearly demonstrate a difference in sleep patterns between narcoleptic dogs and control dogs. Compared with age- and breed-matched dogs, narcoleptic dogs exhibit an increased frequency in sleep state changes, their sleep/wake pattern is more fragmented and shorter, and their wake/sleep bouts are much shorter than those of control dogs (Fig. 1) *(9–11)*.

By adapting and modifying the multiple sleep latency test (MSLT) used in humans to demonstrate shorter sleep latency and occurrence of sleep onset REM periods (SOREMP), we were finally able to demonstrate that narcoleptic Dobermans showed shortened sleep latency and reduced latency to REM sleep during multiple daytime naps (Fig. 1) *(9)*, suggesting that these dogs have a very similar phenotype to those in human narcolepsy.

Considering that sleep in most vertebrates, including dogs and mice *(12)* is not consolidated as in humans (and thus shorter sleep and REM sleep latency is not obvious in some

Fig 1. (*opposite page*) Percent of time spent in, mean frequency of, and mean duration for each vigilance state of narcoleptic and control canines during daytime 6-h recordings (10:00 to 16:00). **(A,B)** No significant difference was found in percent of time spent in each vigilance state between narcoleptic and control dogs. However, the mean durations of wake, drowsy, and deep sleep episodes were significantly shorter in the narcoleptics, suggesting a fragmentation of the vigilance states (wake and sleep) in narcoleptic dogs. To compensate for the influence of cataplectic episodes on wake and drowsy states, those episodes interrupted by the occurrence of cataplexy were excluded. **(C)** Mean latency (min) to each sleep stage and occurrences (number/total sessions) of cataplexy and sleep onset REM periods (SOREMPs) during the multiple sleep latency test (MSLT) in narcoleptic and control canines. Drowsy and light sleep occurred in all sessions. Deep sleep, REM sleep, or cataplexy (for narcoleptic dogs) occurred in some sessions, and the numbers of sessions in which each state occurred/total number of sessions are shown in parentheses. Student's t-test were used to compare the sleep latency of narcoleptic and control canines. Narcoleptic dogs exhibited cataplexy in 9 of 100 sessions, and these events were differentiated from REM sleep episodes. Narcoleptic dogs showed a significantly shorter latency to drowsy and light sleep in overall sessions. Note that narcoleptic dogs exhibited SOREMPs (i.e., REM sleep occurring within 15 min of sleep onset) significantly more often than control animals, although both narcoleptic (36.0% of total session) and control dogs (21.7%) showed REM sleep during the MSLT.

animal species in their living environment), we believe that cataplexy and sleep/wake fragmentation are the primary symptoms of narcolepsy across different species. In other words, narcoleptic subjects could not maintain long bouts of both wakefulness and sleep, which also explains why most narcoleptic humans are insomniacs at night and have excessive daytime sleepiness (EDS) during daytime *(13)*.

3. INHERITANCE OF NARCOLEPSY IN CANINES

Unrelated narcoleptic poodles donated to the Stanford Canine Narcolepsy Colony were bred, but none of the offspring from two primary crosses or a backcross were affected *(14)*. Similar attempts have been made with affected beagles and dachshunds, but none of the offspring developed narcolepsy. Thus, it appears that in these breeds narcolepsy is sporadic and likely to be polygenic and/or environmentally influenced. In humans, about 95% of narcoleptic subjects have sporadic diseases, but familial occurrences were noted in about 5% *(15)*. In 1976, the Stanford Sleep Center received four affected Dobermans (including two litter mates) *(2)*. These dogs were bred, and it was discovered that all offspring from the two affected parents developed cataplexy around 2 mo of age, marking the start of Stanford's genetically transmitted canine narcolepsy colony. In 1978, familial narcolepsy in a Labrador retriever was also discovered, and this animal was donated to Stanford *(2)*. Thereafter, more sporadic cases of canine narcolepsy were identified in collies, dachshunds, beagles, fox terriers, and several mixed breeds *(2)*.

Genetic transmission in Dobermans and Labradors has been well established as autosomal recessive with full penetrance *(2,16)*. Puppies born from narcoleptic Doberman pinscher-Labrador retriever crosses are all affected; thus, both breeds are likely to have mutations at the same locus, termed *canarc-1 (2,16)*. It was impossible, however, to know whether the mutation occurred independently in these two breeds or occurred once in their common ancestry. As in human cases *(17)*, the disease onset in familial cases is earlier than in sporadic cases *(14,18)*. In Dobermans, affected dogs display spontaneous complete cataplexy as early as 4 wk, but almost always by 6 mo *(2,18)* (Table 1). Symptom severity increases until 5–6 mo of age (with females experiencing more severity during development); the disease appears to decrease slowly and then stabilizes through old age *(18,19)*. In sporadic cases, the disease (cataplexy onset) begins as early as 7 wk and as late as 7 yr old *(14)*, suggesting the acquired nature of the disease in these cases.

Genetic canine narcolepsy was thought to be an invaluable model in narcolepsy research, since it is possible that the canine gene (its equivalent or genes with a functional relationship with the canine narcolepsy gene) may also be involved in some human cases.

4. THE ROLE OF DLA (DOG LEUKOCYTE ANTIGEN) IN CANINE NARCOLEPSY

Since human narcolepsy-cataplexy is specifically associated with the HLA gene HLA DQB1*0602 (and DR15; *see* Chap. 17, on human narcolepsy), three populations of narcoleptic dogs (Dobermans, Labradors, and small-breed sporadic dogs) were tested to determine whether a specific dog leukocyte antigen (DLA) allele was present in affected animals as in narcoleptic humans *(14)*. Mixed leukocyte cultures were performed to identify whether any disease-associated DLA-D antigen (analogous to HLA-D) was present. Unlike humans, narcoleptic dogs tested do not share any single DLA locus reactivity, suggesting that a specific MHC class II haplotype is not a requirement for the disease *(20)*. In further experiments, a human HLA-DRb hybridization probe was used on DNA from narcoleptic dogs to determine

Table 1
Characterization of Narcolepsy in Humans, Dogs, and Mice

		Onset	Symptoms/ phenotype	Transmission	Association with HLA/DLA	Abnormality in hypocretin system
Human	Sporadic	Adolescent	EDS, cataplexy, SP, HH, SOREMPs, sleep fragmentation obesity (+)	?	DQB1*0602 (+) (90–95%)	Hypocretin ligand deficiency (~90%)
	Familial	Earlier onset than sporadic cases	EDS, cataplexy, SP, HH, SOREMPs, sleep fragmentation obesity (+)	?	DQB1*0602 (+) (75–80%)	Hypocretin ligand deficiency (~75%)
	Hypocretin mutant (only one case identified)	Extremely early onset (6 mo)	EDS, severe cataplexy	De novo mutant (?), dominant	DQB1*0602(–)	Mutation in preprohypocretin gene, hypocretin ligand deficiency
Dog	Sporadic (17 breeds)	7 wk to 7 yr	Cataplexy, short sleep latency, SOREMPs	?	(–)	Hypocretin ligand deficiency
	Familial Dobermans, Labradors, dachshunds	Earlier than 6 mo	Cataplexy, short sleep latency, SOREMPs	Autosomal recessive, 100% of penetrance	(–)	Mutation in Hcrtr2 gene
Mice	Hypocretin KO	~4 wk	Cataplexy, SOREMPs, short sleep latency, obesity (?)	Autosomal recessive, 100% of penetrance		Hypocretin ligand deficiency
	Hypocretin cell death Hypocretin/ Ataxin-3 transgenic	~6 wk	Cataplexy, SOREMPs, short sleep latency, obesity (++)	Autosomal dominant, 100% of penetrance		Hypocretin/dynorphin deficiency
	Hcrtr1 KO		Fragmented sleep, obesity (?)			Absence of *Hcrtr1*
	Hcrtr2 KO		Cataplexy, SOREMPs, short sleep latency, obesity (?)	Autosomal recessive, 100% of penetrance		Absence of *Hcrtr2*
	Double receptor KO		Same sleep phenotype as that of preprohypocretin KO mice, obesity (?)	Recessive for each receptor gene		Absence of *Hcrtr1* and *Hcrtr2*

Abbreviations: EDS, excessive daytime sleepiness; SP, sleep paralysis; HH, hypnagogic hallucination; SOREMPs, sleep onset REM periods; Hcrtr1, hypocretin receptor 1; Hcrtr2, hypocretin receptor 2; KO, knockout; HLA, human leukoantigen; DLA, dog leukocyte antigen.

whether there was an association between the DLA allele and susceptibility to narcolepsy *(21)*. This probe detected polymorphisms in both Labrador retrievers and Doberman pinschers. Results of this study excluded the possibility of a tight linkage between DLA and the *canarc-1* locus *(21)*. However, it now appears that more extensive searches, including other DLA regions, are required to examine the involvement of the histocompatibility molecules/mechanisms in the development of narcolepsy in sporadic narcoleptic dogs (i.e., hypocretin ligand deficient; *see* below).

5. POSITIONAL CLONING STUDIES IN CANINE NARCOLEPSY AND THE DISCOVERY OF CANARC-1

Screening of genetic markers, including minisatellite probes and functional candidate gene probes, revealed that *canarc-1* cosegregates with a homolog of the switch region of the human immunoglobulin μ heavy-chain gene (Sμ) *(16)*. The genuine Sμ segments are involved in a complex somatic recombination process, allowing individual B cells to switch immunoglobulin classes upon activation *(16)*. Fluorescence *in situ* hybridization indicates that *canarc-1* is located on a different canine chromosome from the canine immunoglobulin switch loci *(22)*. Sequence analysis of the Sμ-like marker indicates that the Sμ-like marker has high homology to the true gene but is not a functional part of the immunoglobulin switch machinery *(23)*. Thus, positional cloning of the region where the Sμ-like marker is located was initiated.

After 10 yr of work, *canarc-1* was finally identified, and narcolepsy in Dobermans and Labradors was found to be caused by a mutation in *Hcrtr 2* (Fig. 2). The mutations in Dobermans and Labradors were found in the same gene but different loci. Both mutations cause exon skipping deletions in the *Hcrtr 2* transcripts and the loss of function of Hcrtr 2, resulting in the impairment of postsynaptic hypocretin neurotransmission. From this study, it appears that the two mutations occur independently in both breeds. Another mutation in *Hcrtr 2* was also found in affected members of a new narcoleptic dachshund family *(24)*, but the reason that the *Hcrtr 2* mutation often occurs in canines (no human case has been identified) is as yet unknown.

Almost simultaneously with the discovery of the canine narcolepsy gene, Dr. Yanagisawa's group reported on that preprohypocretin (prepro-orexin) knockout mice with narcolepsy-like phenotypes (including shorter sleep latency and episodes of behavioral arrest similar to cataplexy in canine narcolepsy) *(4)*. Considering how similar human and canine narcolepsy is at the phenotypic level, it was thought that abnormalities in the hypocretin system are likely to be involved in some human cases, either at the functional or the genetic level. Subsequent neurochemical screening revealed that the hypocretin ligand deficiency is indeed found in most human narcolepsy-cataplexy by cerebrospinal fluid (CSF) hypocretin measures and postmortem studies *(5,7; see* Chap. 17).

6. HYPOCRETIN DEFICIENCY IN SPORADIC CASES OF CANINE NARCOLEPSY

In parallel with studies in human narcoleptic subjects, hypocretin content in the brains and CSF of sporadic narcoleptic dogs (as well as in *Hcrtr 2-mutated* narcoleptic Dobermans) was also examined *(25)*. Hypocretin neurons and contents were found not to be altered in adult *Hcrtr 2-mutated* narcoleptic Dobermans *(25)*. A slight upregulation of ligand production was observed in affected young animals around disease onset *(25)*. These results suggest that the

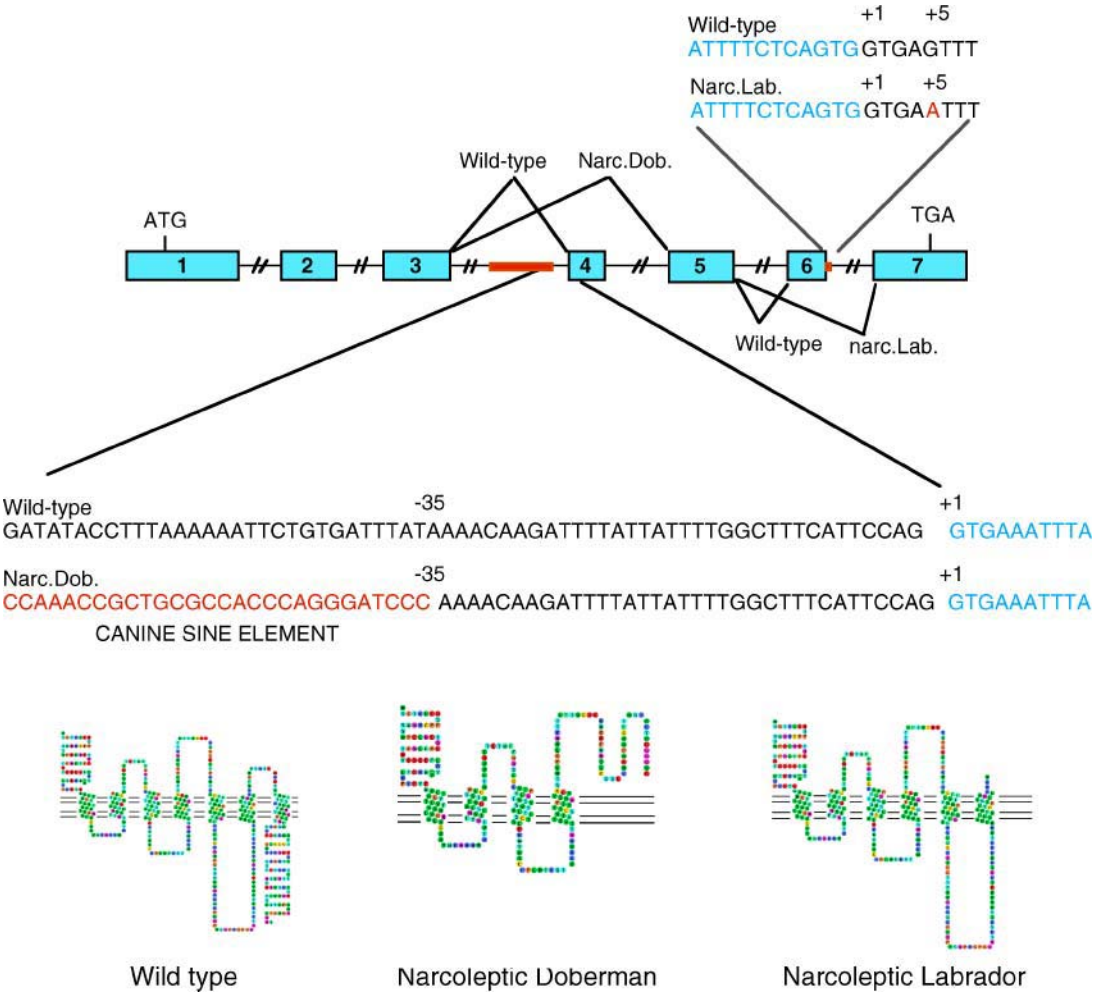

Fig. 2. Genomic organization of the canine *Hcrtr 2* receptor locus. The *Hcrtr 2* is encoded by seven exons. Sequencing of the exon-intron boundary at the site for the deletion of the transcript revealed that the canine short interspersed nucleotide element (SINE) was inserted 35 bp upstream of the 5′ splice donor site of the fourth encoded exon in narcoleptic Doberman pinschers. This insertion falls within the 5′ flanking intronic region needed for pre-mRNA Lariat formation and proper splicing, causing exon 3 to be spliced directly to exon 5 and exon 4 to be omitted. This mRNA potentially encodes a nonfunctional protein with 38 amino acids deleted within the fifth transmembrane domain, followed by a frameshift and a premature stop codon at position 932 in the encoded RNA. In narcoleptic Labradors, the insertion was found 5 bp downstream of the 3′ splice site of the fifth exon, and exon 5 is spliced directly to exon 7, omitting exon 6.

remaining hypocretin neurotransmission mediated by hypocretin receptor 1 (Hcrtr 1) is not sufficient to prevent narcoleptic symptoms. We also examined the brains and/or CSF from four sporadic narcoleptic dogs and found that hypocretin content in all four sporadic narcoleptic dogs was undetectably low *(25)*. Three additional sporadic narcoleptic dogs were newly identified, and they were also confirmed to be hypocretin ligand deficient by CSF hypocretin measures *(26–28)* suggesting that the sporadic narcoleptic dogs share similar pathophysiological mechanisms with most human narcoleptic-cataplexy subjects (Table 1).

7. CANINE NARCOLEPSY AS A SCREENING TOOL FOR UNDERSTANDING THE PHARMACOLOGICAL CONTROL OF CATAPLEXY AND EXCESSIVE DAYTIME SLEEPINESS

Beside the discovery of *canarc-1*, biomedical research in narcolepsy has been also greatly facilitated by using narcoleptic canines. Based on the data obtained from the canine model, our current understanding of the neuropharmacological control of cataplexy and excessive sleepiness (as well as some prospects for the new treatment of narcolepsy) is discussed here. For more details on neuropharmacological results, *see* the review article by Nishino and Mignot *(13)*.

7.1. REM Sleep/Cataplexy and Narcolepsy: Findings in Canine Narcolepsy

After the discovery of SOREMPs in narcoleptic patients *(29)*, narcolepsy has often been regarded as a disorder of REM sleep generation. REM sleep usually appears 90 min after sleep onset and reappears every 90 min in humans (30 min in dogs). Therefore, it was thought that in narcolepsy, REM sleep can intrude in active waking or at sleep onset, resulting in cataplexy, sleep paralysis, and hypnagogic hallucinations; these three symptoms are often categorized as "dissociated manifestations of REM sleep" *(13)*. Abnormal generation of REM sleep might therefore be central to narcolepsy, but this has not been previously demonstrated experimentally. We have therefore analyzed the REM sleep and cataplexy cyclicity in narcoleptic and control canines to observe whether the cyclicity at which REM sleep occurs is disturbed in narcoleptic canines *(9)*. Interval histograms for REM sleep episodes revealed that a clear 30-min cyclicity exists in both narcoleptic and control animals, suggesting that the system controlling REM sleep generation is intact in narcoleptic dogs (Fig. 3). In contrast to REM sleep, cataplexy can be elicited any time upon emotional stimulation (i.e., no 30-min cyclicity is observed) *(9)*.

These results, taken together with the results of extensive human study, show that cataplexy is tightly associated with hypocretin-deficient status (cataplexy now appears to be a unique pathological condition caused by a loss of hypocretin neurotransmission) *(30)* and suggest that mechanisms for triggering of cataplexy and REM sleep are distinct. However, previous electrophysiological data have also demonstrated various similarities between REM sleep atonia and cataplexy *(31)*. Since H-reflex activity (one of the monosynaptic spinal electrically induced reflexes) profoundly diminishes or disappears during both REM sleep and cataplexy, it is likely that the motor inhibitory components of REM sleep are also responsible for the atonia during cataplexy *(31)*. Thus, the executive systems for the induction of muscle atonia during cataplexy and REM sleep are likely to be the same. This interpretation is also supported by the pharmacological findings that most compounds that significantly reduce or enhance REM sleep reduce and enhance cataplexy, respectively. However, some exceptions, such as discrepant effects of dopaminergic D_2/D_3 antagonists on REM sleep and cataplexy, also exist (*32; see* next Section).

7.2. Monoaminergic and Cholinergic Interactions in the Control of Cataplexy

The importance of increased cholinergic activity in triggering REM sleep or REM sleep atonia is well established *(33)*. Similarly, activation of the cholinergic systems using the acetylcholinesterase inhibitor physostigmine also greatly exacerbates cataplexy in canine narcolepsy *(34)*. This cholinergic effect is mediated via muscarinic receptors since muscarinic stimulation aggravates cataplexy, whereas its blockade suppresses it, and nicotinic stimulation or blockade has no effect *(34)*.

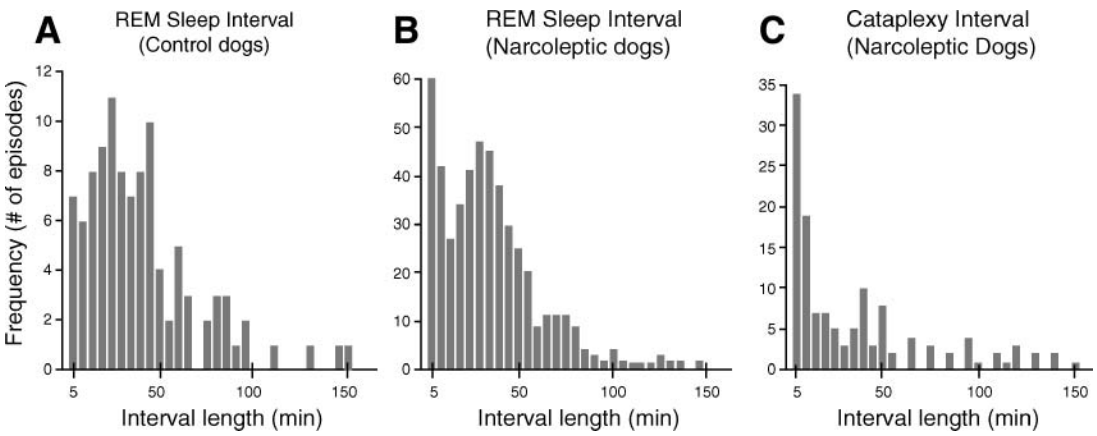

Fig. 3. Frequency of interval lengths between consecutive REM sleep episodes in narcoleptic and control dogs and cataplexy interval lengths in narcoleptic canines. REM sleep and cataplexy intervals are shown in 5-min bins. **(A,B)** A clear 30-min interval between consecutive REM sleep episodes is present in both narcoleptic and control animals. **(C)** No such cyclicity is present for cataplexy.

Monoaminergic transmission is also critical for the control of cataplexy. All therapeutic agents currently used to treat cataplexy (i.e., antidepressants or monoamine oxidase inhibitors [MAOIs]), are known to act on these systems. Furthermore, whereas a subset of cholinergic neurons is activated during REM sleep, the firing rate of monoaminergic neurons in the brainstem (such as in the locus coeruleus [LC] and raphe magnus) is well known to be dramatically depressed during this sleep stage *(35,36)*. Using canine narcolepsy, it was recently demonstrated that adrenergic LC activity is also reduced during cataplexy *(37)*. In contrast, dopaminergic neurons in the ventral tegmental area (VTA) and substantia nigra (SN) do not significantly change their activity during natural sleep cycles *(38,39)*.

Since cataplexy in dogs can be easily elicited and quantified, the canine narcolepsy model has been intensively used to dissect the mode of action of currently used anticataplectic medications. The most commonly prescribed anticataplectic mediations in humans are tricyclic antidepressants. These compounds have a complex pharmacological profile that includes monoamine uptake inhibition (dopamine [DA], epinephrine, norepinephrine [NE], and serotonin) and anticholinergic, α_1-adrenergic antagonistic, and antihistaminergic effects, making it difficult to determine which one of these pharmacological properties is actually involved in their therapeutic effects.

To determine which property was most relevant, we first studied the effects of a large number (a total of 17 compounds) of uptake blockers/release enhancers specific for the adrenergic, serotonergic, or dopaminergic system. Adrenergic uptake inhibition was found to be the key property involved in the anticataplectic effect *(40)*. Serotonergic uptake blockers were only marginally effective at high doses, and the dopaminergic uptake blockers were completely ineffective. Interestingly, it was later found that these DA uptake inhibitors had potent alerting effects in canine narcolepsy *(13)*.

We also compared the effects of several antidepressants with those of their demethylated metabolites. Many antidepressants (most typically tricyclics) are known to metabolize significantly by a hepatic first pass into their demethylated metabolites, which, have longer half-lives and higher affinities for adrenergic uptake sites *(41)*. During chronic drug

administration, these demethylated metabolites accumulate *(41)* and can thus be involved in the drug's therapeutic action. The effects of five available antidepressants (amitriptyline, imipramine, clomipramine, zimelidine, and fluoxetine) were compared with those of their respective demethylated metabolites (nortriptyline, desipramine, desmethylclomipramine, norzimelidine, and norfluoxetine) *(42)*. In all cases, the demethylated metabolites were found to be more active on cataplexy than were the parent compounds. We also found that the active dose of all anticataplectic compounds that tested positively correlated with the in vitro potency of each compound to the adrenergic transporter but not with that of the serotonergic transporter *(42)*. In fact, the anticataplectic effects were negatively correlated with the in vitro potency for serotonergic uptake inhibition, but this may be a bias since potent adrenergic uptake inhibitors included in the study have a relatively low affinity to serotonergic uptake sites. Although most of these results were obtained from inbred *Hcrtr 2-mutated* narcoleptic Dobermans, similar findings (the preferential involvement of adrenergic system) have also been obtained in more diverse cases of sporadic canine narcolepsy in various breeds donated to our colony *(34)*.

In order to dissect receptor subtypes that significantly modify cataplexy, more than 200 compounds with various pharmacological properties (cholinergic, adrenergic, dopaminergic, serotonergic, prostaglandins, opioids, benzodiazepines, γ-aminobutyric acid [GABA] ergics, and adenosinergics) have also been studied in the narcoleptic canine model (*see* ref. *13* for details). Although many compounds (such as muscarinic M_2 antagonists, adrenergic alpha-1 agonists, adrenergic alpha-2 antagonists, dopaminergic D_2/D_3 antagonists, serotonergic 5HT1a agonists, thyroid-releasing hormone [TRH] analogs, prostaglandin E_2, and L-type Ca^{2+} channel blockers) reduce cataplexy), very few compounds significantly aggravate cataplexy (cataplexy-aggravating effects are assumed to be more specific, since cataplexy can be nonspecifically reduced by unpleasant drug side effects) *(13)*. Stimulation of M_2 (non-M_1) receptors significantly aggravates cataplexy. Among the monoaminergic receptors, blockade of the postsynaptic adrenergic α-1b receptors *(43)* and stimulation of presynaptic α-2 autoreceptors *(44)* were also found to aggravate cataplexy, a result consistent with a primary adrenergic control of cataplexy. We also found that small doses of D_2/D_3 agonists significantly aggravated cataplexy and induced significant sleepiness in these animals *(45,46)*. These pharmacological findings parallel neurochemical abnormalities previously reported in canine narcolepsy, namely, significant increases in α-2 receptors in the LC *(47)*, D_2 receptors in the amygdala and nucleus accumbens *(48)*, and M_2 receptors in the pons *(49)*. To date, no other receptor ligands (i.e., adenosinergic, histaminergic or GABAergic) have been found to aggravate cataplexy. Thalidomide (an old hypnotic with an immunomodulatory property) was found to aggravate cataplexy significantly but modes of action of thalidomide on cataplexy are not yet known *(50)*.

The sites of action of D_2/D_3 agonists were also investigated by local drug perfusion experiments, and a series of experiments identified acting sites for these compounds. These include dopaminergic nuclei or cell groups, such as the VTA *(46)*, SN *(51)*, and A11 *(52)* (a diencephalic DA cell group that directly projects to the spinal ventral horn), suggesting a direct involvement of DA cell groups and DA cell body autoreceptors for the regulation of cataplexy. The cataplexy-inducing effects of D_2/D_3 agonists are, however, difficult to reconcile considering the fact that dopaminergic uptake blockers have absolutely no effect on cataplexy *(40)*. We believe that D_2/D_3 receptor mechanisms are more specifically involved in the control of sleep-related motor tonus (i.e., cataplexy or muscle atonia without phasic REM events)

than those for active REM sleep. A recent finding in canine narcolepsy that sulpiride (a D_2/D_3 antagonist) significantly suppresses cataplexy but has no effect on REM sleep also supports this notion *(32)*. It should also be noted that D_2/D_3 agonists are clinically used for the treatment of human periodic leg movements during sleep (PLMS) *(53)*. Involuntary leg movements during sleep are often associated with restless leg syndrome (RLS) and disturbed nighttime sleep *(54)*, both of which occur in narcoleptic humans and Dobermans *(55)*. It thus appears that the dopaminergic system (i.e., D_2/D_3 receptor mechanisms) may be more specifically involved in sleep-related motor control than REM sleep.

The effects on cataplexy by cholinergic stimulation in various brain regions were also examined in narcoleptic and control canines. Local injection or perfusion of carbachol (a predominantly muscarinic agonist) into the pontine reticular formation (PRF) was found to aggravate canine cataplexy in a dose-dependent fashion *(56)*. The results obtained in the PRF with cholinergic agonists were somewhat expected considering the well-established role of the pontine cholinergic systems in the regulation of REM sleep and REM sleep atonia *(33)*. Surprisingly, we also found that local injection/perfusion of carbachol unilaterally or bilaterally into the basal forebrain (BF; rostral to the preoptic area, in the vertical or horizontal limbs of the diagonal band of Broca and the medial septum) dose-dependently aggravated cataplexy and induced long-lasting episodes of muscle atonia accompanied by desynchronized EEGs in narcoleptic canines *(57)* (Fig. 4). Physostigmine was also found to aggravate cataplexy when injected into the same site, thus suggesting that fluctuations in endogenous levels of acetylcholine in this structure may be sufficient to induce cataplexy *(57)*. The carbachol injections did not induce cataplexy in normal animals, but rather induced wakefulness. Thus altered sensitivity of more global cholinoceptive sites (than those for REM sleep induction) may be involved in induction of cataplexy.

Furthermore, we found that bursts of rapid eye movements (phasic phenomena of REM sleep) in 30-min cycles that can be observed at the baseline were preserved during carbachol-induced long episodes of cataplexy *(57)* (Fig. 4). This suggests the possibility that the brain site(s) for triggering cataplexy are different from those for REM sleep and that more global brain structures may be involved in the induction of cataplexy (although they are likely to share the common executive system for inhibition of the muscle tonus).

The BF is anatomically connected with the limbic system, which is regarded as a critical circuit for integrating emotions. Furthermore, BF neurons are known to respond to the arousing property of appetitive stimuli *(58)*, which we use to induce cataplexy in narcoleptic dogs. Considering the fact that emotional excitation is an alerting stimulus in normal animals but induces cataplexy in narcoleptic animals, the BF may be involved in triggering a paradoxical reaction to emotions (atonia rather than wakefulness) in narcoleptic animals.

Global and persistent cholinergic/monoaminergic imbalance, owing to impaired hypocretin neurotransmission, may be required for the occurrence of cataplexy. This could not be induced only by an increase in REM sleep propensity and/or vigilance state instability that occurs in various disease conditions (such as depression) *(59)* or in some physiological conditions (such as REM sleep deprivation). REM sleep abnormalities and sleep fragmentation are often seen in other sleep disorders (such as "narcolepsy without cataplexy," sleep apnea), and even in healthy subjects when their sleep patterns are disturbed. The fact that these subjects never develop cataplexy also supports this hypothesis.

The mechanism for emotional triggering of cataplexy remains to be studied, but multiple brain sites and multiple functional and anatomical systems may also be involved.

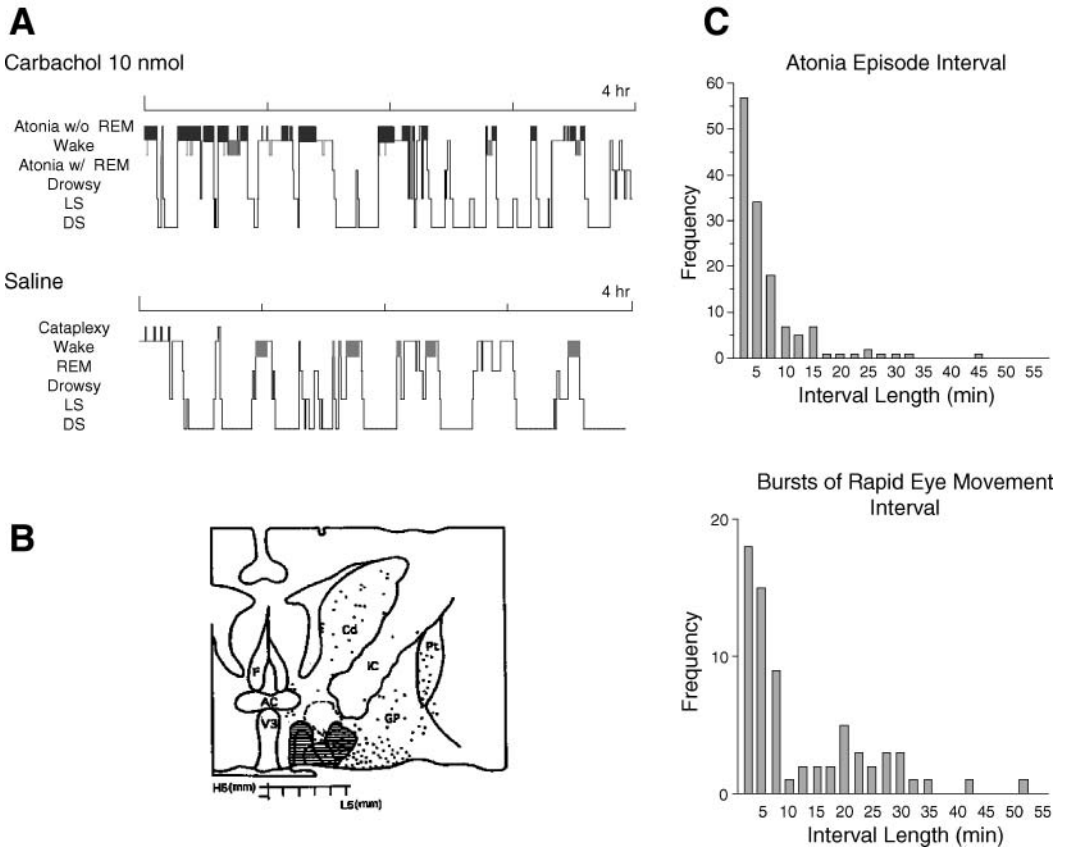

Fig. 4. Effect of carbachol injections in the basal forebrain (BF) of narcoleptic Dobermans, injection sites of carbachol in the BF of narcoleptic dogs, and atonia and REM sleep interval length frequency in narcoleptic dogs after BF carbachol injections. **(A)** BF carbachol injections (10 nmol) induce long-lasting muscle atonia in narcoleptic dogs. Typical hypnograms obtained from one narcoleptic animal are shown. **(B)** Map of BF injection sites. The acting site for carbachol injections is located in the magnocellular BF. AC, anterior commissure; cd, caudate nucleus; F, fornix; GP, globus pallidus; IC, internal capsule; Pt, putamen; V3, third ventricle. **(C)** Although carbachol injection induced long-lasting muscle atonia (with no discernible cyclic pattern), 30-min cyclicity of rapid eye movement bursts, one of the phasic phenomena of REM sleep, is still observed during carbachol-induced long-lasting muscle atonia. These results suggest that sites and mechanisms for the induction of cataplexy are different from those for REM sleep (*see* also Fig. 6 and its legend).

7.3. Dopaminergic Transmission and EEG Arousal

Narcoleptic Dobermans were also used for a series of pharmacological experiments to dissect modes of action of wake-promoting compounds. Amphetamine-like central nervous system (CNS) stimulants (currently used clinically for the management of sleepiness in narcolepsy) presynaptically enhance monoaminergic transmission; however, these compounds also lack pharmacological specificity. In order to study the mode of action of these compounds on daytime sleepiness, the stimulant properties of several dopaminergic and adrenergic uptake inhibitors were quantified and compared with the effects of amphetamine and modafinil using 4-h daytime polygraphic recordings *(60)*. Despite their lack of effect on cataplexy (*see* cataplexy section), all dopaminergic uptake inhibitors induced significant EEG arousal (Fig. 5). In contrast, nisoxetine and desipramine, two potent adrenergic

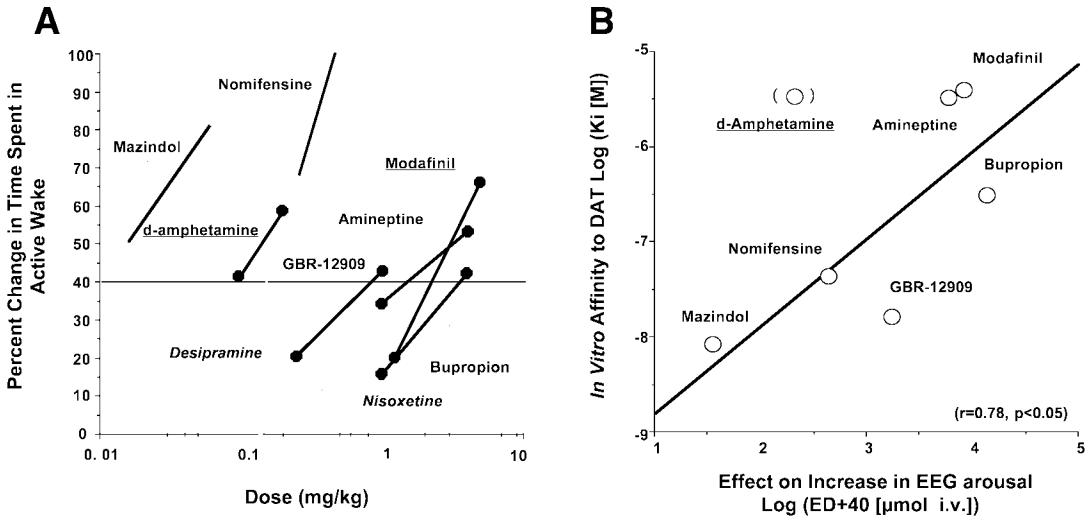

Fig. 5. Effects of various DA and NE uptake inhibitors and amphetamine-like stimulants on the electroencephalographic (EEG) arousal of narcoleptic dogs and correlation between in vivo EEG arousal effects and in vitro dopamine transporter (DAT) binding affinities. The effects of compounds on daytime sleepiness were studied using 4-h daytime polygraphic recordings (10:00–14:00) in four to five narcoleptic animals. Two doses were studied for each compound. All DA uptake inhibitors and CNS stimulants dose-dependently increased EEG arousal and reduced slow-wave sleep (SWS) compared with vehicle treatment. In contrast, nisoxetine and desipramine, two potent NE uptake inhibitors, had no significant effect on EEG arousal when doses that completely suppressed REM sleep were injected. **(A)** The effects of the two doses studied for each stimulant were used to construct a rough dose-response curve. The drug dose that increased the time spent in wakefulness by 40% more than the baseline (vehicle session) was then estimated for each compound. The order of potency of the compounds obtained was: mazindol > amphetamine > nomifensine > GBR 12,909 > amineptine> modafinil > bupropion. In vitro DAT binding was performed using [^{3}H]WIN 35,428 onto canine caudate membranes and demonstrated that the affinity of these DA uptake inhibitors varied widely between 6.5 and 3.3 m*M*. In addition, it was also found that both amphetamine and modafinil have a low, but significant affinity (same range as amineptine) for DAT. **(B)** A significant correlation between in vivo and in vitro effects was observed for all five DA uptake inhibitors and modafinil. Amphetamine, which showed a potent EEG arousal property, has a relatively low DAT binding affinity, suggesting that other mechanisms, such as monoamine release or monoamine oxidase inhibition by amphetamine, are also involved in the mechanism of EEG arousal. These results suggest that presynaptic enhancement of DA transmission is a key pharmacological property mediating the EEG arousal effects of many stimulant compounds available.

uptake inhibitors, had little effect on EEG arousal but significantly suppressed REM sleep. Furthermore, the in vivo potency of DA uptake inhibitors on EEG arousal correlates well with their in vitro affinity to the dopamine transporter (DAT) but not to the norepinephrine transporter (NET).

These results are consistent with the hypothesis that presynaptic modulation of dopaminergic transmission is a key property mediating the EEG arousal effects of these compounds. Interestingly, we also found that modafinil binds to the DAT site with low affinity *(60,61)*, similar to the affinity range for amineptine (a DA uptake inhibitor that also enhances EEG arousal in our model). Furthermore, we recently demonstrated that the

wake-promoting effects of modafinil and amphetamine were completely absent in mice lacking DAT (i.e., DAT knockout mice) *(62)*. These results clearly indicate that DAT is a key target molecule and is required for the mediation of the wake-promoting effect of modafinil and amphetamine.

Amphetamine (which showed a potent EEG arousal property) has, however, a relatively low DAT binding affinity *(63)*, suggesting that other mechanisms, such as monoamine release (exchange diffusion through the DAT) by amphetamine, are also involved in the mechanism of EEG arousal. To assess further the net effects of amphetamine on monoaminergic neurotransmission, we measured DA and NE efflux together with the wake-promoting effects of amphetamine analogs and isomers in the canine narcolepsy model *(64)*. Polygraphic recordings demonstrated that d-amphetamine was about twice as potent as l-amphetamine and was six times more potent than l-methamphetamine in increasing wakefulness, whereas d-amphetamine and l-amphetamine were equipotent in reducing REM sleep and cataplexy, and l-methamphetamine was about half as potent as l- and d-amphetamine *(64)*. By measurements of extracellular levels of DA and NE, we found that d-amphetamine was more potent in increasing DA efflux than l-amphetamine, and l-methamphetamine had little effect. In contrast, there was no significant difference in the potencies of these three derivatives on NE efflux. Thus, the potencies of amphetamine isomers/analogs on wakefulness correlated well with DA and further exemplify the importance of the DA system for the pharmacological control of EEG arousal *(64)*.

It is, however, the involvement of the dopaminergic systems in the regulation of the sleep/wake process that has not been given much attention. This is mostly because of early electrophysiological findings demonstrating that dopaminergic neurons in the VTA and SN do not change their activity significantly during the sleep cycle *(39)*, in contrast to noradrenergic cells of the LC or serotonergic cells of the raphe, which increase firing in wake vs sleep. A recent microdialysis study of measures of DA efflux in the terminal areas also failed to demonstrate state-dependent changes in DA levels *(65)*. These experimental results led most investigators to believe that adrenergic tone was more important than dopaminergic transmission for the control of EEG arousal.

The dopaminergic system may thus not be so critical for the normal sleep-wake cycle regulation, but it may be critical for forced wakefulness by motivation and/or by some wake-promoting compounds. In clinical conditions, a disturbance of this alerting system may also be troublesome, since this may induce intolerable sleepiness when subjects need to be kept wake.

Indeed, an involvement of the dopaminergic system in intolerable sleepiness is also noted in some pathological conditions, such as Parkinson's disease. Frequent sleep attacks and associations with accidents are reported by patients with Parkinson's disease treated with DA D_2/D_3 agonists *(66,67)*. (Low doses of this class of compounds induce drowsy states and cataplexy in the canine model of narcolepsy, but have no behavioral effect on control Dobermans at the same doses).

7.4. Imbalance Within Monoaminergic Systems

After discovery of the involvement of the hypocretin system in narcolepsy, we focused on how these deficits in hypocretin neurotransmission induce narcolepsy. One of the keys to solving this question is revealing the functional differences between Hcrtr 1 and Hcrtr 2, since it is evident that Hcrtr 2-mediated function plays a critical role in generating narcoleptic symptoms in our model *(68)*. *In situ* hybridization experiments in rats demonstrated that

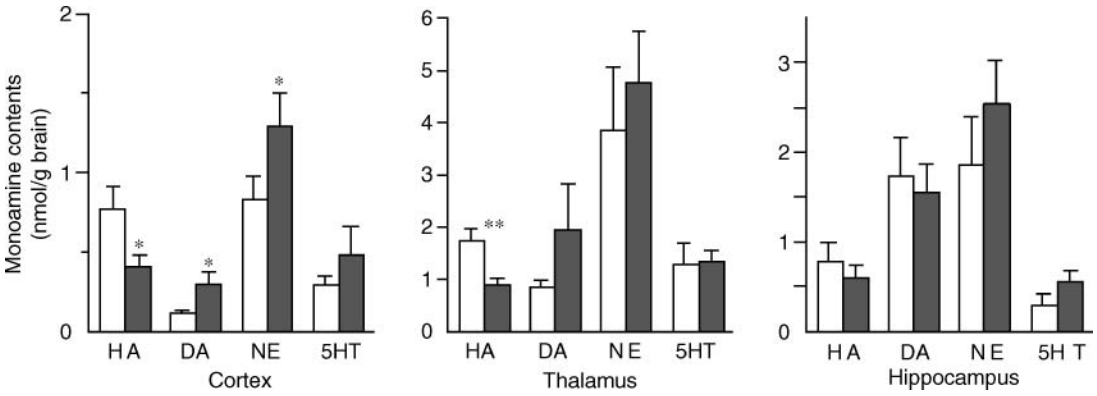

Fig. 6. Histamine, dopamine (DA), norepinephrine (NE), and serotonin (5-HT) contents in the cortex, thalamus, and hippocampus in *Hcrtr2*-mutated narcoleptic and control Dobermans. Histamine content in the cortex and thalamus was significantly lower in narcoleptic Dobermans (dark bars) compared with control Dobermans (open bars), whereas DA and NE were higher in these structures. Increases were statistically significant in cortex (by Student's *t*-test).

Hcrtr 1 is enriched in the ventromedial hypothalamic nucleus, taenia tecta, hippocampal formation, dorsal raphe, laterodorsal tegmental nucleus, and LC *(69)*. In contrast, Hcrtr 2 is enriched in the paraventricular nucleus, magnocellular preoptic area, cerebral cortex, nucleus accumbens, VTA, SN, and histaminergic tuberomamillary nucleus (TMN) *(69)*. These findings are consistent with our pharmacological understanding of the regulation of cataplexy and EEG arousal discussed above, and the lack of input to Hcrtr 2 on the monoaminergic/cholinergic nucleus/receptive sites may result in cholinergic/monoaminergic imbalance.

Histamine is another important wake-promoting monoamine, but the roles of the histaminergic system in the pathophysiology of narcolepsy had not received much attention before the discovery of hypocretin involvement. Histamine neurons are typically wake-active neurons *(70)*, located exclusively in the TMN of the hypothalamus, from where they project to practically all brain regions, including areas important for vigilance control, such as the hypothalamus, BF, thalamus, cortex, and brainstem structures (*see* ref. *71* and *72* for review). Since histaminergic TMN neurons exclusively express hypocretin-2, we initiated a study of the roles of the histaminergic system in canine narcolepsy.

As a first step, we measured histamine content in the brain of *Hcrtr 2-mutated* and control Dobermans *(73)*. DA, NE, and their metabolites were also measured. We found that the histamine content in the cortex and thalamus was significantly lower in narcoleptic Dobermans compared with controls (Fig. 6). In contrast to histamine content, DA and NE levels in familial narcolepsy was high in these structures. Two independent studies previously reported altered catecholamine levels in the brains of narcoleptic dogs *(74,75)*. Since it was recently reported that hypocretins strongly excite TMN histaminergic neurons in vitro, through Hcrtr 2 stimulation *(76–79)*, the decrease in histaminergic content found in narcoleptic dogs may thus be owing to the lack of excitatory input of hypocretin on TMN histaminergic neurons. Although loss of hypocretin input through Hcrtr 2 may also lead to decreases in catecholamine content, DA and NE contents were instead high in several brain structures tested (Fig. 6).

Thus, the imbalance in the neurotransmitter system may also be evident among different monoamine systems. Considering the fact that catecholamine turnover was not reduced in

these animals, but rather high *(73)*, and that compounds that enhance DA and NE transmission significantly improve symptoms of narcolepsy in these animals *(13)*, the increase in DA and NE contents may be compensatory, mediated either by Hcrtr 1 or by other neurotransmitter systems. Uncompensated low histamine levels in narcolepsy may therefore suggest that the hypocretin system through Hcrtr 2 is the major excitatory input to histaminergic neurons, which is consistent with the findings of hypocretin receptor distribution: TMN histaminergic neurons exclusively express Hcrtr 2, whereas LC NE neurons express Hcrtr 1 exclusively, and VTA DA neurons express both Hcrtr 1 and Hcrtr 2 *(69)*.

We also measured histamine in the brain of three sporadic narcoleptic dogs and found that histamine levels in these animals were also as low as in *Hcrtr 2-mutated* narcoleptic Dobermans *(73)*, thus suggesting that a decrease in histamine neurotransmission may also exist in ligand-deficit narcolepsy. Furthermore, two independent human studies showed a decease in histaminergic content in the CSF in hypocretin-deficient narcolepsy *(80,81)*, suggesting that altered histaminergic neurotransmission may not be limited to the *Hcrtr 2-mutated* narcolepsy.

Thus, the histaminergic system may be a new target site for the treatment of EDS and other symptoms associated with narcolepsy, and the centrally active histaminommic compounds such as H_3 autoreceptor antagonists may have therapeutic effects in these conditions *(82)*.

8. HYPOCRETIN DEFICIENCY AND NARCOLEPTIC PHENOTYPE AND HYPOCRETIN REPLACEMENT THERAPY

Finally, a canine narcolepsy model (both familial and sporadic narcoleptic dogs) was used for evaluation of replacement/supplement therapies of hypocretin ligands *(27)*. Our results using canine narcolepsy suggest that development of centrally penetrable hypocretin agonists (i.e., small-molecule synthetic agonists) is likely to be necessary for human application. Details of these experiments are summarized in Chapter 27, on hypocretin replacement therapy.

Human studies have demonstrated that the occurrence of cataplexy is tightly associated with hypocretin deficiency *(30)*. Furthermore, the hypocretin deficiency was already observed at very early stages of the disease (just after the onset of EDS), even before occurrences of definite cataplexy *(83,84)*. Occurrences of cataplexy are rare in acute symptomatic cases of EDS associated with a significant hypocretin deficiency (*see* more details in ref. *85*). Thus, it appears that chronic and selective deficit of hypocretin neurotransmission may be required for the occurrence of cataplexy. The possibility of an involvement of secondary/associated neurochemical changes (other than ligand deficiency) for the occurrence of cataplexy cannot be ruled out in humans. If some of these changes are irreversible, hypocretin supplement therapy may only have limited effects on cataplexy.

Sleepiness in narcolepsy is most likely owing to difficulty in maintaining wakefulness as normal subjects do. The sleep pattern of narcoleptic subjects is also fragmented, with bouts of insomnia (frequent wakening) at night. This fragmentation occurs across 24 h, and thus, the loss of hypocretin signaling should play a role of this vigilance stage stability, although other mechanisms may also be involved in EDS in narcoleptic subjects *(86)*.

One of the most important characteristics of EDS in narcolepsy is that sleepiness is reduced, and patients feel refreshed after a short nap, but this does not last long, as they become sleepy again within a short period. In experiments measuring diurnal fluctuation of hypocretin tonus and in relation to vigilance changes in animals, we have noted that hypocretin levels in the extracellular space and in the CSF of rats significantly fluctuate across 24 h, and build up toward the end of the active periods *(87)*. Several manipulations (such as sleep

deprivation, exercise, and long-term food deprivation) have also been shown to increase hypocretin tonus *(87–89)*. The fact that hypocretins promote wakefulness in experimental conditions suggests that the lack of hypocretin buildup mediated by circadian and alerting stimulation may also play a role in EDS associated with hypocretin-deficient narcolepsy.

9. CONCLUSIONS

Although the canine narcoleptic model contributed significantly to our understanding of the etiological and pathophysiological aspects of the disease, one of the major missions (identifying the narcolepsy gene) of this animal research was over. The value of the sporadic hypocretin ligand-deficit narcoleptic dog is high, but it is difficult to obtain enough of these animals for systematic studies. *Hcrtr 2-mutated* narcoleptic dogs are still useful for various pharmacological and physiological experiments, but some of the studies will be taken over by experiments using rodent models (especially experiments focusing on cell transplantation or gene therapy).

Before closing this chapter, I would like to emphasize again that establishment of the animal model of narcolepsy was a long and steady effort (especially maintaining the colony and cloning the gene), which finally led to the first major breakthrough in over 100 yr of narcolepsy research.

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19
Rodent Models of Human Narcolepsy-Cataplexy

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1. DISCOVERY OF MOUSE NARCOLEPSY-CATAPLEXY

Orexin/hypocretin peptides (orexin-A and -B, or hypocretin-1 and -2) were initially purified from rat brain extracts as endogenous ligands for an orphan G-protein-coupled receptor (GPCR) *(1)*. A subsequent search of expressed sequence tag (EST) databases led to the identification of another novel GPCR as the second receptor for orexin peptides (orexin receptor-1 and -2: OX_1R and OX_2R). Orexin-A and -B are produced by cleavage of a single precursor polypeptide, prepro-orexin, and neurons expressing orexins (orexin neurons) are located exclusively in the perifornical area of the hypothalamus.

When orexin knockout mice (*orexin*$^{-/-}$ mice) were created, intensive studies of their behavior using conventional methods failed to reveal any overt abnormalities during the daytime, their inactive phase *(2)*. To assess behavior at night, when mice are normally most active, mice were recorded by infrared videophotography. The videotapes revealed frequent periods of obvious behavioral arrest in *orexin*$^{-/-}$ mice during the dark phase with full penetrance, which were totally unexpected (Fig. 1A). No sign of serum electrolyte imbalance or hypoglycemia was observed in *orexin*$^{-/-}$ mice. Bodily collapse associated with episodic rocking behavior initially suggested the possibility of a seizure disorder in the *orexin*$^{-/-}$ mice. However, electroencephalograph/electromyograph (EEG/EMG) recordings from *orexin*$^{-/-}$ mice showed no evidence of seizure activity, rather, these EEG/EMG recordings revealed abnormal intrusions of REM sleep into wakefulness and fragmentation of the sleep/wake cycle (Figs. 2 and 3). Reduced latency to REM sleep and increase in REM sleep during the dark phase were also observed (Fig. 4). Overall, these behavioral and electrophysiological characteristics of *orexin*$^{-/-}$ mice were strikingly similar to human narcolepsy-cataplexy. Human narcolepsy-cataplexy is a debilitating neurological disease characterized by cataplexy (sudden bilateral skeletal muscle weakness without impairment of consciousness), premature transitions to REM sleep (sleep-onset REM [SOREM] periods), and excessive daytime sleepiness. An independent discovery around the same time that mutations in the *OX2R* gene are responsible for familial narcolepsy-cataplexy in dogs, which is an accepted animal model of human narcolepsy-cataplexy, further supported rodent narcolepsy-cataplexy in *orexin*$^{-/-}$ mice *(3)*.

Subsequently, OX_2R knockout mice (*OX2R*$^{-/-}$ mice) were created and analyzed *(4)*. Since mutations of the *OX2R* gene are responsible for canine narcolepsy-cataplexy, it was expected that *OX2R*$^{-/-}$ mice would exhibit cataplexy-like abrupt behavioral arrests (abrupt arrest), as observed in *orexin*$^{-/-}$ mice and narcoleptic dogs. In infrared videophotographic studies in the dark phase, we indeed saw that kind of behavioral arrest in *OX2R*$^{-/-}$ mice. However, its fre-

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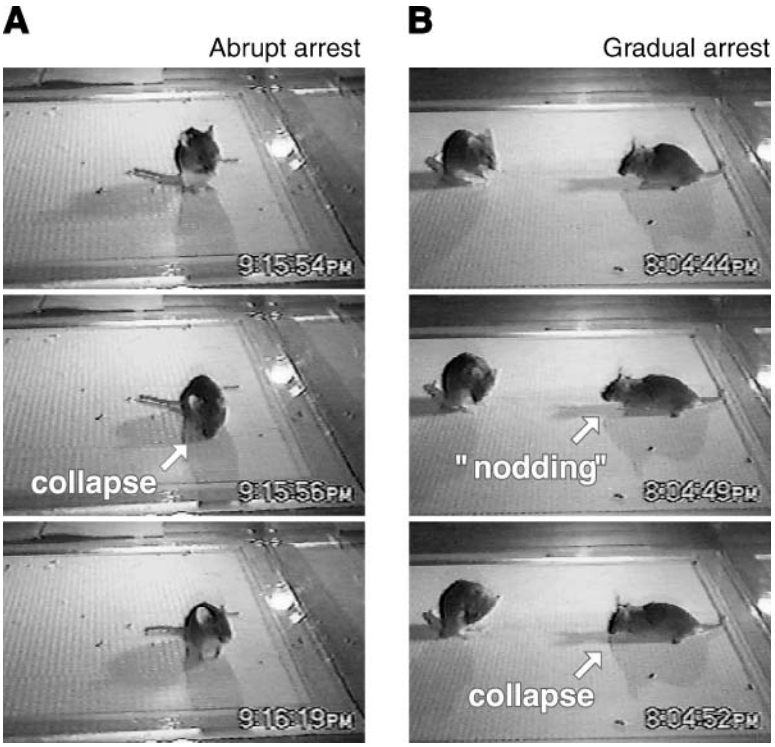

Fig. 1. Comparison of behavioral arrests in knockout mice by infrared videophotography. **(A)** Time-lapse images portraying an abrupt arrest, rarely observed in an *OX2R*$^{-/-}$ mouse. Note the collapsed posture in the second panel. **(B)** Time-lapse images portraying a gradual arrest in an *OX2R*$^{-/-}$ mouse. "Nodding" behavior (second panel) occurs just prior to postural collapse (third panel). (Modified from ref. *4*.)

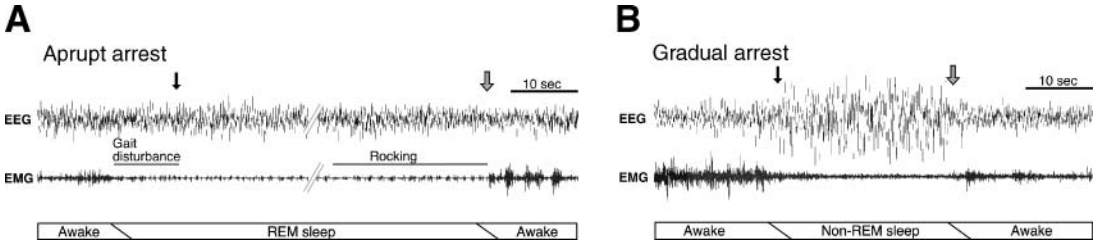

Fig. 2. Typical EEG/EMG traces during behavioral arrests in *orexin*$^{-/-}$ and *OX2R*$^{-/-}$ mice. Solid and gray arrows demarcate onsets and terminations of arrests, respectively. Gray bars reflect the timing of gait disturbances and rocking behavior associated with arrests. Behavioral states are classified as awake, non-REM sleep, or REM sleep based on EEG/EMG features. **(A)** Abrupt arrest in *orexin*$^{-/-}$ mouse. Excited ambulation (high-amplitude nuchal EMG) accompanied by an EEG typical of normal active wakefulness (low-amplitude, mixed frequency activity) gives way to rapid onset of ataxic gait, reduced neck tone, and an EEG resembling REM sleep. Postural collapse is accompanied by neck atonia and continued REM sleep EEG. Rocking behavior from limb movement occurs exclusively during periods with EEG/EMG indistinguishable from REM sleep pattern. Residual low-amplitude noise remaining in the EMG during atonia consists primarily of electrocardiographic contamination. **(B)** Gradual arrest in *OX2R*$^{-/-}$ mouse. Feeding behavior with high-amplitude EMG and a waking EEG gives way to gradual onset of postural collapse with reduced but not atonic nuchal EMG and transition to an EEG indistinguishable from non-REM sleep. The mouse remains immobile until sudden recovery of waking EEG and purposeful behavior. (Modified from ref. *4*.)

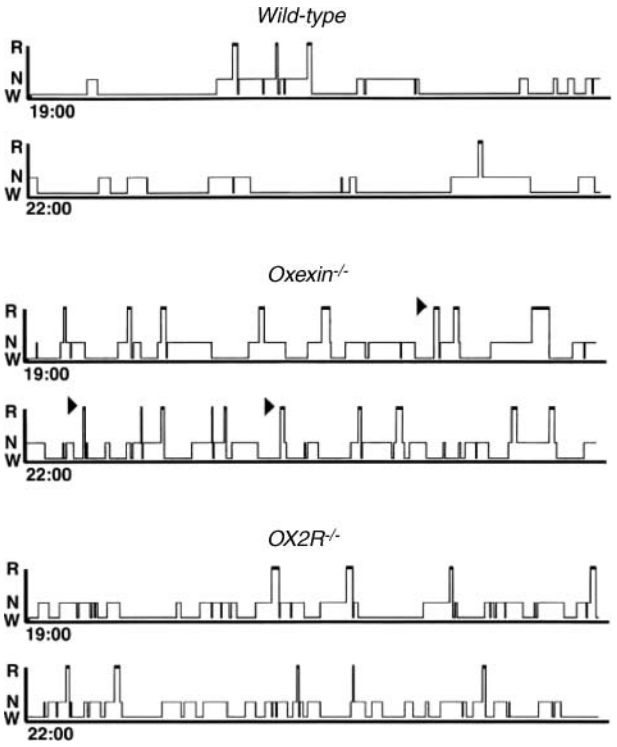

Fig. 3. Representative hypnograms of wild-type, *orexin*$^{-/-}$, and *OX2R*$^{-/-}$ mice over the first 6 h of the dark phase (19:00–01:00) obtained by concatenating 20-s epoch EEG/EMG stage scores. The height of the horizontal line above baseline indicates the vigilance state of the mouse at the time (min) from the beginning of the dark phase. Baseline (W) represents periods of wakefulness; N, non-REM sleep; R, REM sleep. Arrowheads highlight direct transitions from wakefulness to REM sleep. *Orexin*$^{-/-}$ and *OX2R*$^{-/-}$ mice show similar fragmentation of vigilance. (Modified from ref. *4*.)

quency was much less than in *orexin*$^{-/-}$ mice (31-fold lower frequency in *OX2R*$^{-/-}$ mice compared with *orexin*$^{-/-}$ mice). Instead, *OX2R*$^{-/-}$ mice showed a distinct variety of behavioral arrests with onsets that were more gradual in nature (gradual arrest; Fig. 1B), and it turned out that *orexin*$^{-/-}$ mice also exhibited gradual arrests, with a frequency similar to that of *OX2R*$^{-/-}$ mice, in addition to many abrupt arrests. Detailed characterization of behavioral, pharmacological, and electrophysiological features of *orexin*$^{-/-}$ and *OX2R*$^{-/-}$ mice defined abrupt and gradual arrests as the presumptive mouse correlates of cataplexy and sleep attack respectively, in human narcolepsy-cataplexy. In the following sections, we describe the narcoleptic phenotype of *orexin*$^{-/-}$ and *OX2R*$^{-/-}$ mice in detail and discuss them in comparison with symptoms in human and canine narcolepsy-cataplexy.

2. REM SLEEP-RELATED SYMPTOMS (CATAPLEXY)

The characteristics of abrupt arrests were very different from those of quiet behavioral states with decreased overt activity, as well as from normal transitions into sleep *(2,4)*. They were specifically recognized by the abrupt cessation of purposeful motor activity associated with sudden, sustained change in posture that was maintained throughout the episode, ending abruptly with complete resumption of purposeful motor activity (Fig. 1A). Essentially, the episodes looked as if a behavioral switch had been turned "off" and then "on." Each episode

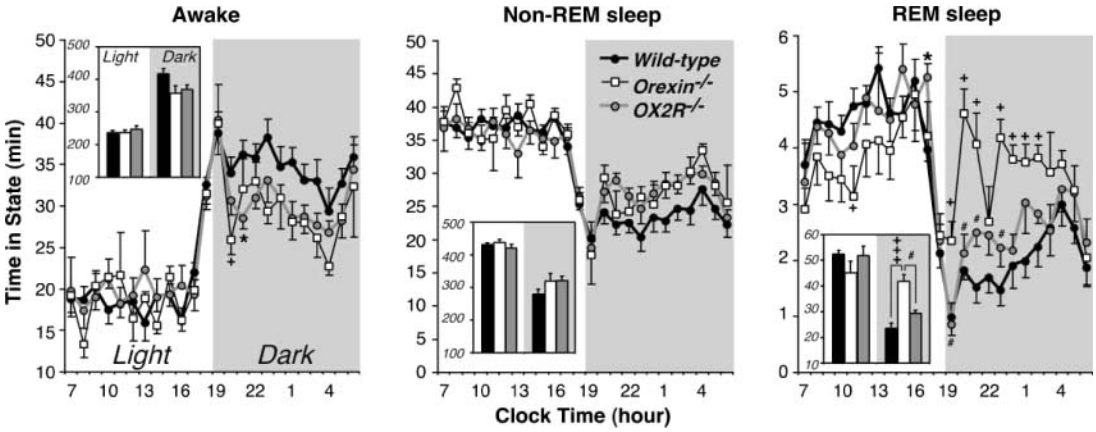

Fig. 4. Hourly amounts of awake, non-REM sleep, and REM sleep states (means and SEM) plotted over the 24-h day for each group. Data collapsed over 12-h light and dark phases are displayed as graph insets. Data for the light and dark phases are displayed on white and light gray backgrounds, respectively. Wakefulness and non-REM sleep are disrupted to a similar degree in *orexin*$^{-/-}$ and *OX2R*$^{-/-}$ mice, especially during the dark phase. *orexin*$^{-/-}$ mice consistently show significantly increased REM sleep times during the dark phase compared with normal mice; *OX2R*$^{-/-}$ mice do not. Significant differences between *OX2R*$^{-/-}$ and wild-type mice: *, $p < 0.05$; **, $p < 0.005$. Significant differences between *orexin*$^{-/-}$ and wild-type mice: +, $p < 0.05$; ++, $p < 0.005$; +++, $p < 0.0005$. Significant differences between *orexin*$^{-/-}$ and *OX2R*$^{-/-}$ mice: #, $p < 0.05$. (Modified from ref. *4*.)

lasted for a very short period, mostly less than a minute. Occasionally, gait disturbance lasting for 1–3 s was observed immediately before episodes. Also grossly observable motor activity causing side-to-side rocking, without change in overall posture, frequently occurred several seconds after the start of the arrest.

Detailed observations of behaviors during EEG/EMG recordings revealed that abrupt arrests in *orexin*$^{-/-}$ mice occurred during direct transitions from wakefulness to REM sleep (Fig. 2A) or during the pre-REM phase immediately after a waking period: the pre-REM phase shows an EEG with high-amplitude spindle oscillations superimposed on a non-REM sleep background, and these spindles are observed only during the transition phase immediately prior to REM sleep in wild-type mice. Indeed, direct or very rapid transitions from wakefulness to REM sleep are the most intriguing characteristic of EEG/EMG in *orexin*$^{-/-}$ mice, which were observed almost exclusively in their active phase, i.e., the dark phase, and were never exhibited by wild-type mice (Fig. 3).

Abrupt arrests in *orexin*$^{-/-}$ mice were suppressed by systemic administration of clomipramine, an anticataplectic agent used for treatment of human narcolepsy-cataplexy. Administration of caffeine, a psychostimulant used to treat excessive sleepiness in human narcolepsy-cataplexy, tended to produce a mild exacerbation of abrupt arrest frequency.

These behavioral, electrophysiological, and pharmacological characteristics of abrupt arrests observed in *orexin*$^{-/-}$ mice are very similar to those found in cataplexy of human narcolepsy-cataplexy. An accepted definition of cataplexy in humans includes sudden bilateral weakness involving skeletal muscles, provocation by sudden strong emotions, lack of impairment of consciousness and memory, short duration (less than a few minutes), and responsiveness to treatment with clomipramine or imipramine *(5,6)*. Some studies performed during cataplectic attacks in humans as well as dogs found REM sleep characteristics in the EEG, whereas other studies found characteristics of wakefulness in the EEG during cataplexy

(7–10). In most cases, consciousness and memory are preserved in cataplexy in human *(6)*. Thus, we cannot conclude definitively that the abrupt behavioral arrests observed in *orexin*$^{-/-}$ mice are the equivalent of cataplexy in humans until we confirm preservation of consciousness during the arrests in these mice. In relation to this discussion, we have occasionally observed apparent consciousness shortly after arrest onset in *orexin*$^{-/-}$ mice, as well as wake-like EEG during abrupt behavioral arrests in transgenic rats lacking orexin neurons (*orexin/ataxin-3* transgenic rats, described below) *(11)*.

Abrupt arrests in *orexin*$^{-/-}$ mice share several further features with cataplexy in humans and dogs. Cataplexy has been well known to be triggered by strong emotions such as laughing, anger, fear, surprise, and excitement in human narcolepsy-cataplexy patients *(5,12)*. Also, in narcoleptic dogs, food and play with other dogs are the well-documented paradigms used to trigger cataplexy *(13)*. In *orexin*$^{-/-}$ mice, purposeful motor activity always precedes episodes of abrupt arrests; excited ambulation, grooming, burrowing, and climbing were most frequently associated with abrupt arrests. The dramatic increase in the number of abrupt arrests noted in the group-housed mice compared with the individually housed litter mates suggests that social interaction may significantly enhance this phenotype. Chasing, tail biting, and social grooming were often observed to immediately precede abrupt arrests in the group-housed mice. The facts that abrupt arrests are observed mainly in their active phase and that novel environment enhances occurrence of abrupt arrests further support the possibility that emotional activation triggers these arrests in *orexin*$^{-/-}$ mice.

Only approximately one-third of human patients experience full loss of muscle tone causing collapse to the floor; most have partial cataplexy, evidenced by jaw sagging, head bobbing, arm dropping, ptosis, or dysarthria *(5)*. Partial cataplexy in the dog is often evidenced by hindlimb buckling. Unambiguous full postural collapse was frequently observed in young *orexin*$^{-/-}$ mice, whereas adults tended to collapse onto their ventral surface at odd angles, suggesting some residual muscle tone. Cataplexy is not always instantaneous in human patients but can progress over several seconds, with some patients experiencing a gait disturbance known as "zig-zag walking" *(5)*. The finding of gait ataxia immediately preceding 27% of abrupt arrests in adult *orexin*$^{-/-}$ mice is intriguing.

3. NON-REM SLEEP-RELATED SYMPTOMS (EXCESSIVE SLEEPINESS AND SLEEP ATTACKS)

Increased daytime sleepiness is well described in human patients *(6,12)* and in the narcoleptic dog *(14,15)*. Human patients often complain of involuntary or irresistible daytime "sleep attacks" that can occur while they are talking, standing, walking, eating, or driving. In addition to REM sleep-related abnormalities, another prominent feature of sleep/wake patterns in *orexin*$^{-/-}$ mice was shortened durations of both wakefulness and non-REM sleep in the dark phase, causing increased fragmentation of the sleep/wake cycle *(2,4)* (Fig. 3). *OX2R*$^{-/-}$ mice also showed sleep/wake fragmentation, whereas the occurrence of REM sleep-related abnormalities was very rare compared with *orexin*$^{-/-}$ mice (Fig. 3). This fragmentation was accompanied by a statistically insignificant tendency toward reduced amounts of wakefulness and increased amounts of non-REM sleep during the dark phase (Fig. 4). Fragmentation of vigilance states is an indication of inability to maintain sleep and wakefulness, suggesting increased sleepiness.

Presumptive excessive sleepiness in *orexin*$^{-/-}$ mice was further clarified by detailed analyses of gradual arrests in *orexin*$^{-/-}$ and *OX2R*$^{-/-}$ mice, which can be considered as analogous to sleep attacks in human narcolepsy-cataplexy. In contrast to abrupt arrests, such arrests have

more gradual onsets (Fig. 1B). Gradual arrests typically began during quiet wakefulness and could be easily distinguished from the normal onset of resting behavior by *(1)* the absence of stereotypic preparation for sleep (e.g., nesting and/or assumption of a curled or hunched posture, with limbs drawn under the body); and *(2)* a characteristic ratchet-like "nodding" of the head over a period of several seconds, with a transition to a collapsed posture.

Systemic administration of caffeine, which is a psychostimulant used for treating excessive sleepiness in human narcolepsy-cataplexy, dose-dependently suppressed gradual arrests, whereas administration of the anticataplectic agent clomipramine did not affect the frequency of gradual arrests in both *orexin*$^{-/-}$ and *OX2R*$^{-/-}$ mice.

EEG/EMG recordings concurrent with videotaping further differentiated gradual arrests from abrupt ones. As described above, abrupt arrests were accompanied either by direct transition from wakefulness to REM sleep or to the pre-REM stage with atonia in both *orexin*$^{-/-}$ and *OX2R*$^{-/-}$ mice (Fig. 2A). In contrast, EEG/EMG correlates of gradual arrests in both *orexin*$^{-/-}$ and *OX2R*$^{-/-}$ mice invariably revealed the onset of attenuated muscle tone, but not atonia, and an EEG transition from wakefulness to non-REM sleep (Fig. 2B). Gradual arrests were occasionally accompanied by apparent automatic behavior (continuation of semipurposeful motor activity after the onset of light sleep such as stereotypic chewing of food) in both genotypes. Although the brevity of many episodes (typically <10 s) precluded detailed analysis, a particularly long (42-s) episode demonstrated that the EEG features of this example conform unambiguously to the spectral features of non-REM sleep. In *orexin*$^{-/-}$ mice, a large proportion of sleep attacks transitioned prematurely from non-REM sleep to REM sleep, whereas such rapid transitions to REM sleep were observed only rarely in *OX2R*$^{-/-}$ mice.

Overall, gradual arrests in both *orexin*$^{-/-}$ and *OX2R*$^{-/-}$ mice resemble sleep attacks in human narcolepsy-cataplexy. They are associated with impaired consciousness and memory but not with strong emotions or abrupt muscle weakness. The identification of sleep is based on a behavioral context: patients report sudden irresistible sleepiness occurring during unusual circumstances (e.g., meals, conversations, driving), and such attacks may also be associated with automatic behavior *(6,16)*. Sleep attacks are generally associated with the onset of early stages of non-REM sleep, reflect a compression of the normal process of entering sleep, and mimic the effects of sleep deprivation in normal humans *(17,18)*. In the context of narcolepsy-cataplexy but not other disorders of excessive sleepiness, sleep attacks may also give way rapidly to REM sleep periods (SOREM periods) that can be accompanied by sleep paralysis or hallucinations *(15,19)*. Unlike cataplexy, sleepiness is reduced by psychostimulants such as amphetamines, modafinil, and caffeine *(20)*.

Similarly, in canine narcolepsy-cataplexy, sleepiness based on increased tendencies to fall asleep and more fragmented wakefulness patterns are seen *(14,21)*. Behavioral attacks of "drowsiness" associated with "stop motion" and nodding of the head and neck are observed in genetically narcoleptic Dobermans, although these have not been reliably distinguished from cataplexy by EEG/EMG, possibly owing in part to the more staged EEG patterns of non-REM sleep in canines (S. Nishino, personal communication).

4. DIFFERENTIAL REGULATION OF SLEEP/WAKE STATES BY OREXIN RECEPTORS

As described above, *orexin*$^{-/-}$ and *OX2R*$^{-/-}$ mice are indistinguishable with respect to parameters of wakefulness and non-REM sleep. Both mice exhibit gradual arrests and fragmentation of the sleep/wake cycle. In contrast, *orexin*$^{-/-}$ and *OX2R*$^{-/-}$ mice are distinct from each other in the expression of abnormalities of REM sleep. o*rexin*$^{-/-}$ mice without exception

displayed abrupt arrests and direct transitions from wakefulness to REM sleep, the hallmarks of rodent narcolepsy-cataplexy, whereas *OX2R*$^{-/-}$ mice exhibited no such transitions or did so with far less frequency. o*rexin*$^{-/-}$ mice exhibited a striking 75% increase in the amount of time spent in REM sleep over the entire dark phase, increased frequency of REM sleep in the dark phase, and reduced latency to REM sleep in both phases. Whereas in comparison with *orexin*$^{-/-}$ and wild-type mice, *OX2R*$^{-/-}$ mice tended to exhibit intermediate values for some of these same parameters of REM sleep, only a reduced REM sleep latency during the dark phase differed significantly from patterns in wild-type mice. Overall, the general patterns of REM sleep of *OX2R*$^{-/-}$ mice more closely resemble those of wild-type mice than those of *orexin*$^{-/-}$ mice. Thus, OX_2R deficiency causes abnormal transitions from wakefulness to non-REM sleep, whereas lack of orexin peptides results in abnormal transitions from wakefulness not only to non-REM sleep but also to REM sleep *(4)*.

OX1R knockout mice (*OX1R*$^{-/-}$ mice), as well as *OX1R, OX2R* double-knockout mice (*OX1R*$^{-/-}$*;OX2R*$^{-/-}$ mice) were also created to complete analyses of every component of the orexin signaling pathway *(22)*. *OX1R*$^{-/-}$ mice did not have overt behavioral abnormalities and exhibited only mildly increased fragmentation of sleep/wake states. *OX1R*$^{-/-}$*;OX2R*$^{-/-}$ mice appeared to be a phenocopy of *orexin*$^{-/-}$ mice. Overall, it should be concluded that normal regulation of wake/non-REM sleep transitions depends critically on OX_2R activation, whereas the profound dysregulation of REM sleep control unique to the narcolepsy-cataplexy syndrome emerges from loss of signaling through both OX_1R- and OX_2R-dependent pathways.

A complementary experiment we carried to confirm this conclusion further was examination of the arousal effect of icv orexin-A administration in each strain of knockout mice (M.M. and M.Y., unpublished observations). As previously reported in rodents, icv administration of orexin-A in wild-type mice increased wakefulness and suppressed both non-REM and REM sleep in a dose-dependent manner. The effects of orexin-A on sleep/wake patterns were not significantly different in *OX1R*$^{-/-}$ mice, whereas the effects on wakefulness and non-REM sleep in *OX2R*$^{-/-}$ mice were statistically significant but dramatically attenuated compared with wild-type and *OX1R*$^{-/-}$ mice. These data suggest that OX_1R is dispensable for increase of wakefulness and suppression of non-REM sleep by orexin-A administration in the presence of OX2R, although stimulation of OX_1R in the absence of OX_2R is capable of promoting wakefulness and suppressing non-REM sleep with an efficiency much lower than that of stimulation of OX_2R. As for suppression of REM sleep by orexin-A administration, there was no significant difference among wild-type, *OX1R*$^{-/-}$, and *OX2R*$^{-/-}$ mice, although suppression tended to be less efficient in *OX2R*$^{-/-}$ mice, suggesting that both OX_1R and OX_2R mediate REM sleep suppression. Furthermore, we obtained data suggesting that activation of OX_1R directly suppresses REM sleep. Importantly, no significant effect of orexin-A administration on sleep/wake patterns was observed in *OX1R*$^{-/-}$*;OX2R*$^{-/-}$ mice, implying that the arousal effect of orexin-A is mediated by these two receptors. Overall, these results are consistent with the model described above derived from comparison of behavioral and baseline sleep/wake cycle characteristics of *orexin*$^{-/-}$, *OX1R*$^{-/-}$, and *OX2R*$^{-/-}$, and *OX1R*$^{-/-}$*;OX2R*$^{-/-}$ mice.

5. RODENT MODELS OF THE PATHOPHYSIOLOGY OF HUMAN NARCOLEPSY-CATAPLEXY

We have shown that behavioral and electrophysiological abnormalities in *orexin*$^{-/-}$ mice are strikingly similar to those of human narcolepsy-cataplexy. However, unlike *orexin*$^{-/-}$ mice, as well as OX_2R-dificient narcoleptic dogs, familial transmission of human narcolepsy-cataplexy

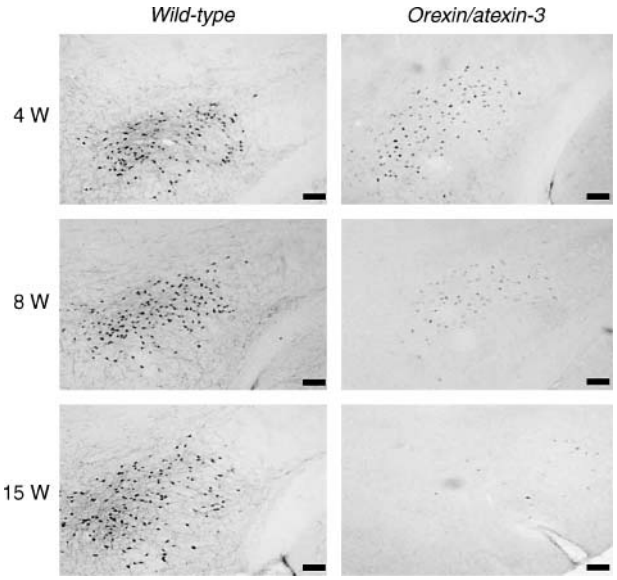

Fig. 5. Ablation of orexin neurons in *orexin/ataxin-3* transgenic mice. Matched brain sections (bregma 22.1 mm) from 4-, 8-, and 15-wk-old *orexin/ataxin-3* transgenic mice (right panels) and their transgene-negative litter mates (left panels) were stained with anti-orexin antiserum. Scale bars=100 μm. (Modified from ref. *27*.)

is rare; even in these rare cases penetrance is far from 100% *(23)*. No mutation has been found either in the *prepro-orexin* or orexin receptor genes of human narcolepsy-cataplexy patients except for an unusually severe, early-onset case associated with a mutation in the *prepro-orexin* gene that impairs peptide trafficking and processing *(24)*. In most cases of human narcolepsy-cataplexy, symptoms start to appear during adolescence. Nevertheless, dramatic reduction of orexin levels in the cerebrospinal fluid (CSF) and postmortem brains of narcolepsy-cataplexy patients has been reported *(24–26)*. Thannickal et al. *(26)* also reported that global loss of orexin neurons was accompanied by residual gliosis in the perifornical area. Based on these observations, as well as a strong association of human narcolepsy-cataplexy with certain HLA alleles *(23)*, it has been speculated that narcolepsy-cataplexy may result from selective autoimmune degeneration of orexin neurons.

In order to mimic the pathophysiological conditions of human narcolepsy-cataplexy, Hara et al. *(27)* generated transgenic mice (*orexin/ataxin-3* transgenic mice) in which orexin neurons are ablated by orexinergic-specific expression of a truncated Machado-Joseph disease gene product (ataxin-3) with an expanded polyglutamine stretch (Fig. 5) *(27)*. At postnatal d 5, the number of orexin neurons was not different between *orexin/ataxin-3* transgenic mice and their wild-type littermates. Thereafter, the number of orexin neurons gradually decreased, and at 12 wk of age, more than 99% of orexin neurons were lost. o*rexin/ataxin-3* transgenic mice exhibited behavioral and electrophysiological defects that were essentially same as those displayed by *orexin*$^{-/-}$ mice: abrupt and gradual arrests, direct transitions from wakefulness to REM sleep, shortened latency to REM sleep, fragmentation of vigilance states, and increases in REM sleep time and duration in the dark phase. The only exception was that the behavioral arrests seen in *orexin/ataxin-3* transgenic mice typically began at about 6 wk of age, whereas arrests were observed in some *orexin*$^{-/-}$ mice earlier than 3 wk of age. Thus, *orexin/ataxin-3* transgenic mice have an etiology and course of disease similar to those of

human narcolepsy-cataplexy, and *orexin/ataxin-3* transgenic mice may represent the most accurate pathophysiological model of narcolepsy-cataplexy available.

Another conclusion derived from analyses of *orexin/ataxin-3* transgenic mice is that although orexin neurons produce other neurotransmitters such as glutamate and dynorphins *(28,29)*, it is orexin that is important for the regulation of the sleep/wake state by these neurons. Indeed, we utilized this mouse model of narcolepsy-cataplexy to rescue its narcoleptic phenotype by genetic (overexpression of orexin peptides throughout the brain) and pharmacological (icv administration of orexin-A) means *(30)*, demonstrating that mice retain the ability to respond to orexin neuropeptides even if they lack endogenous orexin neurons and that a temporally regulated and spatially targeted secretion of orexins is not necessary to prevent narcoleptic symptoms. Thus, orexin receptor agonists would be of potential value for treating human narcolepsy-cataplexy.

Very recently, Beuckmann et al. *(11)* generated transgenic rats that have the same transgene as *orexin/ataxin-3* transgenic mice (*orexin/ataxin-3* transgenic rats). In these rats, the number of orexin neurons was gradually reduced after their birth, and at 17 wk of age hypothalamic orexin expression was undetectable. Again, *orexin/ataxin-3* transgenic rats showed a narcoleptic phenotype, with a decreased latency to REM sleep, increased REM sleep time, direct transitions from wakefulness to REM sleep, and a marked fragmentation of vigilance states. Brief episodes of muscle atonia and postural collapse resembling cataplexy were also observed while rats maintained the EEG characteristics of wakefulness, suggesting that they were conscious during these episodes as human patients are during cataplexy. Since the rat has been more widely used for physiological and pharmacological studies in the field, *orexin/ataxin-3* transgenic rats are likely to be a valuable resource for the study of human narcolepsy-cataplexy.

6. MORE THAN SLEEP/WAKE ABNORMALITIES

The most profound symptoms of human narcolepsy-cataplexy are sleep-related disturbances. However, discovery of the orexin system and analysis of mice with a deficiency in the orexin signaling pathway shed light on another aspect of human narcolepsy-cataplexy: disturbance of energy metabolism. Orexin peptides were originally recognized as feeding peptides; icv administration of orexins increased food intake in rats *(1)*. Consistent with this observation, *orexin*$^{-/-}$ and *orexin/ataxin-3* transgenic mice were hypophasic *(22,27)*. Nevertheless, *orexin/ataxin-3* transgenic mice showed striking late-onset obesity *(27)*. This apparently contradictory phenomenon was likely to result from reduced energy expenditure in these mice since they were less active in the dark phase compared with wild-type mice *(27)*. It has been reported that the frequency of obesity and non-insulin-dependent (type II) diabetes, as well as the body mass index, is increased in human narcolepsy-cataplexy patients *(31–33)*. o*rexin*$^{-/-}$ mice also show mild obesity when fed with high-fat diet *(22);* however, metabolic abnormality seems more severe in *orexin/ataxin-3* transgenic mice than in *orexin*$^{-/-}$ mice, according to initial reports.

Loss of neuropeptides or modulatory factors expressed in orexin neurons in *orexin/ataxin-3* transgenic mice could explain this difference. Another possibility is that compensatory mechanisms may overcome the impact of orexin deficiency on the metabolism of *orexin*$^{-/-}$ mice, in which the orexin gene has been disrupted from the beginning of development. The third and most likely explanation is the difference in genetic backgrounds used in these reports: *orexin/ataxin-3* transgenic mice in 75% C57BL/6:25% DBA1 and *orexin*$^{-/-}$ mice in 50% C57BL/6J:50% 129Sv/Ev. Body weight is determined according to the balance between

food intake and energy expenditure, and metabolic phenotypes are known to be highly sensitive to genetic background. Indeed, neither *orexin/ataxin-3* transgenic nor *orexin*$^{-/-}$ mice showed obesity after they were backcrossed to an almost pure C57BL/6J background (J. T. W. and M. Y. unpublished data; T. S., personal communication).

Pharmacological orexin administration studies, as well as studies of the anatomy of the orexin system, have suggested that orexin peptides are also involved in regulation of the autonomic and endocrine systems (reviewed in refs. *22* and *34*). Defects in these systems could result in a disturbance of metabolism, leading to changes in energy expenditure. In *orexin*$^{-/-}$ mice, it has been reported that basal arterial blood pressure is significantly lower, and the autonomic "fight or flight" response is attenuated compared with wild-type mice *(35)*. Several studies have reported disturbance of autonomic regulation in human narcolepsy-cataplexy patients *(36–40)*; more systematic investigations should be carried out.

7. CONCLUSIONS

Behavioral and electrophysiological analysis of *orexin*$^{-/-}$ mice, together with studies on narcoleptic OX_2R-deficient dogs, has greatly increased our understanding of narcolepsy-cataplexy, as well as regulation of the sleep/wake cycle. Mouse molecular genetics were used to explore non-REM and REM sleep regulatory processes by comparing mutants of every component of the orexin signaling pathway. Furthermore, obesity in *orexin/ataxin-3* transgenic and *orexin*$^{-/-}$ mice directed researchers' interest toward metabolic abnormalities in narcolepsy-cataplexy patients. One of the big advantages in utilizing rodents is the relative ease with which large numbers of animals are prepared for systematic study. The detailed analyses of mouse and rat models of narcolepsy-cataplexy described have not only deepened our understanding of disturbances of sleep regulation in narcolepsy-cataplexy but have also revealed novel aspects of physiological abnormalities in human narcolepsy-cataplexy patients.

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V

Pathophysiology: Narcolepsy and Orexin/Hypocretin Deficiency

B. Orexin/Hyprocretin Deficiency in Human Narcolepsy and Other Conditions

20

Hypocretin Deficiency in Human Narcolepsy

Mutation Screening, Neuropathology, and CSF Hypocretin-1 Level

Patrice Bourgin, MD, PhD, Jamie M. Zeitzer, PhD, and Emmanuel Mignot, MD, PhD

1. INTRODUCTION

Narcolepsy-cataplexy is a neurological disorder affecting 0.02–0.18% of the general population. The syndrome is characterized by excessive daytime sleepiness (EDS), fragmentation of nocturnal sleep, and intrusion of REM sleep events into wakefulness (e.g., cataplexy, sleep paralysis, and hypnagogic hallucinations). Daytime sleepiness and cataplexy are the most consistent symptoms of the syndrome. Sleepiness is the most disabling symptom. It is characterized by unpredictable and irresistible sleep attacks on a background of constant daytime sleepiness. Cataplexy, the sudden occurrence of muscle atonia triggered by emotions such as laughing or anger, is almost pathognomonic for the disorder. Nocturnal polysomnography is conducted to exclude other causes of daytime sleepiness, and multiple sleep latency tests (MSLTs) are carried out to demonstrate excessive daytime sleepiness and sleep onset REM periods, which are also characteristics of the disorder. Current therapeutic options are purely symptomatic. Modafinil is the most common treatment for sleepiness, and antidepressant compounds have some therapeutic effect on cataplexy and REM-related symptoms. Sodium oxybate (GHB) is a sedative hypnotic that improves disturbed nocturnal sleep and reduces cataplexy and sleepiness after long-term administration.

2. A COMPLEX GENETIC DISORDER INFLUENCED BY ENVIRONMENTAL FACTORS

A genetic component for narcolepsy was first suggested by Westphal (1877). In this first report of narcolepsy, both the proband and his mother were affected. Further evaluation of the transmission of the disease indicated that narcolepsy-cataplexy is, however, rarely a familial disorder when other causes of daytime sleepiness, most notably sleep apnea, are excluded. Most cases are sporadic and only 25–31% of monozygotic twin pairs are concordant for the disorder, suggesting a role for environmental triggers *(1)*. Although only 1–2% of first-degree relatives develop the disorder, this corresponds to a 20–40-fold increase over the prevalence in the general population (0.05%) *(1)*.

Genetic predisposition to narcolepsy involves the human leukocyte antigen (HLA) system. Almost all (90–95%) patients with a severe form of the disease and cataplexy carry the HLA

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allele DQB1*0602 *(2)*. Rare multiplex families with possible autosomal dominant transmission have been reported, many of which are HLA-DQB1*0602 negative. The association of HLA with narcolepsy was first reported in Japanese patients with the HLA-DR2 allele *(3)*. It was quickly confirmed in Caucasian subjects *(5–8)* but was found to be lower in African-American patients *(9)*. Further characterization indicated that the association extends to the DR15, DQ6 (DR2, DQ1-Dw2) haplotype in Japanese and Caucasian subjects. In contrast, African-American patients were DR2 negative but DQ1 positive *(9)*. High-resolution typing as well as sequencing of HLA-DRB1, DQA1, and DQB1 have demonstrated that narcoleptics of all ethnicities carry HLA-DQB1*0102 and HLA-DQB1*0602. In almost all Japanese and Caucasian patients, DQB1*0602 is found in the context of a DRB1*1501, DQA1*0102, DQB1*0602 haplotype. These results provide evidence that DQB1*0602, an allele found in 12, 25, and 38% of the control Japanese, Caucasian, and African-American populations, respectively, is the major HLA susceptibility allele for narcolepsy across ethnic groups (for review; *see* ref. *87*).

The HLA association observed in narcolepsy is complex and is not only the result of a dominant effect of DQB1*0602. Indeed, the relative risk for narcolepsy varies depending on the trans-located alleles associated with DQB1*0602 *(10,11)*. DQB1*0602 homozygotes are at two to four times increased risk *(12)*. Co-expression of DQB1*0301 increases the risk for narcolepsy, whereas subjects carrying DQB1*0601 or DQB1*0501 have a lower risk. Interestingly, DQB1*0602 is found in only 40% of narcoleptics without cataplexy, suggesting disease heterogeneity when cataplexy is not present *(2)*. The prevalence and etiologies of narcolepsy without cataplexy are unknown and may involve other genes. The fact that up to 30% of familial narcolepsy-cataplexy cases are HLA negative, together with the high incidence of HLA negativity in concordant twin pairs *(1)*, also suggests the existence of highly penetrant non-HLA narcolepsy genes. The existence of other genetic factors has also been suggested by genetic linkage and association studies *(13–22)*.

3. ANIMAL STUDIES: A LINK BETWEEN HYPOCRETINS AND ANIMAL MODELS OF NARCOLEPSY

The discovery of a possible link between narcolepsy and hypocretin/orexin was made in 1999 by two different groups, only one year after hypocretins were first discovered *(23,24)*. Lin and colleagues *(25)* reported that the sleep disorder canine narcolepsy is caused by mutation in the hypocretin receptor 2 gene with a loss of ligand binding and subsequent intracellular transduction signal *(25)*. Chemelli et al. *(26)* reported that according to behavioral and electroencephalographic criteria, hypocretin knockout mice exhibit a phenotype strikingly similar to that of human narcolepsy. Subsequent studies have led to the establishment of multiple rodent models with hypocretin ligand or receptor abnormalities *(27–29)*. Of special interest is the mouse and rat ataxin-3 transgenic model, which causes loss of hypocretin cells *(30,31)*.

4. HYPOCRETIN, HCRTR1 AND HCRTR2 MUTATION SCREENING

The discovery of a link between narcolepsy and hypocretin in animal models led to the immediate testing of the involvement of this system in human pathology. Three possible approaches were available to explore human hypocretinergic neurotransmission in vivo: measuring hypocretin peptide levels in human cerebrospinal fluid (CSF), neuropathological analysis of the hypocretin system in human postmortem brains, and mutation screening of the preprohypocretin and hypocretin receptor (hcrtr) genes in narcoleptic patients.

The exons and exon-intron boundaries of the preprohypocretin and hypocretin receptor genes were cloned. A total of 74 patients and 118 controls (all Caucasians) were studied, with special emphasis on rare patients with a family history ($n = 27$) or lacking HLA DQB1*0602 ($n = 29$) *(32)*. The hypocretin gene has two exons and one intron, and each of the two receptors (hcrtr1 and hcrtr2) has seven coding exons.

Only one case, clinically different from those usually observed, was found to have a mutation likely to cause narcolepsy *(32)*. This case is unusual because of the very early onset of cataplexy at 6 mo of age. Sleep onset REM periods during the MSLT, period leg movements (PLMs), and nocturnal bulimia were also reported. The patient is HLA negative and has undetectable CSF hypocretin-1. The mutation was not present in the unaffected mother, and the father's genomic DNA was unavailable. To understand the functional significance of this latter mutation, we developed a DNA construct fused to a green fluorescent protein and transfected it into neuroblastoma cells. The mutant peptide did not go through the trans-Golgi network into mature secretory vesicles, rather, the mutant construct accumulated in a compartment likely to be the smooth endoplasmic reticulum, demonstrating that the mutation results in an abnormal trafficking of the peptide precursor. We hypothesized that this effect may be toxic to the cell, leading to the neurodegeneration of hypocretin cells after the onset of hypocretin transcription in this young child.

Fourteen polymorphisms were found in the *Hcrtr1* and *Hcrtr2* loci, including at least two in each loci with a high allele frequency (HCRTR1: 111T/C; Val408Ileu; HCRTR2: -25 A/C, 49 T/C; Val308Ileu; Table 1). None of these polymorphisms were associated or linked with narcolepsy, and all were presumed to be benign. Two rare coding HCRTR2 polymorphisms, Pro11Thr and Pro10Thr, were also detected but were probably not functionally significant *(32)*. We also detected a rare noncoding 5′UTR polymorphism likely to be benign and later identified a common polymorphism (–909 C/T) in a repeat rich region located 5′ of the preprohypocretin gene. The preprohypocretin polymorphism was not associated with narcolepsy in a large number of subjects *(33)*.

Three other studies have examined the possibility of hypocretin gene mutations or association in narcolepsy *(34–36)*. Gencik et al. *(34)* screened the entire preprohypocretin gene in 130 patients with narcolepsy-cataplexy and in two patients without cataplexy. Ethnicity was not reported. A rare sequence variation in the upstream regulatory sequence of the gene (3250T/C) without clear functional relevance was reported in 6 of 178 narcoleptic patients and in one of 189 control subjects. No particular clinical features were associated with this polymorphism. This finding was not replicated in three large studies in the United States *(32,33,35)*, suggesting that the polymorphism is more likely ethnic specific.

In the Olafsdottir et al. study *(35)*, 47 patients (with and without cataplexy) from a more diverse ethnic pool were included, together with 75 control individuals. Coding exons of the three genes were all sequenced, and the common polymorphisms reported in the Peyron et al. *(32)* study were all confirmed. A few additional polymorphisms, many of which were ethnic specific, were also reported. None were associated with narcolepsy, and all were presumed to be benign. Finally, Thompson et al. *(36)* recently reported on single-stranded conformation polymorphism (SSCP) analysis of all HCRTR2 coding exons in 28 subjects with EDS and who were HLA negative patients, in 28 narcolepsy subjects, in 70 Tourette's syndrome subjects, and in 110 controls. Three coding and one noncoding variants were identified, including the Val308Ile HCRTR2 variant found in all groups with a similar frequency. A rare Pro10ser variant previously reported by Peyron et al. *(32)* in two sporadic HLA-positive narcolepsy patients (presumed to be benign) was also observed in a subject with

Table 1
Allelic Variance of Hcrt, Hcrtr1, and Hcrtr2 Loci in Narcoleptic and Control Subjects

			Peyron et al., 2000[1] (32)		Olafsdottir et al., 2001[2] (35)		Gencik et al., 2001[3] (34)		Hungs et al., 2001[4] (33)		Thompson et al., 2004 (36)		
DNA change	Domain	A.A change	Narcolepsy	Control	Narcolepsy	Control	Narcolepsy	Control	Narcolepsy	Control	Narcolepsy	Control	Comments
Preprohypocretin (hcrt)													
(-)909T_C[4]	Alu repeat/ promoter	Noncoding							0.764 (185)	0.73 (107)			Benign
(-)593A insertion[3]	Promoter	Noncoding					0.002 (178)	0.00 (189)	n.t.	n.t.			Benign
(-)22C_T[3]	5´UTR	Noncoding	0.00 (72)	0.00 (24)	0.00 (57)	0.00 (85)	0.017 (178)	0.002 (189)	0.00 (68)	0.00 (77)	n.t.	n.t.	Benign
(-)20C_A[1]	5´UTR	Noncoding	0.014 (72)	0.00 (24)	0.00 (57)	0.00 (85)	0.00 (178)	0.00 (189)	0.00 (68)	0.006 (68)	n.t.	n.t.	Benign
IVS +16C_T[2]	Intron	Noncoding			0.114(57)	0.065(85)	0.00 (178)	0.00 (189)					Benign
47T_G[1]	Exon2 signal peptide	L16R	0.007 (72)	0.00 (106)	0.00 (57)	0.00 (85)	0.00 (178)	0.00 (189)					**Mutation**
Hypocretin receptor 1 (Hcrtr 1)													
(-)123C_A[2]	5´UTR	Noncoding			0.00 (96)	0.018 (83)							Benign
111T_C[1]	N-terminus extracellular	Synonymous	0.382 (68)	0.36 (40)	0.520 (48)	0.048 (82)							Benign
IVS +21G_T[2]	Intron1	Noncoding			0.00 (47)	0.007 (75)							Benign
201C_T[2]	Exon2	Synonymous			0.009 (54)	0.00 (86)							Benign
210C_T[2]	Exon2	Synonymous			0.00(56)	0.006 (86)							Benign
237G_A[2]	Exon2	Synonymous			0.00 (52)	0.006 (86)							Benign
499G_A[2]	Exon3 transmembrane domain IV	G167S			0.00 (55)	0.006 (88)							Benign
793C_A[1]	Exon5 (IL3 domain)	L265M	0.007 (68)	0.00 (45)									Benign
836 G_A[2]	Exon5 (IL3 domain)	R279Q			0.185 (54)	0.011 (87)							Benign

842G_A[1]	Exon5 (IL3 domain)	R281H	0.00 (68)	0.01 (45)	0.010 (52)	0.006 (88)			Benign
IVS +6C_T[1]	Intron6	Noncoding	0.029 (68)	0.06 (40)	0.059 (51)	0.052 (86)			Benign
1222G_A[1]	Exon7 s (C terminu cytoplasmic)	V408I	0.375 (68)	0.34 (46)	0.577 (52)	0.559 (76)			Benign
1307G_A[2]	3´UTR	Noncoding			0.073 (48)	0.051 (78)			Benign
Hypocretin receptor 2 (Hcrtr2)									
28C_T[1]	N-terminus extracellular	P10S	0.007 (70)	0.00 (90)	0.00 (51)	0.006 (88)	0.00 (28)	0.00 (110)**	Benign
31C_A[1]	N-terminus extracellular	P11T	0.014 (70)	0.006 (90)	0.020 (51)	0.00 (88)	0.00 (28)	0.00 (110)***	Benign
IVS -26A_C[1]	Intron1	Noncoding	0.150 (67)	0.16 (58)	0.202 (47)	0.187 (83)			Benign
IVS +49C_T[1]	Intron2	Noncoding	0.25 (70)	0.17 (61)	0.00 (51)	0.00 (88)			Benign
577 T_A[1]	Transmembrane domain IV	C193S	0.00 (70)	0.01 (39)	0.00 (51)	0.00 (88)			Benign
846 G_A[2]	Exon 5	Synonymous			0.020 (49)	0.006 (80)			Benign
853 C_A[2]	Exon 5	Synonymous			0.00 (47)	0.006 (77)			Benign
877 G_A[2]	Exon 5 (IL3)	I293V			0.00 (49)	0.006 (80)			Benign
922G_A[1]	Transmembrane domain VI	V308I	0.157 (70)	0.19 (35)	0.108 (48)	0.154 (78)			Benign
942A_G[1]	Transmembrane domain VI	Synonymous	0.014 (70)	0.01 (35)	0.043 (46)	0.020 (76)			Benign
IVS -87 G_A[2]	Intron 6	Noncoding			0.00 (51)	0.024 (85)			Benign
1202C_T[1]	C terminus cytoplasmic	T401I	0.007 (70)	0.00 (99)	0.00 (51)	0.00 (88)			Benign

[a]For each study the allele frequencies are indicated as well as the number of patients in parentheses. *, Originally identified the corresponding polymorphism. Amino acid (AA) changes are counted from the ATG (Met residue)-codon. UTR, untranslated region; IVS, intervening sequence (intron) with position relative to adjacent exon; IL, intracellular loop; n.t., not tested because the primer was over this site; **, the Pro11Thr variant of the Hcrtr2 was found in two patients with excessive daytime sleepiness ($N = 28$); ***, the Pro10Ser variant of the Hcrtr2 was found in one Tourette's syndrome patient ($N = 70$) (Data from ref. *36*.)

Tourette's syndrome. This variant was also reported in a single control subject by Olafsdottir et al. *(35)*, making it unlikely to be involved in narcolepsy susceptibility.

In the Thompson et al. study *(36),* a Pro11Thr polymorphism was observed in two EDS patients and in no subjects of the other groups. This polymorphism had previously been reported by Peyron et al. *(32)* in one control subject and in a single HLA-negative narcolepsy-cataplexy proband of a multiplex family. In the Peyron et al. study *(32)*, this polymorphism was unlinked with narcolepsy in two multiplex HLA-negative families and was thus presumed to be benign. This hypothesis is reinforced by the lack of conservation of this residue in other species *(36)*. The authors nonetheless functionally characterized both the HCRTR2 Pro11Thr and Pro10Thr polymorphisms after transfection of the polymorphic and wild-type HCRTR2 constructs in COS-7 cells and study of calcium mobilization after ligand stimulation. In both variants, alterations in the response of the cells after application of high ligand concentration was observed, raising the possibility of a partial impairment of hypocretin transmission. It is, however, still unknown whether this abnormality is functionally significant, especially in heterozygous subjects.

In summary, extensive screening studies of the three known hypocretin genes has shown that these loci are exceptionally mutated and do not contribute significantly to the pathophysiology of human narcolepsy. Additional studies focusing on rare and atypical cases, for example with early onset, may reveal additional, rare mutations.

5. NEUROPATHOLOGICAL STUDIES OF POSTMORTEM NARCOLEPTIC HUMAN BRAINS

Following on the observation that human narcolepsy is associated with low CSF hypocretin-1 *(37)*, two studies examined hypocretin expression in 10 postmortem narcoleptic human brains and controls *(32,38)*. These studies confirmed the specific distribution of hypocretin-containing neurons in the tuberal region of the hypothalamus. Hypocretinergic cells were localized throughout the dorsal area of the perifornical nucleus and the bordering parts of the dorsomedial and ventromedial hypothalamic nuclei as well as within the tuberal lateral hypothalamic nucleus *(32,39)*. Peyron et al. *(32)* examined the presence of hypocretin mRNA by *in situ* hybridization, and Thannickal et al. *(38,39)* examined the presence of the hypocretin peptide by immunocytochemistry. Both papers reported a dramatic decrease in signal for hypocretin in narcoleptic brains compared with brains from control subjects (Fig. 1). In the Thannickal et al. studies *(38,39)*, 74–97% of the hypocretin cells were lost in five brains studied to date *(38,39)*. In the Peyron et al. *(32)* study, no detectable preprohypocretin signal was observed in the perifornical area of two brains examined. Both studies used another peptide expressed in the perifornical area, melanin-concentrating hormone (MCH), as control. The expression of MCH neurons was similar in control and narcoleptic brains, confirming the specificity of the hypocretin signal loss. Hypocretin-1 and -2 concentrations were also determined by radioimmunoassay in areas (pons and cortex) receiving a dense innervation from the hypocretin-containing cells. The peptide levels were undetectable or very low in both structures, in contrast to control brains *(32)*.

These results indicate that in HLA DQB1*0602-positive narcoleptic patients, either a lack of transcription of the preprohypocretin gene or a destruction of hypocretinergic cells causes narcolepsy-cataplexy. In favor of a destruction of hypocretin-containing cells, a narcoleptic brain was also evaluated for dynorphin, a peptide that colocalizes with hypocretin in the human perifornical area *(102)*. A loss of both the hypocretin and dynorphin signal was noted, suggesting that either these cells are completely quiescent or, more probably, that they have

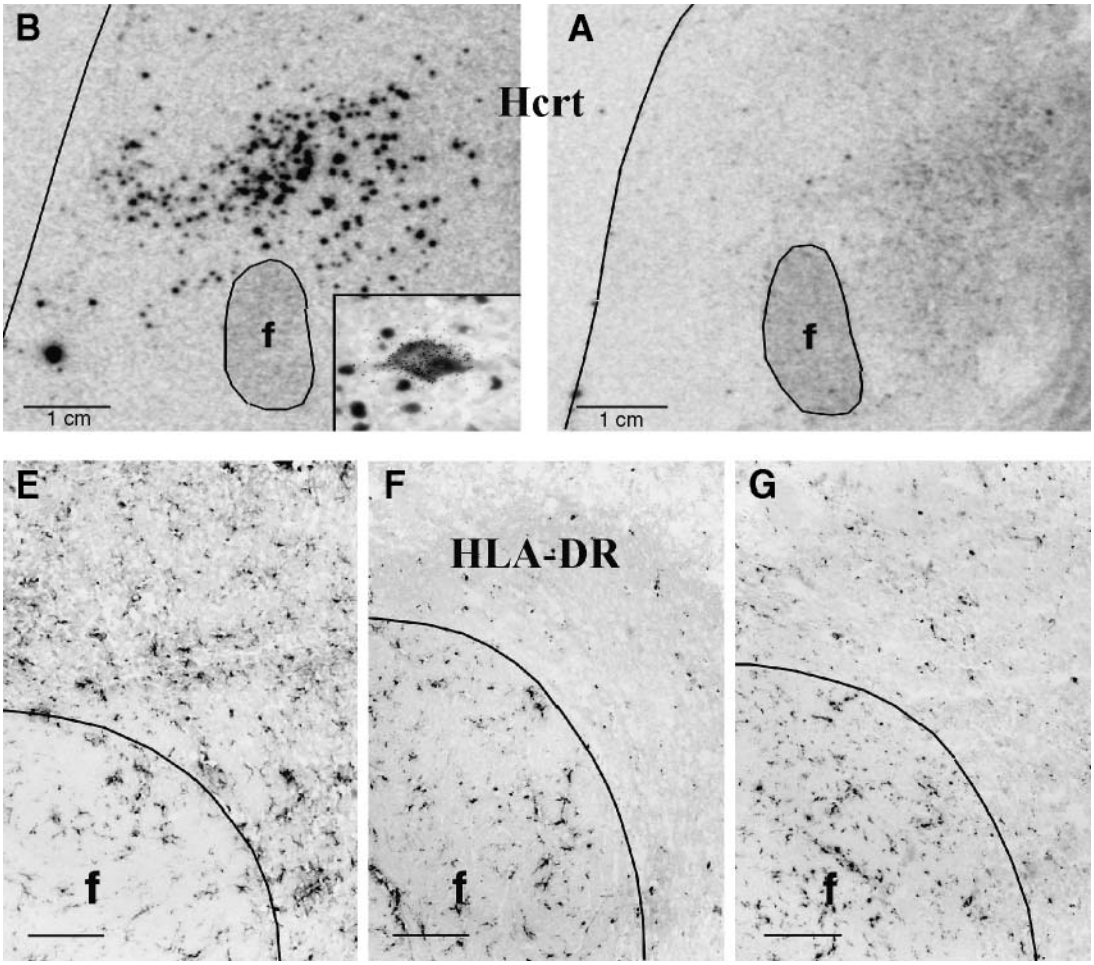

Fig. 1. Hypocretin (Hcrt) and HLA-DR labeling in the hypothalamus of narcoleptic and control subjects. *In situ* hybridization shows the presence of *preprohypocretin* mRNA in the hypothalamus of control **(B)** but not narcoleptic subjects **(A)**. Immunohistochemical staining of HLA-DR-positive microglia do not differ in white or gray matter between control **(G)** and narcoleptic brains **(E, F)**. f, fornix. (Data from ref. *32*.)

been destroyed. The possibility of local inflammation and gliosis, indirect markers of a possible autoimmune destruction, was also evaluated by both Thannickal et al. *(38,39)* and Peyron et al. *(32)*. In two brains examined, Peyron et al. *(32)* did not observe a significant upregulation of MHC microglial class II expression, although the subjects examined had developed narcolepsy 30 yr prior to death, making it unlikely that the disease process was still active. Thannickal et al. *(38,39)* found an elevated level of hypothalamic gliosis, as measured by glial fibrillary acidic protein (GFAP) staining. They also evaluated hypocretinergic axon loss as well as the message for hcrtr2 in different brain projections areas *(39)*. They found the highest percentage of depletion of hypocretin cells and GFAP staining in the posterior and tuberomammillary hypothalamus, suggesting that this region may be a focus of inflammation and damage in narcolepsy. They also reported that the percentage of hypocretin axon loss and the percentage of increase in gliosis were significantly correlated with hcrtr2 message density, these two correlations being independent *(39)*. Thanickal et al. *(39)* hypothesized that the

functional loss of hypocretin in narcolepsy is the consequence of a cytotoxic or immunological process targeting the hcrtr2 or a potent antigen related to hcrtr2. However, because these latter correlations are relatively low and derived from a small sample of patients, this hypothesis remains speculative.

6. CSF HYPOCRETIN-1 LEVELS: FROM TYPICAL NARCOLEPSY TO THE NARCOLEPSY BORDERLAND

The involvement of the hypocretin system in the pathophysiology of human narcolepsy was first demonstrated though studies of human CSF samples *(37)*. Measuring CSF hypocretin-1 is becoming an established diagnostic procedure *(40)*, and it is possible that, in the future, hypocretin-1 will be measurable in plasma.

6.1. Typical Cases: Narcolepsy with Definite Cataplexy and HLA Positivity

Nishino et al. *(37)* were the first group to measure hypocretin-1 levels in narcolepsy-cataplexy patients and in control subjects. Hypocretin-2 was found to be undetectable in all samples, an observation likely to be caused by the biological instability of the hypocretin-2 peptide in vivo. Nishino et al. *(37)* reported undetectable hypocretin-1 levels (<40 pg/mL) in the CSF of seven of the nine narcolepsy cataplexy cases tested. All cases with undetectable levels were HLA DQB1*0602 positive. This finding has now been replicated by multiple groups. Undetectable CSF hypocretin-1 levels are found in more than 90% of sporadic narcoleptic patients with cataplexy, and such cases are almost always HLA DQB1*0602 positive *(37,41–48)*. Subjects with low hypocretin-1 levels more frequently display typical cataplexy and abnormal MSLTs and are more often HLA DQB1*0602 positive *(42)*. Other characteristics typical of narcoleptics do not statistically influence the probability of low hypocretin-1 level. Typical cataplexy has a better predictive value for low hypocretin-1 levels than abnormal MSLT results *(42)*. Overall, these results indicate that narcolepsy with typical cataplexy and HLA positivity is strongly associated with a hypocretin deficiency, as reflected by low CSF hypocretin-1.

6.2. Atypical Cases: Narcolepsy with Atypical Cataplexy or Without Cataplexy and Familial and HLA Negative Cases

Results are more difficult to interpret in the atypical forms of the disease. Only 20% of cases with atypical cataplexy (not triggered by laughter or joking) have low CSF hypocretin-1, emphasizing the importance of a careful definition of cataplexy *(42)*. Narcolepsy without cataplexy and HLA DQB1*0602-negative cases are also generally associated with normal levels of hypocretin-1 in the CSF. More rarely, low levels of hypocretin-1 can also be observed in narcoleptic patients who do not have cataplexy (approximately 10–30%, especially children) or who are HLA DQB1*0602 negative (only a few cases known in the entire world), but not both. *(41–44,47,48)*. The observation that only a minority of narcoleptic patients without cataplexy have low CSF hypocretin-1 corroborates neuropathological data indicating a 74% decrease in the number of hypocretin-containing neurons of the hypothalamus in the only narcoleptic patient without cataplexy tested in postmortem studies *(32,38)*. It has been speculated that narcolepsy without cataplexy may represent a milder form of the disease, with partial lesion of the hypocretinergic system and normal or low CSF hypocretin-1 *(42)*.

In familial cases, concentrations of CSF hypocretin-1 are also tightly associated with the presence of the HLA DQB1*0602 allele; all DQB1*0602-negative narcoleptic-cataplectic

patients in multiplex families tested to date had normal levels of hypocretin-1 in the CSF *(41,42,44,48)*. In the DAN family, a large African American lineage with both HLA-positive and HLA-negative cases, and the EIC pedigree, a family with only HLA-negative cases, all HLA-negative narcoleptic patients had normal CSF hypocretin-1 levels *(42)*. These findings suggest that the DQB1*0602 allele may have a major role in conferring low or absent hypocretin neurotransmission. In addition, in the DAN pedigree, normal or intermediate hypocretin levels of CSF hypocretin-1 were reported among younger HLA-positive narcoleptic patients or in relatives affected with isolated daytime naps or lapses into sleep. This suggests that hypocretin deficiency as measured by CSF hypocretin levels is more weakly associated with the familial form of the disease and that, in the DAN family, complete hypocretin deficiency may occur more slowly than in most sporadic cases. This observation does not exclude the involvement of the hypocretin system in the physiopathology of familial forms of the disease, even if a patient is HLA negative, since the function of the hypocretin receptors or the efficacy of the signal transmission might be altered. As mentioned above, however, hypocretin receptor mutations have also been screened in familial cases without success.

The recent report of CSF hypocretin-1 measurements in two monozygotic twin pairs further illustrates the complexity of the relationship between narcolepsy and low hypocretin levels. One monozygotic twin pair concordant for narcolepsy-cataplexy and HLA DQB1*0602 positive had normal CSF hypocretin-1 levels, suggesting the existence of a genetic form of typical narcolepsy without hypocretin neurotransmitter abnormality *(49)*. It is interesting to speculate that perhaps this concordant twin pair is HLA positive by chance and is pathophysiologically related to other, HLA-negative cases (more often familial in nature and with normal CSF hypocretin-1; *see* above). On the other hand, another monozygotic twin pair was HLA-DQB1*0602 positive and discordant for narcolepsy, with undetectable CSF hypocretin-1 in the affected twin and normal levels in the unaffected twin, suggesting that environmental factors may play a key role in the development of hypocretin deficiency and resulting narcolepsy *(50)*.

In conclusion, whereas HLA-positive sporadic cases with typical cataplexy are likely to be etiologically homogeneous and typically associated with low CSF hypocretin-1, only a minority of atypical cases have low CSF hypocretin-1 (Fig. 2). A possible explanation may be etiological heterogeneity in these other situations. However, an alteration of the hypocretin system is still possible in these atypical forms not associated with a low level of hypocretin-1. For example, some of these cases may be secondary to hypocretin receptor-1 or-2 defects, or any abnormality downstream of the hypocretin pathway. It is also possible that partial lesions of hypocretin neurons may affect specific projections and lead to sleep abnormalities, without noticeably low CSF hypocretin-1 *(42)*.

6.3. Idiopathic Hypersomnia and Secondary Cases

Idiopathic hypersomnia is characterized by excessive daytime somnolence and no REM sleep abnormalities. In the classic form, excessive amounts of nocturnal and diurnal sleep are reported. All patients with monosymptomatic or polysymptomatic idiopathic hypersomnia tested to date had normal CSF hypocretin-1 levels *(42,43,47,48)*, demonstrating that hypocretin-1 dysfunction does not appear to be the final, common pathway of the pathophysiology of most hypersomnias.

Secondary narcolepsy and hypersomnia cases are discussed elsewhere in this book. Hypersomnia and narcolepsy-like symptoms have been reported in various disorders. Thus, hypocretin-1 has been measured in such cases after traumatic brain injury *(42,48)*, acute

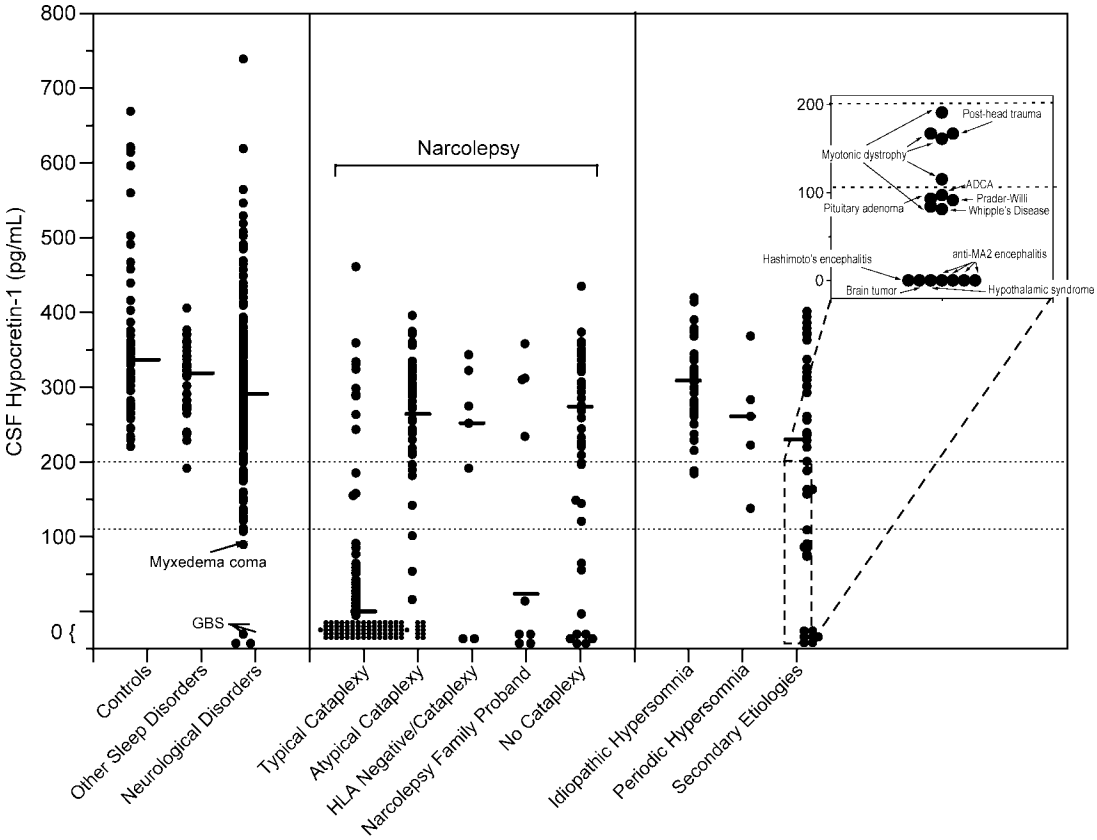

Fig. 2. Lumbar cerebrospinal fluid (CSF) hypocretin-1 concentrations in controls, narcoleptics, and other pathologies. Each point is the concentration of hypocretin-1 in the crude (unfiltered) lumbar CSF of a single individual. Represented are controls (samples taken during night and day) and narcoleptics, including those with typical cataplexy, with atypical cataplexy, with cataplexy but who are HLA negative, and without cataplexy, as well as narcolepsy family probands. Individuals with hypersomnia owing to idiopathic hypersomnia, periodic hypersomnia, or hypersomnia caused by secondary etiology are also shown, as are those with other diagnostically described sleep disorders (obstructive sleep apnea [$n = 17$], restless legs syndrome [$n = 12$], insomnia [$n = 12$]) and those with a variety of neurologic disorders. Specific pathologies are described for individuals with low (<110 pg/mL) or intermediate (110–200 pg/mL) concentrations of hypocretin-1. (Data from ref. *42*.)

disseminated encephalomyelitis *(51,52)*, hypothalamic sarcoidosis *(53,54)* or histiocytosis X, multiple sclerosis (MS) *(44,48,55,56),* and Parkinson's disease *(42,48,57,58)*. In some cases, lesions of the hypothalamic hypocretin centers have been clearly identified using magnetic resonance imaging, as in bilateral MS plaques in the hypothalamus and tumors of the third ventricle *(59–62)*. Cataplexy may not be present in these cases, and the CSF hypocretin-1 levels may be either in the narcolepsy range (<100 pg/mL) or in the intermediate range *(40)*. The disorders may cause damage to nearby hypocretin projection sites, with adequate preservation of cell bodies to maintain detectable levels, or may be simply coincidental *(40)*.

Genetic disorders such as Niemann-Pick disease type C, Coffin-Lowry syndrome *(63–66),* and Norrie's disease *(67)* have been reported to be associated with daytime sleepiness and/or cataplexy. CSF hypocretin-1 has been measured in cases of Neimann-Pick disease type C, and intermediate levels have been found in some cases with comorbid cataplexy

(42,68,69). Some diseases are associated with the development of both narcolepsy and sleep-related breathing disorder, such as myotonic dystrophy and Prader-Willi syndrome *(40)*; in these patients, some but not all have very low CSF hypocretin-1 levels (<110 pg/mL), suggesting hypocretin deficiency *(40,42,48,70)*. Similarly, in one case of late-onset congenital hypoventilation syndrome (a disorder with reported hypothalamic abnormalities), very low CSF hypocretin-1 levels were found in an individual with otherwise unexplained sleepiness and cataplectic-like episodes *(42)* and who had an excellent response to anticataplectic therapy. CSF hypocretin-1 levels are thus potentially helpful in complex clinical situations in which the history, polysomnographic, and/or MSLT data are difficult to interpret *(40)*.

6.4. Indications and Significance of Hypocretin Measurement in Narcolepsy

Hypocretin measurements have been performed in CSF and blood. Plasma levels of hypocretin-1 are very low in comparison with CSF concentrations and are typically similar between control subjects and narcoleptic subjects, even though these two groups have dramatically different CSF concentrations of hypocretin-1 *(71–73)*. These results may suggest that the hypocretin deficiency in narcolepsy is restricted to the central nervous system (CNS). Alternatively, methodological issues pertaining to measuring hypocretin-1 levels in plasma may be involved. Indeed, plasma hypocretin-1 levels are close to the detection limit of the assay, and the signal may be partially masked by the background noise of the assay. CSF hypocretin-1 is measured by direct radioimmunoassay (RIA) and after peptide extraction. Only hypocretin-1 can be detected. Hypocretin-1 levels do not differ significantly by age or sex *(74)*. Normal levels are higher than 200 pg/mL, and decreased (narcolepsy) levels are less than 110 pg/mL. The significance of intermediate (110–200 pg/mL) and high (>500 pg/mL) hypocretin-1 levels is still unclear and needs further investigation.

As lumbar CSF is typically the measured source of hypocretin-1 concentrations, the question arises as to the functional correlation between lumbar CSF concentrations of hypocretin-1 and hypothalamic hypocretin neuron status. Based on studies in narcolepsy-cataplexy, it is clear that undetectable CSF hypocretin-1 reflects a lack of hypocretin in the brain *(32,38)*. In rats, an average loss of 73% of hypocretin neurons, caused by the injection of hypocretin-2-saporin into the hypothalamus, produces a 50% decrease in CSF hypocretin levels *(75)*. This result suggests some degree of compensation in cases with partial lesions. As the spinal cord receives robust innervation by hypocretin neurons *(76)*, it is also possible that lumbar CSF concentrations of hypocretin-1 reflect spinal release, rather than cortical levels, of hypocretin-1.

Cisternal CSF concentrations of hypocretin-1 display a robust daily rhythm in nocturnal rodents and diurnal primates *(77,78)*. Concentrations of lumbar CSF in humans also exhibit a diurnal fluctuation, but the fluctuation amplitude is only 6% *(79)*, indicating that sampling lumbar CSF at any time of day is likely to be an accurate diagnostic measure. Lesions of the suprachiasmatic nucleus (SCN), the locus of the mammalian circadian clock, results in a loss of rhythmic CSF hypocretin-1 in rats *(98)*. Importantly, however, the dynamic range of hypocretin is the same in animals lacking their SCN as in controls. Thus, even in individuals with circadian rhythm disturbance, sampling at any time of day should yield diagnostically relevant results.

Sleep deprivation can also alter CSF hypocretin-1 levels. Sleep deprivation has been shown to increase CSF hypocretin in a variety of species, including canines, rats, and monkeys *(78,80,81)*. The effects of sleep deprivation are, however, typically limited to increasing CSF hypocretin concentrations to a peak that is normally observed during wake time, and not

above this normal peak. As chronic sleep deprivation is often observed in disorders in which the measurement of CSF hypocretin-1 could be useful, it is important to recognize that CSF concentrations are probably slightly elevated, but as the variation in human lumbar CSF is small, it is unlikely to impact the diagnostic value of a lumbar tap.

The CSF hypocretin-1 measurement can provide valuable information to aid in the diagnosis and characterization of narcolepsy, especially when presenting as an atypical pattern or in pediatric and secondary forms. Low hypocretin-1 concentrations are highly (99%) specific in cases of narcolepsy with atypical cataplexy, but the sensitivity is low (16%) *(42)*. This indicates, of course, that measurement of CSF hypocretin cannot preclude the clinical and electrophysiological evaluation of the disease as well as HLA subtyping. Evaluation of hypocretin-1 levels may also be of critical importance at the onset of the disease, as a measure to evaluate the retardant effect of immunosuppressive treatment *(82)*.

7. HYPOCRETIN DEFICIENCY IN NARCOLEPSY: AN AUTOIMMUNE PROCESS?

Narcolepsy was early shown to be tightly associated with HLA-DR2 and -DQB1 *(3)*, suggesting a possible autoimmune mechanism. Major histocompatibility complex genes play a key role in recognition and processing of foreign antigens by the immune system. HLA molecules are glycoproteins expressed on the surface of macrophages, monocytes, and lymphocytes that present processed antigens to antigen-specific T lymphocytes to induce T-lymphocyte activation and multiplication. Most of the diseases known to be HLA associated (e.g., rheumatoid arthritis, insulin-dependent diabetes mellitus, celiac disease, and MS) are autoimmune disorders. MS, in fact, shares with narcolepsy the same HLA DQB1*0602 association.

Among HLA-associated diseases, narcolepsy shows one of the tightest positive associations with specific HLA alleles (DQB1*0602 and DQA1*0102). DQB1*0602 confers disease susceptibility. A similar HLA allele, DQB1*0601, is, however, protective. The P4 and P9 pockets differ significantly between DQ*0602 and DQ*0601, suggesting that differential peptide binding between these two is critical for a positive or negative risk to develop narcolepsy *(99)*. Hypocretin itself may fit the P4 pocket, but no antibodies directed against hypocretin-1, hypocretin-2, or preprohypocretin have been detected in narcoleptic patients (Mignot, unpublished data). Most investigations, however, have failed to provide conclusive evidence of a causal role of HLA alleles in the pathogenesis of narcolepsy *(83–86)*. This led to the hypothesis that HLA-DR2 was a linkage marker for a gene that caused narcolepsy. Analysis of microsatellite polymorphisms in the area immediately flanking DQB1*0602 and DQA1*0102, however, have not revealed any narcolepsy-related genes *(87)*. Low hypocretin levels have been reported in some patients with Guillain-Barre syndrome, a postinfection immune disorder in which circulating antibodies against neuronal gangliosides are found. Screening of serum from narcolepsy patients has failed to find antiganglioside antibodies, indicating that gangliosides are probably not potent antigens involved in the pathophysiology of narcolepsy *(100)*.

HLA class II-associated autoimmune diseases commonly show a female preponderance, but this is not observed in narcolepsy *(88,89)*. CD4/CD8 lymphocytes subpopulations, erythrocyte sedimentation ratio, complement levels, C-reactive protein level, cytokine levels, and T-cell activity, all markers of immune function, are in a normal range in narcoleptics. Moreover, in contrast to other autoimmune disorders, narcolepsy is not associated with detectable circulating autoantibodies against neuronal or nonneuronal molecules, nor is it associated with specific CSF oligoclonal bands *(90–93)*. Even locally within the hypothalamus, narcolepsy is not clearly

associated with increased microglial HLA class II expression *(32)*. It is important to recognize, however, that most of studies that have examined immune function have done 20 months or years after the likely cessation of the presumptive disease onset, and any autoimmune attack is likely to have abated or desisted. The DQB1*0602 association, the peripubertal onset of the disease, the low concordance rate in monozygotic twins, and the complex genetic susceptibility in family studies provide strong support for the hypothesis of an autoimmune mechanism. A recent study *(82)* has indicated a possible limited efficacy of immunosuppressive therapy during the onset of narcolepsy, adding credence to an autoimmune mechanism. This study also emphasizes the potential importance of early recognition of the disease.

Polymorphisms in other genes have been associated with narcolepsy, including α-interferon *(16)*, monoamine oxidase A (MAO-A) *(94)*, catechol-*o*-methyl transferase (COMT) *(13)*, and tumor necrosis factor-α (TNF-α) *(18)*. A polymorphism in α-interferon, a protein that can decrease the promotor activity of preprohypocretin *(101)*, is associated with DR2-negative narcolepsy, suggesting that interferons may have a role in the pathophysiology of the disease in this narcolepsy subgroup *(16)*. MAO-A and COMT are enzymes involved in the degradation of dopamine and noradrenaline, two wake-promoting systems that are partially controlled by hypocretin neurons *(95,96)*. Polymorphisms in MAO-A are associated with narcolepsy, although not differentiated between DR2-positive and-negative narcolepsy *(94)*. Although polymorphisms in COMT are not predictive of the presence of narcolepsy, they do, in a sexually dimorphic manner, influence the severity of the narcolepsy phenotype. This suggests that monoaminergic systems may be able to compensate somewhat for the loss of hypocretin signaling in some narcoleptics. A rare chromosomal recombinant of TNF-α, a cytokine involved in inflammatory processes, and a polymorphism in one of its receptors (TNF-α receptor 2) have been shown to be associated with the DRB1*1501 Japanese subgroup and without relation to DRB1*1501 *(18)*.

Many inflammatory processes are precipitated by environmental factors. Stress, a change in the sleep/wake cycle, accident, illness, or pregnancy are all events that have been described as preceding the onset of narcolepsy by a few weeks or months *(97)*. Birth seasonality is also observed in narcolepsy and supports a role of environmental factors *(4)*. This latter finding, however, does not indicate whether the environmental influence is prenatal or postnatal, although the most likely hypothesis would involve the annual viral epidemic cycles. Identifying an antigen capable of inducing such an autoimmune attack against hypocretin neurons would be a critical step in our understanding of the pathophysiology of narcolepsy.

8. PERSPECTIVES FOR THERAPEUTIC MANAGEMENT AND CONCLUSION

After nearly a century of narcolepsy research, the field was revolutionized by the discovery of hypocretin in 1998 and the subsequent association of hypocretin dysfunction with narcolepsy. Although the loss of hypocretin function as the cause of most typical cases of narcolepsy with cataplexy has been fairly well established, the role of hypocretin in most other forms of narcolepsy (the narcolepsy "borderland") has yet to be clarified. The cause of the hypocretin dysfunction, whether by autoimmune destruction or otherwise, is still unknown and is a critical component in our treatment or prevention of the disease. The partial efficacy of IVIG treatment early in the course of the disease, if confirmed, may open a new field of therapy for patients diagnosed close to the onset. Although narcolepsy is currently treated symptomatically and specific hypocretin receptor agonists are probably forthcoming, replacement of lost hypocretin neurons would presumably be an ideal technique to cure existing narcolepsy patients.

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Hypocretin Status in Neurological Disorders in Relation to Excessive Sleepiness and Cataplexy

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1. INTRODUCTION

A tight association (90–95%) between human narcolepsy-cataplexy and hypocretin orexin deficiency has recently been revealed *(1–8)*. Hypocretins are hypothalamic neuropeptides involved in various fundamental hypothalamic functions including sleep-wake control, energy homeostasis, and autonomic and neuroendocrine functions *(9–11)*. Hypocretin-containing neurons are located exclusively in the lateral hypothalamic area (LHA). Narcolepsy is a chronic sleep disorder characterized by excessive daytime sleepiness (EDS), cataplexy, hypnagogic hallucinations (HH), and sleep paralysis (SP) (i.e., the narcolepsy tetrad) *(5,12)*. Since hypocretin deficiency in narcolepsy is also tightly associated with human leukocyte antigen (HLA) DR2/DQ6 (DQB1*0602) positivity, an acquired cell loss of hypocretin-containing neurons along with the autoimmune process is suggested in so-called idiopathic cases of narcolepsy *(4,5)*. The term *idiopathic narcolepsy* has been used for cases of narcolepsy unassociated with apparent radiographical or clinical evidence of brain pathology apart from sleep-related abnormalities. Hypocretin deficiency in the brain can be determined clinically via cerebrospinal fluid (CSF) hypocretin-1 measures; CSF hypocretin-1 levels in healthy subjects are above 200 pg/mL regardless of gender, age (from neonatal to the 70s), and time of CSF collection *(2,4,12)*. Owing to the specificity and sensitivity of low CSF hypocretin-1 levels (<110 pg/mL or one-third of the mean normal value), narcolepsy-cataplexy is high among various sleep disorders *(5,13,14)*, CSF hypocretin measures will be included in the diagnostic criteria for narcolepsy-cataplexy in the second revision of the International Classification of Sleep Disorders (ICSD).

Impaired hypocretin systems may also be observed in some neurological disorders affecting the LHA (the location of hypocretin cell bodies) and hypocretin projection pathways. Indeed, an earlier study by Ripley et al. *(13)* measured CSF hypocretin levels in 235 neurological patients and showed that a subset of subjects with acute or subacute neurological disorders (i.e., intracranial tumors, cerebrovascular events,craniocerebral trauma, central nervous system [CNS] infections, and Guillain-Barré syndrome [GBS]) had decreased CSF hypocretin-1 levels, although such levels in most patients with chronic neurological conditions, such as Alzheimer's and Parkinsons disease (AD and PD), are not significantly reduced (Fig. 1). Arii et al. *(15)* also recently studied CSF hypocretin-1 levels in 132 pediatric neurological conditions. The results are consistent with those of Ripley et al.'s study *(13)*, and in only a limited number

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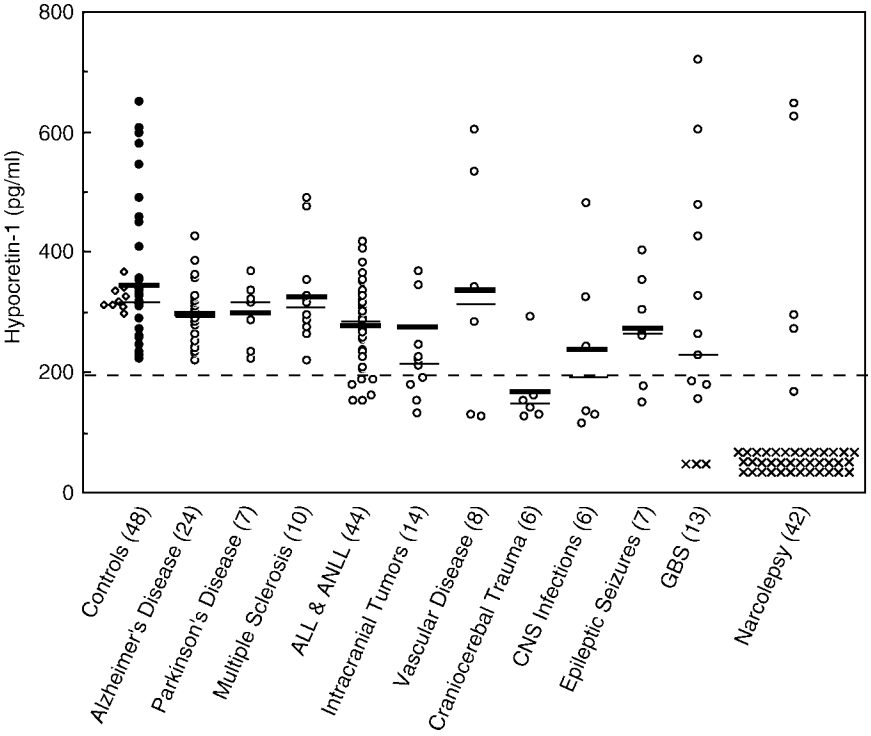

Fig. 1. Hypocretin-1 levels in controls and major neurological disorder groups. The number of subjects in each group is given in parentheses. Thick bars represent mean values, and thin bars represent median values. Control subjects who were measured at night (6 PM to 2 AM) are indicated with diamonds. ALL, acute lymphocytic leukemia; ANLL, acute nonlymphocytic leukemia; CNS, central nervous system GBS; Guillain-Barré syndrome (from ref. *13.*).

of neurological conditions beside narcolepsy were levels reduced. These include intracranial tumors *(15)*, craniocerebral trauma, autoimmune and postinfectious disease (GBS and acute disseminated encephalomyelitis [ADEM]), and some inherited disorders, such as Niemann-Pick disease type C [NPC] and Prader-Willi syndrome [PWS] *(15)*.

The findings by Ripley et al. *(13)* and Arii et al. *(15)* are particularly interesting since these neurological conditions are often associated with acutely disturbed consciousness, lethargy, sleepiness, and/or residual sleep disturbances. In rare cases, symptoms of narcolepsy can be seen during the course of a neurological disease process (i.e., symptomatic narcolepsy). Interestingly, involvements of hypothalamic structures in symptomatic narcoleptic cases were repeatedly emphasized several decades ago *(16,17)*, and an impaired hypocretin system may also be involved in some symptomatic narcolepsy cases.

Association with EDS/cataplexy in some inherited neurological diseases (such as NPC, PWS, or myotonic dystrophy) is also known *(18–20)*. An impaired hypocretin system may thus also be involved in these sleep-related symptoms in conjunction with these neurological conditions.

In this chapter, we first overview cases of symptomatic narcolepsy reported in the literature. Since EDS cases without other narcolepsy symptoms can also occur with a variety of neurological disorders and are not usually an indication of narcolepsy, we also extend our discussion on the roles of hypocretin system in EDS disorders associated with various neurological conditions. We use hypersomnia and EDS synonymously in this review as defined by the inability to stay alert and awake during the major waking episodes of the day, resulting in

unintended lapses into sleep. Sleepiness may vary in severity and is more likely to occur in boring, monotonous situations that require no active participation. In some cases, sleepiness is associated with large increases in total daily amount of sleep without any genuine feeling of restoration. In other cases, as seen in idiopathic cases of narcolepsy, sleepiness can be alleviated temporarily by naps but reoccurs shortly thereafter. We also discuss the rare cases of symptomatic cataplexy (i.e., isolated cataplexy).

Since data on CSF hypoocretin-1 measures are available for some recent symptomatic narcolepsy and/or EDS cases, we focus on these cases and discuss the roles of hypocretin status in these disorders (Table 1). For this purpose, we categorized the cases as follows: (I) symptomatic narcolepsy-cataplexy associated with focal/generalized CNS invasion, such as cerebral tumors, vascular diseases, and neurodegenerative disorders (Sections 2 and 4.1), (II) symptomatic cataplexy with (IIa) focal/generalized CNS invasion (Section 3.1) and (IIb) cataplexy-like attacks in inherited/congenital disorders (Sections 3.2 and 4.2), and (III) hypersomnia associated with (IIIa) focal/generalized CNS invasion, such as cerebral tumors, brain infections, vascular diseases, head trauma and neurodegenerative disorders (AD and PD) (Section 5.1), and (IIIb) with CNS diseases mediated with neuroimmune mechanisms, such as inflammatory and demyelinating diseases (Section 5.2). The latter hypersomnia categories (IIIa, b) include less defined EDS cases and probably consist of heterogeneous conditions. This is partially because applying standardized polygraphic assessments (all-night polygraphic recordings followed by multiple sleep latency test [MSLT]) was often difficult in these neurological conditions. However, since the prevalence of these hypersomnia cases appeared to be much higher than that of symptomatic narcolepsy, we believe that a discussion of the roles of the hypocretin system in less well-defined EDS cases can also have clinical implications.

2. SYMPTOMATIC NARCOLEPSY: DEFINITION AND OVERVIEW

Symptoms of narcolepsy can be sometime seen during the course of a neurological disease process. In such instances, the term symptomatic narcolepsy is used, implying that narcolepsy is a symptom of the underlying process rather than an idiopathic condition. In this case, the signs and symptoms of narcolepsy should be temporally associated with an underlying neurological process. Many authors use symptomatic narcolepsy and secondary narcolepsy indiscriminately, even though they have apparently different meanings. We suggest the use of symptomatic narcolepsy/EDS, since "secondary EDS" has also been used for EDS associated with sleep apnea and restless leg syndrome. Symptomatic narcolepsy/EDS must be distinguished from the category of associated narcolepsy/EDS, i.e., cases in which narcolepsy/EDS is associated with epileptic seizure and in which the two conditions may be secondary to a common process (such as brain tumor and injury).

Although several important original studies and extensive reviews *(21–29)* of symptomatic narcolepsy are available, many older cases have no objective measures for sleepiness, and the diagnosis of these cases is mostly based on the clinical criteria *(30–38)*. Furthermore, some of these reports did not provide the symptomatology and course of the assumed causal disease. In addition, the term narcolepsy itself was sometimes used loosely (i.e., for lethargy or occurrence of long episodes of sleep periods), and occurrences of cataplexy or rapid eye movement (REM) sleep abnormalities were not emphasized. It is therefore very difficult to review these cases in a uniform way.

The current diagnostic criteria for idiopathic narcolepsy include (1) EDS occurring almost daily for at least 3 mo or short sleep latency (SL) (typically less than 5 min [less than 8 min will be used in the second revision of the ICSD]) by MSLT together with (2a)

Table 1
Symptomatic Narcolepsy or EDS with Hypocretin Measurements

Neurological condition	Category	Location	Age (yr)	Gender	EDS	Sleep latency (min/MSLT)	CA	SOREMP	HLA	Hypocretin (pg/mL)		Notes	Author
Tumors (n = 10)									DR2/DQB1 *0602(DQw1)				
Tumor resection	E	Hypothalamus	16	F	+	1.7	–	–	–	Low	104	–	Arii 2001 *(55)*
Astrocytoma resection	N	Suprasellar	11	M	+	?	–	+	–	Low	<40		Marcus 2002,2003 *(52,112)*
Choroid plexus carcinoma resection	N	Pineal gland, thalamus	28	F	+	7.5	–	+	–	Normal	518		Krahn 2002 *(56)*
Craniopharyngioma	E	Hypothalamus	11–19	M:2, F:3	+	Mean:10	–	?	?	Control level	Mean:133		Snow 2002 *(53)*
Craniopharyngioma	E	Hypothalamus	11–19	M:2, F:3	+	Mean:10	–	?	?	Control level	Mean:133		Snow 2002 *(53)*
Craniopharyngioma	E	Hypothalamus	11–19	M:2, F:3	+	Mean:10	–	?	?	Control level	Mean:133		Snow 2002 *(53)*
Germinoma	E	Hypothalamus	11–19	M:2, F:3	+	Mean:10	–	?	?	Control level	Mean:133		Snow 2002 *(53)*
Arachnoid cyst	E	Thalamus	11–19	M:2, F:3	+	Mean:10	–	?	?	Control level	Mean:133		Snow 2002 *(53)*
Adenoma	NC	Pituitary, hypothalamus	60	M	+	6.4	+	+	–	Normal	275	Acromegaly+	Dempsey 2003 *(57)*
Tumor	NC	Hypothalamus	65	F	+	?	+	+	?	Low	61		Nokura 2004 *(58)*
Head trauma (n = 3)													
Head trauma	E	Nonspecific	23	M	+	4.5	–	–	–	Int.	176		Dauvilliers 2003 *(66)*
Head trauma	E	Nonspecific	21	M	+	3	–	–	+	Normal	503		Dauvilliers 2003 *(66)*
Head trauma	E	Base of skull	15	M	+	2	–	–	?	Int.	151	GCS12	Arii 2004 *(67)*
Vascular disorder (n = 5)													
Infarction	NC	Hypothalamus	23	M	+	0.5	+	+	–	Int.	167		Scammell 2001 *(100)*
Infarction	E	Thalamus	34	M	+	9	–	–	?	Normal	265		Bassetti 2003 *(7)*
Infarction	E	Pontomedullary	40	M	+	1	–	–	?	Normal	316		Bassetti 2003 *(7)*
Infarction	N	Thalamus	45	M	+	5	–	+	?	Normal	312		Nokura 2004 *(58)*
Infarction	E	Thalamus	15	M	+	?	–	–	?	Normal	274		Tohyama 2004 *(114)*
Encephalopathies (n = 3)													
Rasmussen's syndrome	NC	Left fronto-temporal and insular	40	M	+	1.6	+	+	+	Low	<40		Lagrange 2003 *(118)*
Wernicke's encephalitis	E	Hypothalamus	5	F	+	?	–	–	?	Low	<40		Kashiwagi 2004 *(117)*

Limbic encephalitis	E	Limbic, hypothalamus	65	M	+	?	–	–	?	Low	87		Yamato 2004
Degeneration (*n* = 33)													
PD	E	Nonspecific	69	M	+	6.1	–	–	–	Normal	253		Overeem 2002 *(119)*
PD	E	Nonspecific	64	M	+	4.9	–	+	–	Normal	307		Overeem 2002 *(119)*
PD	E	Nonspecific	52	M	+	4.4	–	–	–	Normal	319		Overeem 2002 *(119)*
PD (*n* = 16)	E	Nonspecific	?	?	+	?	–	?	?	Low	<50-97		Drouot 2003 *(120)*
PD (*n* = 3)	E/REF	Nonspecific	?	?	+/–	?	–	?	?	Int.	138–169		Drouot 2003 *(120)*
Dementia with Lewy bodies (*n* = 10)	E	Cortical atrophy	69–82	M:7, F:3	+	?	–	–	?	Normal	382–667		Baumanna 2004 *(122)*
Progressive supra-nuclear palsy	E	Enlargement of the 3rd V	74	F	+	2.0	–	–	+	Low	<40		Hattori 2003 *(121)*
Heredogenerative disorders (*n* = 1)													
ADCA-DN	NC	Enlargement of the 3rd V, brainstem atrophy	51	M	+	?	+	?	–	Low	96		Melberg 2001 *(98)*
Demyelinating disorders (*n* = 7)													
MS	N	Hypothalamus	22	F	+	2.8	–	+	–	Low	<40		Iseki 2002, Oka 2004 *(73,74)*
MS	E	Hypothalamus	45	F	+	?	–	–	?	Low	<40		Kato 2003 *(126)*
MS	E	Hypothalamus	43	F	+	?	–	–	?	Int.	191		Nozaki 2004 *(131)*
ADEM	E	Hypothalamus	12	F	+	4.5	–	–	–	Low	102		Kubota 2002 *(127)*
ADEM	N	Hypothalamus, coronaradiata, aqueduct, raphe	38	F	+	4.4	–	+	+	Low	87		Gledhill 2004 *(128)*
ADEM	E	Hypothalamus	7	F	+	?	–	–	–	Int.	146		Yoshikawa 2004 *(129)*
ADEM	E	Hypothalamus	0.9	F	+	?	–	–	?	Low	<40		Yano 2004 *(130)*
Paraneoplastic autoimmune syndromes (*n*=6)													
Anti-Ma associated encephalitis	E	Mesiotemporal regions, ventricular enlargement, temporal lobe atrophy	45	M	+	?	–	?	?	Low	<100	Non-semino-matous germ cell tumor of the testis	Overeem 2004 *(137)*

(*Continued*)

Table 1 (*Continued*)

Neurological condition	Category	Location	Age (yr)	Gender	EDS	Sleep latency (min/MSLT)	CA	SOREMP	HLA	Hypocretin (pg/mL)		Notes	Author
Anti-Ma associated encephalitis	E	Left temporal enhancing abnormalities	22	M	+	?	–	?	?	Low	<100	Mixed germ-cell tumor of the testis	Overeem 2004 *(137)*
Anti-Ma associated encephalitis	REF	Brainstem, periventricular region, basal ganglia	82	F	–	?	–	?	?	Normal	237	Poorly differentiated lung cancer	Overeem 2004 *(137)*
Anti-Ma associated encephalitis	E	Thalamus, superior collicular, medial temporal lesions	67	F	+	?	–	?	?	Low	<100	Adenocarcinoma of the lung	Overeem 2004 *(137)*
Anti-Ma associated encephalitis	E	Hippocampus, midbrain	38	M	+	?	–	?	?	Low	<100	Germ-cell tumor of the testis (seminoma)	Overeem 2004 *(137)*
Anti-Ma associated encephalitis	REF	Non-specific	53	F	–	?	–	?	?	Normal	218	Adenocarcinoma of the ovary	Overeem 2004 *(137)*
Immune-mediated polyneuropathy (*n*=2)													
Guillain-Barré syndrome	E	Non-specific	28	M	+	0.7/TNST	–	–	?	Low	<40		Nishino 2003 *(135)*
Guillain-Barré syndrome	E	Non-specific	19	M	+	0.8/TNST	–	–	?	Intermediate	151		Nishino 2003 *(135)*
Genetic/congenital disorders (*n* = 14)													
PWS	E	Nonspecific	16	M	+	3	–	–	–	Low	109	BMI 48.1, AHI 5.6	Mignot 2002 *(5)*
PWS	E	Nonspecific	10	M	+	6	–	–	?	Int.	130	BMI 29.8, AHI 3.1	Nevsimalova 2005 *(106)*
PWS	REF	Nonspecific	23	M	–	?	–	–	?	Int.	191	BMI 49, AHI 46.8	Nevsimalova 2005 *(106)*
PWS	REF	Nonspecific	6	M	–	?	–	–	?	Normal	226	BMI 25.8, AHI 0	Nevsimalova 2005 *(106)*

PWS	REF	Nonspecific	0.5 mo	M	?	?	–	–	?	Int.	192		Arii 2004 *(67)*
NPC	C	Nonspecific	5	M	–	16.5	+	–	–	Int.	142		Kanbayashi 2003 *(89)*
NPC	NC	Nonspecific	14	F	+	5.1	+	+	+	Int.	157		Vankova 2003 *(90)*
NPC	E	Nonspecific	25	F	+	3.5	–	–	–	Normal	226		Vankova 2003 *(90)*
NPC	E	Nonspecific	24	M	+	3.2	–	–	–	Normal	245		Vankova 2003 *(90)*
NPC	E	Nonspecific	31	M	+	10.7	–	–	–	Int.	190		Vankova 2003 *(90)*
MYD	E	Nonspecific	46	?	+	4.7	–	+	–	Low	91		Martinez-Rodriguez 2003 *(20)*
MYD	N	Nonspecific	47	?	+	7	–	–	–	Normal	200		Martinez-Rodriguez 2003 *(20)*
MYD	E	Nonspecific	19	?	+	8	–	–	–	Int.	187		Martinez-Rodriguez 2003 *(20)*
MYD	N	Nonspecific	50	?	+	1.8	–	+	–	Normal	206		Martinez-Rodriguez 2003 *(20)*
MYD	E	Nonspecific	25	?	+	5.7	–	–	–	Normal	235		Martinez-Rodriguez 2003 *(20)*
MYD	E	Nonspecific	68	?	+	5.7	–	–	–	Int.	167		Martinez-Rodriguez 2003 *(20)*
MYD	N	Nonspecific	23	M	+	6.4	–	+	–	Normal	401		Dauvilliers 2003 *(66)*
Others (*n* = 2)													
Hashimoto's encephalopathy	E	?	65	M	+	?	–	–	?	Low	<40	full recovery by steroids	Castillo 2004 *(140)*
Whipple's disease	O	?	53	M	?	?	–	–	?	Int.	113		Voderholzer 2002 *(141)*

Abbreviations: NC, symptomatic narcolepsy-cataplexy; N, symptomatic narcolepsy; E, symptomatic EDS; C, symptomatic cataplexy; O, other; Ref, reference cases (hypocretin-1 levels in subjects without EDS); EDS, excessive daytime sleepiness; CA, cataplexy; V, ventricle; MSLT, multiple sleep latency test; TNST, two-nap sleep test; EEG, electroencephalogram; ADCA-DN, autosomal dominant cerebellar ataxia, deafness and narcolepsy; MS, multiple sclerosis; ADEM, acute disseminated encephalomyelitis; PWS, Prader-Willi syndrome; NPC, Neimann-Pick disease type C; MYD, myotonic dystrophy; PD, Parkinson's disease; AHI, apnea hypoxia index; BMI, body mass index; CA, cataplexy; SOREMP, sleep-onset REM period; +, present; –, absent; ?, not assessed; Int., intermediate.

cataplexy (sudden and transient episodes of loss of muscle tone triggered by emotions; narcolepsy with cataplexy in the second revision of the ICSD), or (2b) with abnormal REM sleep features documented by polygraphic measures (more than two sleep onset REM periods [SOREMPs] by MSLT; narcolepsy without cataplexy in the second revision of the ICSD). In our review, symptomatic narcolepsy is defined as the cases that met the criteria (if MSLT data were not available, equivalent polygraphic REM sleep abnormalities were also considered, and this is noted for each case). In addition, association with a significant underlying neurological disorder accounts for the EDS and temporal associations (narcolepsy onset should be within 3 yr if the causative diseases are "acute" neurologic conditions) (see ref. *63*). In contrast, if neither cataplexy nor polygraphic abnormal REM sleep features are associated with EDS (clinically "or" short sleep latency documented by polygraphic measures (typically less than 10 min [less than 8 min will be used in second revision of ICSD] during MSLT)), the diagnosis of symptomatic EDS was made. In particular cases, such as EDS associated with ADEM, EDS may rapidly disappear with steroid treatments and may not last for 3 mo. We diagnosed these cases arbitrarily as symptomatic EDS, and the duration of the EDS episode is noted. Because the causal relationship between these two conditions were mostly judged by the statements of the authors' original case reports, it may be impossible to exclude cases in which the neurological condition is only a coexistence of idiopathic narcolepsy/idiopathic hypersomnia. In rare cases, isolated cataplexy (without EDS) associated with neurological conditions occurs. If the authors emphasize the occurrence of cataplexy as a significant underlying neurological disorder and no EDS is associated, we classified these as "symptomatic (isolated) cataplexy."

Using these criteria, we counted about 116 symptomatic cases of narcolepsy reported in the literature (Fig. 2A). [The details of all cases will be reported in our review article on symptomatic narcolepsy *(142)*].

As reported previously by several authors, tumors, inherited disorders, and head trauma are the three most frequent causes of symptomatic narcolepsy: 33 cases (28%) of the symptomatic narcoleptic cases were caused by brain tumors, with 55% of them exhibiting cataplexy *(29,39–58)*. The results of HLA typing were reported in 14 cases, and 8 cases were HLA DR2 negative. We also analyzed the brain structures involved in symptomatic cases of narcolepsy with brain tumors and found that hypothalamic lesions (70%) are most often associated (Fig. 2B). The brainstem lesions were much less frequent, found in only 10% of these cases. Other structures were reported in 12% and multiple sites in 9%.

In 38 patients (33%) had inherited diseases, and 58% of them exhibited cataplexy. HLA typing was performed in 19 patients, and 11 of them were HLA negative. The lesions of inherited diseases were not specified by neuroimaging.

Nineteen patients (16%) had head trauma, and 74% of them exhibited cataplexy *(21,27,51, 59–68)*. In contrast to tumor cases, it was often difficult to determine the impaired structure for the symptomatic narcoleptic cases associated with head trauma.

Eleven cases (9%) of symptomatic narcolepsy studied were associated with multiple sclerosis (MS) *(21,51,69–74)*. Most old cases were reported to exhibit both EDS and cataplexy, but many of them lacked clinical details. It was thus difficult to determine whether some of these patients had coexisting idiopathic narcolepsy. HLA typing was performed in four patients, and two of them were HLA DR2 negative.

Six patients (5%) had vascular disorders. The lesions impaired were reported to be the hypothalamus ($n = 1$), thalamus ($n = 1$), brainstem (or both) ($n = 2$), and unspecified ($n = 2$). HLA typing was done in four patients, and two were HLA DR2 negative.

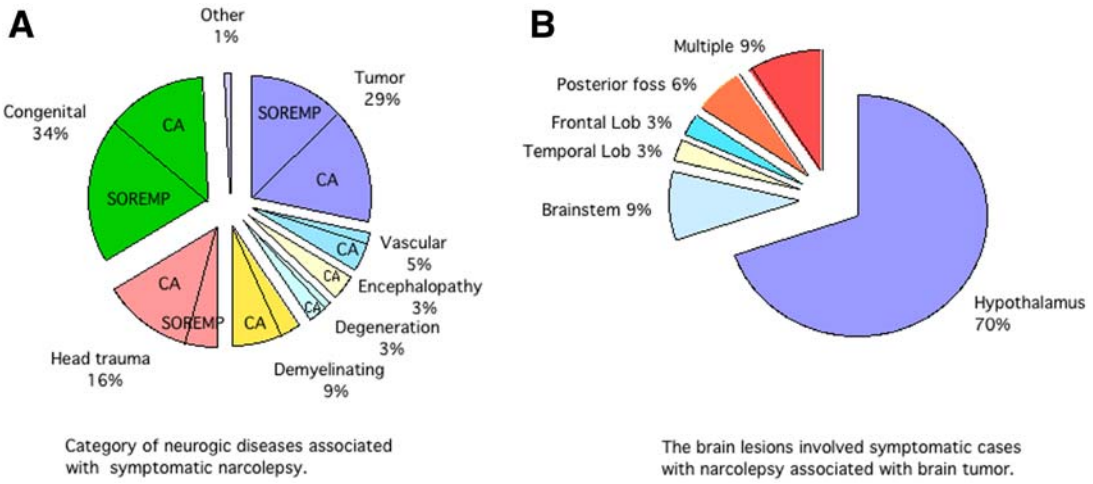

Fig. 2. Categories of neurologic diseases associated with symptomatic narcolepsy(A) and the brain lesions involved in symptomatic cases of narcolepsy associated with brain tumor (B). One hundred and thirteen symptomatic cases of narcolepsy are included. The percentage of each neurologic category (with cataplexy[CA]/ with sleep onset REM periods [SOREMPs]) is shown. **(A)** Tumors, inherited disorders, and head trauma are the three most frequent causes. **(B)** Analysis of symptomatic narcolepsy with tumor cases clearly showed that the lesions were most often in the hypothalamus and adjacent structures (the pituitary or suprasellar or optic chasm).

Analysis of the symptomatic narcolepsy with tumor cases clearly showed that in 70% the lesions were in the hypothalamus and adjacent structures (the pituitary or supraseller or optic chiasm). Von Economo *(75)* was probably the first to suggest that narcolepsy may have its origins in the posterior hypothalamus and in some cases a secondary etiology. Neuropathological studies on the encephalitis lethargica pandemic in 1916–1923 revealed involvement of the midbrain periaqueductal gray matter and posterior hypothalamus in the hypersomnolent variant, with frequent extension to the oculomotor nuclei. This led von Economo to speculate that the anterior hypothalamus contained a sleep-promoting area, whereas an area going from the posterior wall of the third ventricle to the third nerve was involved in actively promoting wakefulness. Along with von Economo's cases, two case reports by Stiefler *(76)* and Adie *(17)* on narcolepsy-cataplexy after encephalitis lethargica were also available.

von Economo *(16)* also speculated that idiopathic narcolepsy involved this general area. A postulated hypothalamic cause of narcolepsy was widespread until the 1940s *(59)* but was ignored during the psychoanalytic boom *(77,78)* and was then replaced by a brainstem hypothesis *(79)*, along with the establishment of the role of the brainstem in generating REM sleep and REM sleep atonia *(80)*. The involvement of the hypothalamus in the occurrence of narcoleptic symptoms was nicely refined by Aldrich et al. *(29)*, who noted that tumors or other lesions located close to the third ventricle were associated with symptomatic narcolepsy and hypothesized that the posterior hypothalamic region may be the culprit. The hypothesis was finally confirmed by the discovery of hypocretin deficiency in idiopathic cases of human narcolepsy *(1,81,82)*. The fact that impairments in the hypothalamus are noted in most symptomatic cases of narcolepsy also suggests a possible involvement of impaired hypocretin neurotransmission for this condition.

3. SYMPTOMATIC CATAPLEXY WITH VARIOUS ETIOLOGIES

3.1. Symptomatic Cataplexy Associated with Focal/Generalized CNS Invasion

Ethelberg *(83)* reported on two cases of cataplexy associated with head trauma. Two subjects (a 25-yr-old man and a 14-yr-old girl) had depressed fractures, making impressions on the lateral surface of the frontal lobe corresponding to the anterior part of the motor cortex. So-called cataplexy or attacks of general limpness developed in both cases, sometimes associated with blunting or loss of consciousness. Emotional provocation of these attacks was not seen in either case. Although the author used the term "cataplexy," the genesis and the pathophysiology of the attacks of limpness are considered to be indicative of epileptic seizures.

Smith *(84)* reported five cases with meningiomas associated with cataplexy. Three of the five tumors were localized frontally and the other two over the convexity of the parietal lobe. The patients were 50–68 yr old at the time of diagnosis. The dominating symptoms were sudden falls or sagging at the knees when standing or walking. There was never loss of consciousness, and the patients were aware of the fall. As in Ethelberg's case, emotional provocation was not seen. Three of the patients had simple partial seizures. These authors suggested that the "cataplexy-like" attacks were caused by "weak" epileptic discharges spreading along the corticoreticular fibers and activating the motor inhibitory reticulospinal pathway.

Four additional symptomatic cases of cataplexy have recently been reported *(85–87)*. Some have similar characteristics to the cataplexy seen in idiopathic narcolepsy, such as emotional provocation and/or response to anticataplectic medications. EDS was excluded by objective polygraph measures in some cases.

D'Cruz et al. *(85)* reported on a 19-yr-old woman with cataplexy. She had a previous diagnosis of chronic progressive MS since the age of 16. Magnetic resonance imaging (MRI) revealed diffuse white matter lesions involving the medial medulla, pons, and subcortical white matter. There were no interval changes in the size and distribution of these lesions in comparison with an earlier scan performed prior to onset of symptoms. However, a temporal association of cataplexy onset with the use of a levonorgestrel implant was reported. The patient was DR2 positive. Protriptyline at 5 mg/d produced almost complete relief from the disabling cataplexy. The patient discontinued the medication after removal of the levonorgestrel implant without exacerbation of cataplexy.

D'Cruz et al. *(85)* also reported on a 6-yr-old girl with a pontomedullary pilocytic astrocytoma who vomited continuously and had cataplexy. After the surgery, residual tumor remained at the dorsal pontomedullary region. The emesis subsided, but the cataplexy episodes continued. The frequency of the attacks increased when the child had disturbed nocturnal sleep. A few months later, nocturnal sleep improved and cataplexy subsided without further intervention. The authors suggested that lesions even more caudal in the brainstem could be associated with symptomatic cataplexy.

Fernandez et al. *(86)* reported on a 28-yr-old man with cataplexy. He was treated with azidothymidine for human immunodeficiency virus (HIV) seropositivity. He also had SP together with daytime hypersomnia but without overt sleep attacks or HH. His father has symptoms suggesting narcolepsy. MSLT failed to show either a decreased sleep latency or SOREMPs. Increased numbers of leukocytes were seen in both the blood and the CSF. MRI showed a small, round lesion with marked enhancement of contrast in the mesencephalic tegumentum and periaqueductal area; there were no signs of hydrocephalus or lesions in other locations, including the hypothalamus. Because the presence of toxoplasmosis was

assumed, treatment was initiated and brought about progressive improvement until all symptoms disappeared after 2 wk of treatment.

Servan et al. *(87)* reported on another symptomatic case of cataplexy. A 23-yr-old man had head trauma with short loss of consciousness. The first episode of cataplexy was 1 yr later. The cataplexy episodes were severe (up to five times/d) and always triggered by strong emotions. The patient displayed no EDS, no irresistible sleep attacks, no HH, and no SP. He was HLA DR2 negative.

3.2. Cataplexy-like Attacks in Inherited Diseases

As mentioned earlier, an association with cataplexy (or cataplexy-like attacks) in some inherited neurological diseases is also known. These diseases include NPC (sometime also associated with EDS), Norrie disease (ND), Coffin-Lowry syndrome (CLS), and Möbius syndrome.

NPC type C is an autosomal recessive congenital neurological disorder characterized by the accumulation of cholesterol and glycosphingolipids in the peripheral tissues and glycosphingolipids in the brain. Classic NPC symptoms include hepatosplenomegaly, vertical supranuclear gaze palsy, ataxia, dystonia, and dementia. Subjects with NPC have been reported to frequently display narcolepsy-like symptoms, including cataplexy *(18,28,88–90)*. This condition is remarkable as cataplexy is often triggered by typical emotions (laughing), and it is responsive to anticataplectic treatments.

ND is an X-linked recessive disorder causing ocular atrophy, mental retardation, deafness, and dysmorphic features. Virtually absent monoamine oxidase (MAO) type A and B activity has been found in some patients with chromosome deletions. Vossler et al. *(91)* reported on three related boys (2, 4, and 7-yr-old) with coexistence of cataplexy and abnormal REM sleep organization.

Crow et al. *(92)* reported an unusual, nonepileptic, cataplexy-like phenomenon in two males and one female with Coffin-Lowry syndrome (CLS). CLS is a rare disorder characterized by moderate to severe mental retardation, facial dysmorphism, tapering digits, and skeletal deformity. Paroxysmal drop attacks occur in patients with CLS, characterized by sudden loss of muscle tone induced by unexpected tactile or auditory stimuli. Fryns and Smeets *(93)* reported marked distal muscle wasting in two affected brothers with CLS at 15 and 14-yr-old. These subjects were followed up, and it became evident that the "epileptic episodes" previously described were sudden, nonepileptic collapses with atonia similar to the case of Crow et al. *(92)*. Fryns and Smeets *(93)* also examined 20 other CLS males and found that in one of these patients the same type of sudden, nonepileptic attacks were noted from the age of 4 yr.

Nelson et al. *(94)* reported on two teenaged boys with CLS and so-called stimulus-induced drop episodes. Attacks in these patients were characterized by abrupt episodes of complete or partial loss of lower extremity tone but were elicited by a loud noise, unexpected light touch stimulation, or visual threat. These events were not associated with impairment of consciousness, and immediate recovery was noted. No epileptiform discharges during attacks were seen in either patient. In one patient loss of tonic EMG activity in paraspinal muscles similar to cataplexy was observed, whereas in the other patient, the episodes had at later years changed to brief myoclonic jerks and tonic spasms (hyperekplexia). Hyperekplexia was also noted by other authors in several other CLS cases *(95,96)*. These results suggest that "cataplexy-like nonepileptic events" are not rare in CLS. However, the attacks in CLS may not be uniform, and most of these may be pathophysiologically different from genuine cataplexy.

Möbius syndrome is a rare congenital disorder with the primary diagnostic criteria of congenital facial and abducens nerve palsy *(97)*. Orofacial anomalies and limb malformations may be associated with the disorder. An involvement of other cranial nerves is also common. Occasionally, cranial nerves V, X, XI, and XII are involved, resulting in difficulty chewing, swallowing, and coughing, which often leads to respiratory complications. Mental retardation and autism have been reported in some cases. The syndrome has been associated with a number of possible causes of damage to chromosome 13. This syndrome also causes a wide variety of sleep disturbances, including parasomnias, EDS, and a condition resembling cataplexy.

Parkes *(19)* reported on five patients with Möbius syndrome who had both EDS and laughter-provoked cataplexy-like conditions, and these cases were diagnosed as symptomatic narcolepsy.

4. HYPOCRETIN STATUS IN VARIOUS NEUROLOGICAL CONDITIONS

4.1. Hypocretin Status in Symptomatic Narcolepsy-Cataplexy Associated with Distinct CNS Lesions

Soon after the involvement of hypocretin impairment in idiopathic narcolepsy was noted, Melberg et al. *(98)* reported a reduced CSF hypoocretin-1 level (96 pg/mL) in a previously reported 51-yr-old male case with autosomal dominant cerebellar ataxia (ADCA) and deafness and narcolepsy (DN). In this Swedish pedigree (ADCA-DN; OMIM, Online Mendelian Inheritance in Man, accession number 604121), four out of five ADCA subjects are affected with narcolepsy-cataplexy *(99)*, and CSF previously collected from one of theses subjects (patients III-2) was available for the hypocretin measures. The patient was negative for HLA DR2. This patient has a heredodegenerative disease with enlargement of the third ventricle, and moderate atrophy of the cerebellum and the cerebral hemispheres were observed by MRI. We listed this case as narcolepsy associated with distinct CNS lesions rather than as narcolepsy associated with inherited disease.

Scammell et al. *(100)* subsequently reported on a 23-yr-old man who developed narcolepsy-cataplexy owing to a large hypothalamic stroke following resection of a craniopharyngioma. This lesion included two-third of the caudal hypothalamus, except for the most lateral component on the right, and extended into the mediodorsal thalamus bilaterally, the left amygdala, and parts of the basal forebrain and rostral midbrain. His postoperative course was complicated by panhypopituitarism, staphylococcal meningitis and hydrocephalus. He experienced HH. He became obese, with a BMI of 31.7. Sleep latency (SL) by MSLT was 0.5 min, and REM latency was 3.5 min. An overnight polysomnography showed 1 min and 1.5 min of SL and REM latency, respectively, without significant sleep apnea. His HLA was negative for DQB1*0602, and his CSF hypocretin level was 167 pg/mL.

Nokura et al. *(58)* reported on one case with narcolepsy and cataplexy-like phenomena, a 66-yr-old woman with hypersomnia owing to a hypothalamic tumor (Fig. 3). She showed EDS and cataplexy-like symptoms, such as abrupt falling without loss of consciousness, but the emotional triggers were unclear. MRI revealed lesions with high signal intensities in the hypothalamus, thalamus, and midbrain bilaterally (Fig. 3). She also had mild anterior hypopituitarism and a SOREMP in a daytime polysomnography. Her hypocretin-1 level was 61 pg/mL. Her symptoms were improved with reduction of the tumor after 46 Gy of radiation and the intravenous administrations of nimustine hydrochloride and interferon-β.

The lesions in these three cases had different etiologies: degeneration, infarction, and tumor. (The number of such cases is limited.) Hypothalamic lesions were noted in all three cases. Moderate reduction in CSF hypocretin levels (two low and one intermediate) also confirmed the functional impairment of the hypothalamus. It is likely that a massive impairment of

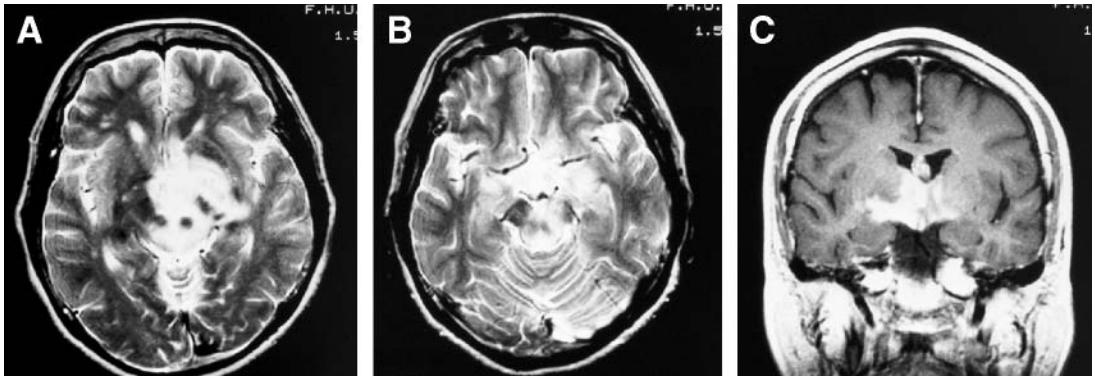

Fig. 3. A narcolepsy-cataplexy case with a hypothalamic tumor and low hypocretin level (61 pg/mL): A 66-yr-old woman with a hypothalamic tumor. **(A,B)** Axial T2-weighted MR image at admission exhibits high signal intensities in the midbrain, hypothalamus, and thalamus. **(C)** Coronal T1-weighted image with gadolinium exhibits enhancement in the same lesion. This case was also accompanied by mild anterior hypopituitarism. Her symptoms and MRI findings were improved with reduction of the tumor after 46 Gy of radiation and nimustine hydrochloride and interferon administered intravenously (from ref. *58*.).

hypocretin projections and projection sites is involved in the second case (hypothalamic stroke after resection of a craniopharyngioma), implying that more severe impairment of hypocretin neurotransmission (than that estimated from CSF hypocretin-1 levels) may exist in this case. Although these results are consistent with the hypothesis of hypothalamic hypocretinergic involvement in symptomatic cases of narcolepsy, it is not certain whether all cases with low hypocretin levels associated with hypothalamic damage develop narcoleptic symptoms.

4.2. Hypocretin Status in Symptomatic Narcolepsy-Cataplexy and/or EDS Associated with Inherited Disorders

As mentioned earlier, there are clusters of cases of genetic or congenital disorders associated with primary central hypersomnolence and/or cataplexy, and CSF hypocretin-1 has also been assessed in several patients with PWS, NPC, and myotonic dystrophy.

4.2.1. Prader-Willi Syndrome (PWS)

PWS is a genetically defined disorder that is characterized by infantile hypotonia and failure to thrive, hyperphagia with early childhood obesity, hypogonadism, temperature instability and developmental delay *(101–103)*. The molecular genetic cause is the nonexpression of the paternal genes in the PWS region on chromosome 15q11-13 *(106)*. EDS is a common symptom in PWS *(101–106)*.

EDS is a common symptom in PWS *(101–103)*. Sleep-disordered breathing (SDB) and narcoleptic traits such as SOREMPs and cataplexy have also been reported in these subjects *(104,105)*. If SDB exists, primary hypersomnia should only be diagnosed if EDS does not improve after adequate treatment of SDB. Mignot and Nevsimalova et al. (*5*) reported on a 16-yr-old boy with EDS. He was HLA-DQB1*0602 positive and obese (BMI = 48.1), with a documented 15q11-13 deletion, limited number of SDB events (apnea hypopnea index [AHI] of 5.6), and no cataplexy; SL was 3.0 min, there were no SOREMPs by MSLT, and hypocretin was 109 pg/mL. Nevsimalova et al. *(106)* also measured CSF hypocretin-1 in three other PWS cases. One subject (10 yr old) exhibited EDS (SL = 6.0 min) with no SOREMPs and an AHI of 3.1. All three subjects were obese and exhibited no cataplexy. CSF hypocretin levels in the PWS case with EDS and DQB1*0602 were low (130 pg/mL, 10 yr old, BMI = 29.8, AHI = 3.1), and in the others without EDS they were intermediate (191 pg/mL, 23 yr old,

BMI = 49, AHI = 46.8) or in the normal range (226 pg/mL, 6 yr old BMI = 25.8, AHI = 0). Interestingly, AHI in these PWS subjects was correlated with age and BMI but not with CSF hypocretin-1 levels and EDS.

Arii et al. *(15)* reported on a 2-wk-old PWS boy with severe hypotonia, poor feeding, and documented 15q11-12 deletion; the hypocretin level was intermediate (192 pg/mL).

These reports raised the possibility that EDS in PWS may also be attributed to the hypocretin system, not to SDB caused by obesity. The latter author also proposed that PWS cases may be a model for congenital dysfunction/developmental failure of the hypocretin system.

4.2.2. Niemann-Pick Type C Disease (NPC)

Kanbayashi et al. *(89)* measured CSF hypocretin levels in two NPC cases with and without cataplexy. The first case was a 5-yr-old boy with NPC, cataplexy, and an intermediate hypocretin level (142 pg/mL). Cataplexy had been evoked by laughter since the age of 2.3 yr. EDS was not claimed by the patient, and normal SL (16.5 min) without SOREMPs was observed by a two-nap sleep test (TNST) *(107)*. No abnormal findings in the hypothalamus were detected by MRI scans. He was negative for HLA DR2. The second case was a 3-yr-old girl with NPC and a normal hypocretin level (299 pg/mL). This patient exhibited neurological symptoms such as tremor, ataxia, and akathisia but did not exhibit cataplexy or EDS.

Vankova et al. *(90)* reported on five patients with juvenile NPC. Deterioration of intellectual function, the presence of pyramidal, dystonic, and cerebellar signs, and splenomegaly were observed in all cases. Cataplexy was reported in one patient. Nocturnal polysomnography revealed disrupted sleep in all patients. Total sleep time, sleep efficiency, REM sleep, and delta sleep amounts were decreased compared with age-matched controls. Shortened mean sleep latencies were observed in three patients during the MSLT, but SOREMPs were observed only in the case with cataplexy, and this case met with the criteria of symptomatic cases of narcolepsy. This patient was HLA DQB1*0602 positive; the other subjects were HLA DQB1*0602 negative. CSF hypocretin-1 levels were reduced in two patients (190 pg/mL and 157 pg/mL in one with cataplexy); in two other patients, CSF hypocretin-1 levels were at the lower level (226 and 245 pg/mL) of the normal range. The authors speculated that lysosomal storage abnormalities in NPC patients may also have an impact on the hypothalamus, including the area where hypocretin-containing cells are located.

In these two reports, all the NPC patients with cataplexy have reduced hypocretin-1 levels, whereas CSF hypocretin-1 levels in the NPC patients without cataplexy are in the lower limit of normal, suggesting that a degree of impairment of the hypocretin system may contribute to the occurrence of cataplexy in this inherited diffuse CNS impairment condition.

4.2.3. Myotonic Dystrophy

Myotonic dystrophy type 1 (MYD1) is a multisystem disorder characterized by myotonia, muscle weakness, cataracts, endocrine dysfunction, and intellectual impairment *(108–110)*. This disorder is caused by a CTG triplet expansion in the 3´ untranslated region of the DMPK gene on 19q13. MYD1 is frequently associated with EDS, sharing with narcolepsy a short sleep latency and the presence of SOREMPs during the MSLT. The disease is also often associated with SDB, which may also account for the appearance of SOREMPs. Martinez-Rodriguez et al. *(20)* reported on six patients with MYD1 complaining of EDS. The mean sleep latency on MSLTs was abnormal in all patients (<5 min in two, and <8 min in four), and two SOREMPs were observed in two subjects, meeting the criteria for symptomatic narcolepsy. It should be noted that these two cases also had SDB. All patients were

HLA-DQB1*0602 negative. Hypocretin-1 levels (181 pg/mL) were significantly lower in patients vs controls (340 pg/mL); one case with two SOREMPs had hypocretin-1 levels in the range generally observed in narcolepsy (<110 pg/mL). Three cases had intermediate levels (110–200 pg/mL). The authors suggested that a dysfunction of the hypothalamic hypocretin system may mediate sleepiness and abnormal MSLT results in patients with MYD1.

In one case of late-onset congenital hypoventilation syndrome, a disorder with reported hypothalamic abnormalities *(111)*, we found very low CSF hypocretin-1 levels in an individual with otherwise unexplained sleepiness and cataplexy-like episodes *(20)*. Excellent response to anticataplectic mediciation was observed in this case.

Although only a limited number of cases with genetic or congenital neurologic conditions associated with EDS and/or cataplexy were studied, moderate decreases in CSF hypocretin levels were observed in almost all patients with EDS and/or cataplexy. However, the degree of reduction was small in contrast to idiopathic narcolepsy-cataplexy. Moreover, because CSF hypocretin-1 levels in other genetic and congenital neurological conditions without EDS/cataplexy have not been systematically studied, the specificity of an impaired hypocretin system in EDS and cataplexy is still uncertain in such neurological conditions.

5. HYPOCRETIN STATUS IN HYPERSOMNIA IN VARIOUS NEUROLOGICAL CONDITIONS

5.1. Focal/Generalized CNS Invasion

Symptomatic narcolepsy is relatively rare, but sleepiness without other narcoleptic symptoms can often occur with a variety of neurological disorders; they are more likely to be caused by multifocal or global disturbances of the brainstem, diencephalon and cerebral cortex. Recently, several clinical studies also suggested that disruption of the hypothalamic hypocretin system in EDS is associated with various neurological conditions.

5.1.1. Cerebral Tumors

Arii et al. *(55)* reported on a 16-yr-old girl with hypersomnia after removal of a hypothalamic suprasellar grade II pilocystic astrocytoma. MRI showed that the bilateral, medial, and lateral hypothalamic areas and right posterior hypothalamus were damaged. This patient had diabetes insipidus, hypothyroidism, weight gain, no cataplexy, sleep latency of 1.7 min by MSLT, no SOREMPs, and a hypocretin-1 level of 104 pg/mL; she was HLA DR2 negative.

Marcus et al. *(52,112)* reported on an 11-yr-old boy in a vegetable state following astrocytoma resection and CNS hemorrhage. MRI revealed a large suprasellar mass that extended into the sella inferiorly and was displaced posteriorly. The boy developed hypothyroidism and syndrome of inappropriate antidiuretic hormone (SIADH). In the nocturnal EEG study, sleep was fragmented, with 16 short REM cycles. The daytime EEG showed frequent REM periods. HLA DR2 and DQB1*0602 were negative. Hypocretin-1 was at an undetectably low level. His EDS improved with 200 mg of modafinil and 5 mg of methylphenidate.

Snow et al. reported that five patients (11–19 yr, mean: 15 yr) with EDS *(53)*. The mean sleep latency by MSLT in the five patients was 10.3 min, but no detailed sleep data were reported for each case. Three patients underwent surgeries for craniopharyngioma, one for germ cell tumor, and one for a thalamic arachnoid cyst. The craniopharyngiomas and germ cell tumor were located in the hypothalamus-hypophysis region, and the arachnoid cyst was in the thalamic region. All patients received relatively extensive surgeries involving the hypophysis and hypothalamus and hormone replacement therapies. Patients had significantly higher BMI (mean: 28), and this was primarily attributable to two morbidly obese patients associated with obstructive sleep apnea.

Although treatment with continuous positive airway pressure resulted in complete resolution of their sleep-disordered breathing in these two cases, no changes in daytime somnolence occurred.

Krahn et al. *(56)* reported on a patient who developed a narcoleptic-like sleep disorder after receiving treatment for a choroid plexus carcinoma of the pineal gland. She underwent a pinealectomy, chemotherapy, and radiation treatment. Immediately after surgery, the patient developed EDS that she attributed to severe insomnia and an irregular sleep/wake rhythm. She had a few episodes of SP and HH but no cataplexy. An increased percentage of REM sleep was seen in nocturnal polysomnography, and three or four SOREMPs were seen during the MSLT. She was negative for HLA DQB1*0602 and had a normal CSF hypocretin level (518 pg/mL). The author proposed that her symptoms were caused by an unknown mechanism unrelated to hypocretin depletion.

Dempsey et al. *(57)* reported on a 60-yr-old man with acromegaly who developed narcolepsy-cataplexy 2 wk after completing radiotherapy (45 Gy) for a pituitary adenoma. He had both HH and SP. Sleep latency by MSLT was 6.4 min, and REM latency was 9 min (3 SOREMPs/5 naps). He was obese (BMI of 35) and his AHI was 17/h. HLA was not typical for narcolepsy. Hypocretin-1 was within the normal range (275 pg/mL). The authors speculated that the radiotherapy or the tumor was associated with damage to a locus rich in hypocretin receptors. In contrast to the case of Nokura et al. *(58)*, the same 45–46 Gy of radiation resulted in an opposite outcome.

Kubota et al. *(113)* reported one typical case of narcolepsy–cataplexy with a ganglioma in the right amygdala in a 7-year-old girl. She showed hypnagogic hallucinations and a SOREMP in the nocturnal polysomnography. Sleep latency by MSLT was 6.5 min without SOREMP. Her HLA was DR2/DQw1 and hypocretin-1 level was 79 pg/mL. This case is likely to be the comorbidity of idiopathic narcolepsy and a brain tumor, since her symptoms were not changed after the resection of the tumor. This case is not listed in Table 1. Overall, three symptomatic cases with EDS had low hypocretin-1 levels; however, two other cases and Snow's five cases had normal levels. It should be noted that all three cases with low CSF hypocretin-1 levels are HLA-DR2 or HLA-DR2 and DQB1*0602 negative. Therefore, EDS in these HLA negative cases are likely to be secondary due to the hypocretin deficiency being caused by the tumors. EDS in the remaining seven cases with normal or high hypocretin-1 levels were thought to be caused by other factors, although there is also a possibility of impaired hypocretin projections, terminals or postsynaptic receptors caused by the tumors in these cases.

5.1.2. Infarctions

Bassetti et al. *(7)* reported on two cases with EDS and cerebral infarction. The first patient was a 34-yr-old man who suffered from thalamic infarction. His mean sleep latency by MSLT was 9 min, and his hypocretin level was 265 pg/mL. The second patient was a 40-yr-old man suffering from pontomedullary infarction. His sleep latency by MSLT was 1 min, and his hypocretin level was 316 pg/mL.

Nokura et al. *(58)* and Tohyama et al. *(114)* independently reported two hypersomnia cases with bilateral paramedian thalamic infarctions. The paramedian thalamus is believed to play an important role in the regulation of sleep, and disturbances of sleep regulation are known to occur in paramedian thalamic stroke *(115,116)*. The first patient was a 45-yr-old man *(58)*. He suffered from bilateral paramedian thalamic infarctions and had EDS with SOREMPs (two times in four naps) by MSLT (meeting the criteria for symptomatic narcolepsy). His hypocretin-1 level was 312 pg/mL. The second patient was a 15-yr-old boy who suffered from bilateral paramedian thalamic infarctions and hypersomnia (Fig. 4). His hypocretin level was 274 pg/mL

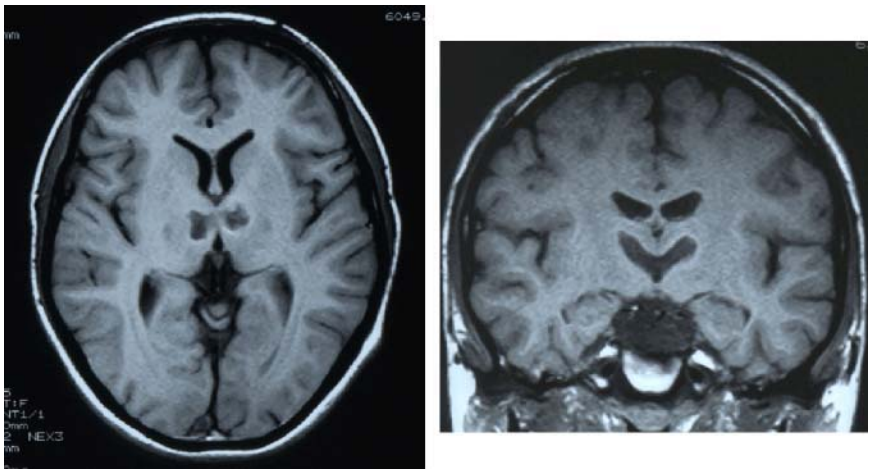

Fig. 4. A case of paramedian thalamic infarctions, EDS, and a normal hypocretin level (274 pg/mL): a 15-yr-old boy with EDS owing to bilateral paramedian thalamic infarctions *(114)*. Patients with bilateral paramedian thalamic lesions are often known to exhibit atypical hypersomnia (i.e,. de-arousal or subwakefulness *(see* ref. *115)*. The LHA, where hypocretin cell bodies are located was not affected, and the CSF hypocretin-1 level was in the normal range. It is not known whether other parts of the hypocretin system (projections or receptive sites) are involved in EDS with paramedian thalamic infarctions.

(from ref. *114* with permission). The lesions from the infarctions did not include the hypocretin cell bodies, and hypocretin levels seemed to be normal in both patients. However, impairment of hypocretin projection could still be involved. It should also be noted that, as pointed out by Guilleminault et al. *(115)*, patients with bilateral paramedian thalamic lesions do not present with a typical hypersomnia but a de-arousal or subwakefulness with an inability to develop sleep outside the normal circadian boundary (pseudo-hypersomnia). Indeed, these patients showed reduced latency to stage 1 during MSLT but did not develop other normal non-REM sleep and REM sleep status during the daytime. It may also be possible that hypocretin deficiency is not involved in so-called pseudo-hypersomnia associated with bilateral paramedian thalamic lesions and that other pathophysiology needs to be considered for these unique sleep symptoms.

5.1.3. Encephalopathies

5.1.3.1. Wernicke's Encephalopathy

Kashiwagi et al. *(117)* reported on a 5-yr-old girl with Wernicke's encephalopathy. She gradually developed sleepiness and an abnormal sleep/wake schedule. Her sleep time was 15–20 h/d, and she fell asleep frequently even while eating. She developed ocular and neurological symptoms (such as involuntary movements, hemiparesis, depression of speech, and global confusional state). MRI revealed lesions in the bilateral hypothalamus in addition to the dorsomedial nucleus of the thalamus and mammillary bodies as well as the periaqueductal gray and floor of fourth ventricle. Vitamin B_1 levels were low (38.7 ng/mL; normal range 52–176 ng/mL), and CSF hypocretin was decreased (<40 pg/mL). Her sleepiness and MRI findings gradually improved with thiamine therapy. Six months after the onset of sleepiness, both lesions on MRI and CSF hypocretin levels (158 pg/mL) had recovered to some degree. Although it is probable that the brain lesions in Wernicke's encephalopathy (like cases with tumors) affect the hypothalamic hypocretin system directly or indirectly, the question of whether the changes in hypocretin neurotransmission are solely responsible for EDS in these cases has not been fully studied.

5.1.3.2. Limbic Encephalopathy

Yamato et al. (personal communication) reported on a patient with non-paraneoplastic immune-mediated limbic encephalitis exhibiting low hypocretin-1 concentrations (87 pg/mL). A 65-yr-old man developed chronic progressive hypersomnia. MRI of the brain showed bilateral signal abnormalities in the medial temporal lobes and the hypothalamus, but systemic examinations for malignant tumors were negative. Acyclovir treatment failed to amend his condition. Subsequent steroid treatment improved his hypersomnia and reduced the extent of abnormal signals on MRI. The CSF hypocretin concentration increased to 148 pg/mL at 23 d after treatment.

5.1.3.4. Rasmussen's Syndrome

Lagrange et al. *(118)* reported narcolepsy and Rasmussen's syndrome in a previously healthy 40-yr-old man. Adult-onset Rasmussen's syndrome is an extremely rare, progressive, central nervous system disorder, characterized by seizures, hemiparesis, inflammation of the brain, and mental deterioration. An autoimmune response to a viral infection has been suggested as a possible cause. He developed severe EDS, cataplexy, HH, and SP over the course of a few months. Brain MRI was normal, and polysomnography with MSLT confirmed a diagnosis of narcolepsy (SL 1.6 min, three SOREMPs in four naps). His HLA haplotype was DQB1*0602, and CSF analysis showed no detectable hypocretin. Approximately 18 mo later, he developed complex partial seizures. Further MRI showed a progressively enlarging lesion involving the left frontotemporal and insular areas. Pathology from partial resection samples was consistent with Rasmussen's syndrome. Evaluation for tumorous, infectious, and paraneoplastic etiologies was negative. There was no further progression of the residual lesion on serial MRI.

Although the pathophysiological basis of Rasmussen's syndrome and its implication in narcolepsy is unknown, the author speculated the possibility of a common underlying disease process related to the autoimmune mechanism for Rasmussen's syndrome and hypocretin deficiency. It is, however, a temporal relationship between the onset of Rasmussen's syndrome and that of hypocretin deficiency that was not well documented. The comorbidity with idiopathic narcolepsy and Rasmussen's syndrome is also a possibility since this subject is HLA positive, and a number of late onset cases (over 35 years old) of idiopathic narcolepsy reported in the literature.

Although it is likely that the brain lesions in the EDS cases with tumors, infarctions and various encephalopathies directly or indirectly affect the hypothalamic hypocretin system, it is not yet fully studied whether the change in the hypocretin neurotransmission is solely responsible for the occurrence of the EDS in these cases.

5.1.4. Neurodegenerative Disorders

5.1.4.1. Parkinson's Disease

Thirty percent of patients with PD have been reported to have EDS. Sleep problems are often related to the disease itself (e.g., difficulties in maintaining sleep because of motor disabilities), but they can also occur secondary to pharmacological treatment, especially with dopamine $D_{2/3}$ agonists. Ripley et al. *(13)* initially reported that CSF hypocretin-1 levels in seven PD subjects were in the normal range, but sleep abnormalities of these subjects were not assessed. Overeem et al. (*119)* measured CSF hypocretin levels in three PD patients with EDS; all were normal.

Drouot et al. *(120)* reported that patients with late-stage PD had low ventricular CSF hypocretin-1 levels ($n = 16$: <50–97 pg/mL; $n = 3$: 138–169 pg/mL). Hypocretin-1 levels decreased with increasing disease severity. The authors speculated that CSF hypocretin-1 levels may reflect the size of the hypocretin neuron pool and that a decrease may indicate degeneration of hypocretin

neurons in PD. The sleepiness of the patients was assessed by the Epworth sleepiness scale (ESS). The mean ESS of these PD patients (11 ± 1) was significantly higher than that of controls (4 ± 1), but hypocretin-1 levels were not correlated with ESS among PD subjects. The discrepancy between this study and that of Overeem et al. *(119)* has not been assessed.

5.1.4.2. Progressive Supranuclear Palsy

Hattori et al. *(121)* reported on a 74-yr-old woman with EDS who was diagnosed with probable progressive supranuclear palsy (PSP). Her EDS mimicked that of narcolepsy without cataplexy: the MSLT showed short latencies (<2 min without SOREMPs), HLA was positive for DR2/DQB1, and the CSF hypocretin-1 concentration was undetectable. It is not clear whether the coincidence of these disorders was owing to a common process or comorbidity. The authors speculated that the existence of neuropathological changes, such as neurofibrillary tangles in the hypothalamus of the patient with PSP, might cause decreased hypocretin neurotransmission.

5.1.4.3. Dementia with Lewy Bodies

EDS, hallucinations, and REM sleep behavior disorder are symptoms reported in both dementia with Lewy bodies (DLB) and narcolepsy. However, Baumann et al. *(122)* reported that patients with DLB had normal hypocretin levels.

5.1.4.4. Alzheimer's Disease

Riply et al. *(13)* also reported that CSF hypocretin-1 levels in 24 patients with AD were normal. Sleep abnormalities are known to occur in this condition *(123)*. Dysfunction of other neurochemical systems, for example, cholinergic systems in AD, may be more directly involved in sleep abnormalities in these subjects.

5.1.5. Head Trauma

The association of narcolepsy/EDS with head injury is controversial. Most people with hypersomnolence after closed head injury do not have narcolepsy *(60)*, but some patients with narcolepsy report that their symptoms began after a head injury *(61–65,68)*. Lankford et al. *(63)* reported nine detailed cases with narcolepsy (five HLA positive, two HLA negative and two undetermined). Hypocretin-1 measurements were not made. Riply et al. *(13)* reported decreased CSF hypocretin-1 levels in (five of six cases) after head trauma.

Dauvilliers et al. *(66)* reported that a brain lesion patient (as determined by MRI) severely affected with posttraumatic hypersomnia had an intermediate CSF hypocretin-1 level (176 pg/mL, HLA negative). Another severely affected patient had normal levels (503 pg/mL, HLA positive). These two patients had no cataplexy but had shortened sleep latencies (4.5 and 3.0 min, respectively) without SOREMPs by MSLT.

Arii et al. *(67)* reported on a 15-yr-old boy affected with posttraumatic hypersomnia who had an intermediate hypocretin-1 level (Fig. 5). His Glasgow Coma scale at 48 h after injury was 12 (E2V4M6). MRI showed severe cerebral contusion of the bilateral basalis of the frontotemporal lobe and medial part of the right occipital lobe, with CSF leakage. One year after injury, he needed more than 9 h of nocturnal sleep and one or two 1–3 h naps daily. The hypocretin-1 level was 151 pg/mL. MRI showed atrophy in the basalis of the temporal lobe and medial part of the right occipital lobe. The hypothalamus had moderate atrophy with dilation of the third ventricle but no localized lesion.

EDS appearing during the first year after a head injury may be considered posttraumatic *(124)*. It typically presents as extended night sleep and episodes of daytime sleep. Sleepiness is usually associated with other characteristics such as headaches, difficulties in concentration, or memory disorder. Radioimaging studies may reveal several possibilities: lesions

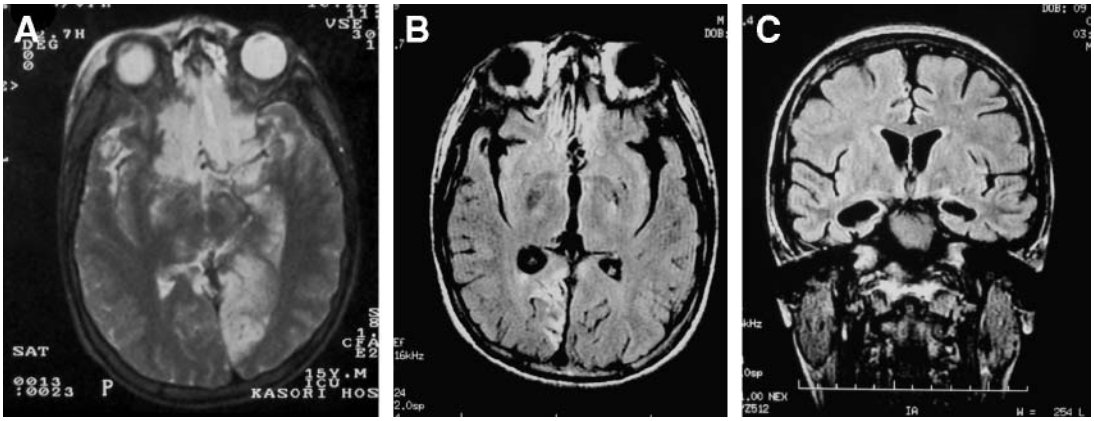

Fig. 5. A case of posttraumatic hypersomnia with atrophy of the hypothalamus and an intermediate hypocretin level (151 pg/mL): a 15-yr-old boy with posttraumatic hypersomnia. One year after injury, he slept more than 9 h at night and had 1–3 h naps every day. His Glasgow Coma scale at 48 h after injury was 12 (E2M6V4). **(A)** MRI showed severe cerebral contusion of the bilateral basalis of the frontotemporal lobe and the medial part of the right occipital lobe with CSF leakage. **(B,C)** MRI showed atrophy in the basalis of the temporal lobe and medial part of the right occipital lobe. The hypothalamus was moderately atrophied with dilation of the third ventricle but no localized lesion (from ref. *67*.).

affecting the hypothalamic region or brainstem, midbrain, or pontine tegmentum, or, more often than not, the absence of any significant lesions. Sleepiness should be objectively evaluated by an MSLT but is often not in clinical situations. Patients with hypersomnia after head or brain trauma associated with sleep apnea syndrome have also been reported *(60)*.

Although two of three patients with posttraumatic EDS had moderately decreased CSF hypocretin-1 levels, it is not known whether all posttraumatic subjects with lower CSF hypocretin-1 levels exhibit EDS. Similarly, the question of whether a more pronounced degree of hypocretin-1 impairment exists in posttraumatic symptomatic narcolepsy has not been studied.

5.2. CNS Diseases Mediated with Neuroimmune Mechanisms

As mentioned earlier, 10 cases of narcolepsy-cataplexy have been reported in MS *(21,51,69–74)*. The question was also raised as to whether the two disorders exist fortuitously or whether a causal relationship exists between them. In some cases with late onset and a regressive course, demyelination is suggested as the cause of narcolepsy, but some authors have also suggested the involvement of a common genetic susceptibility (i.e., HLA DR2) *(125)*. Among these previous cases, a patient reported by Younger et al. *(72)* had a lesion in the cerebral peduncles detected by MRI, but specific lesions were not noted for most other cases. Recently, three EDS cases associated with MS *(58,73,74,126)* with hypothalamic lesions and reduced hypocretin-1 levels were reported; an involvement of the hypothalamus in MS associated with EDS/cataplexy was suggested. EDS in four ADEM cases was recently reported *(127–130)*. All these cases associated with EDS had hypothalamic lesions and low CSF hypocretin-1 levels, suggesting an involvement of the hypothalamic hypocretin system in these conditions.

5.2.1. Demyelinating Diseases

5.2.1.1. Multiple Sclerosis (MS)

Iseki et al. *(73,74)* reported on a 22-yr-old woman with MS and hypersomnia and several SOREMPs secondary to bilateral hypothalamic lesions (Fig. 6). Her nocturnal sleep time

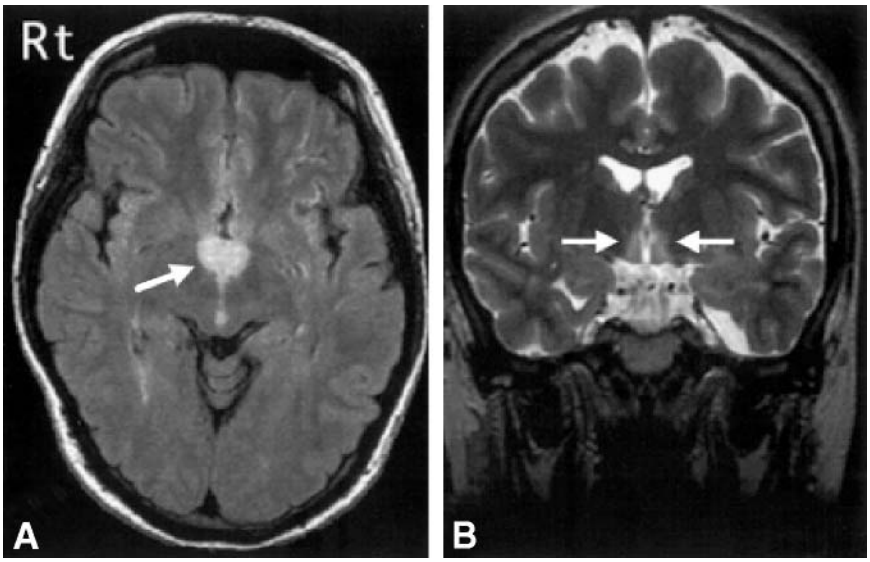

Fig. 6. A multiple sclerosis (MS) case with EDS, SOREMPs, and a low hypocretin level (<40 pg/mL): a 22-yr-old woman with bilateral hypothalamic lesions of MS. Her nocturnal sleep time was 15 h, sleep latency by MSLT was 2.8 min, and REM latency was 4.7 min with five SOREMPs. Her HLA was DR2 negative. MRI revealed FLAIR hyperintensity in the hypothalamus bilaterally. **(A)** Axial section of FLAIR image. **(B)** Coronal section of T2-weighted image. Bilateral hypothalamic plaque is demonstrated as median high-intensity areas (arrows) (from refs. *73,74*.).

was 15 h sleep latency by MSLT was 2.8 min, and REM latency was 4.7 min (five SOREMPs in five naps). She did not experience cataplexy, HH, or SP. Her HLA was DR4 and DR6. MRI revealed fluid-attenuated inversion recovery (FLAIR) hyperintensity in the hypothalamus bilaterally, and her CSF hypocretin-1 level was less than 40 pg/mL. After intravenous methylprednisolone was started, followed by oral predonisolone, her symptoms resolved, and MRI findings improved. Hypocretin-1 levels improved to 167 pg/mL after 2 mo and 221 pg/mL after 4 mo.

Kato et al. *(126)* reported on a 45-yr-old woman with hypersomnia in a relapse of MS. Five days before hospital admission, she had had hypersomnia attacks; she suddenly fell asleep during a conversation and again while cooking. After admission, she slept almost all day. T2 and FLAIR MRI revealed lesions in the hypothalamus bilaterally. The CSF hypocretin-1 level in CSF was below 40 pg/mL. Methylprednisolone pulse treatment was started, followed by oral prednisolone. Three days after initiation of methylprednisolone, her hypersomnia completely resolved. Twenty days later, hypocretin-1 levels recovered to 167 pg/mL.

Nozaki et al. *(131)* reported on a 43-yr-old female MS patient presenting with a period of hypersomnia and fever. T2 MRI revealed lesions of the hypothalamus bilaterally. The CSF hypocretin-1 level was 191 pg/mL. Intravenous methylprednisolone was started, followed by oral predonisolone; her symptoms resolved, and MRI findings improved. The hypocretin-1 level was increased to 291 pg/mL.

Although earlier all patients with symptomatic narcolepsy associated with MS also had cataplexy, two recent MS patients with low hypocretin levels did not exhibit cataplexy or REM sleep abnormalities. In these cases, extended nocturnal sleep as well as hypersomnia was observed. The diagnostic methods and therapies for MS have improved, which may possibly change the symptomatology of sleep abnormalities associated with MS in recently reported cases.

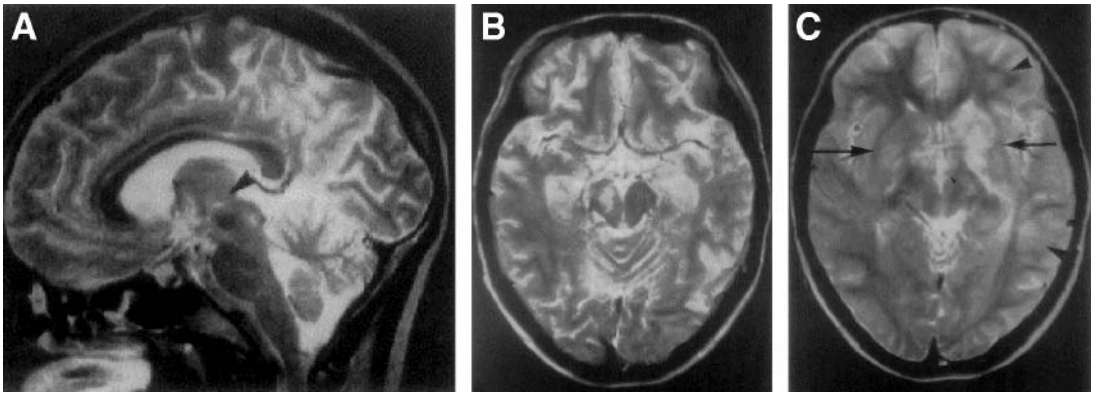

Fig. 7. An ADEM case with EDS and a low hypocretin level (102 pg/mL): a 12-yr-old girl with ADEM. MRI revealed lesions in the bilateral hypothalamus along with other multifocal lesions including cerebral white matter, brainstem, and basal ganglia. MSLT revealed a mean sleep latency of 4.5 min with no SOREMPs. HLA typing of this patient was negative for DQB1*0602. Her hypersomnia and MRI findings including the hypothalamus and other regions improved with steroid treatment. (From ref. *127*, with permission.)

5.2.1.2. Acute Disseminated Encephalomyelitis (ADEM)

Kubota et al. *(127)* reported on a 12-yr-old girl with acute ADEM and hypersomnia (Fig. 7). MRI revealed bilateral hypothalamus lesions in addition to other multifocal sites including cerebral white matter, brainstem, and basal ganglia lesions. MSLT revealed mean sleep latency was 4.5 min with no sleep-onset REM periods This patient was negative for DQB1*0602, and the CSF hypocretin-1 level was 102 pg/mL. Her hypersomnia and MRI findings (including the hypothalamus and other regions) improved with steroid treatment in approx 1 mo.

Gledhill et al. *(128)* reported on a 38-yr-old woman with ADEM and hypersomnia. She had no REM-related symptoms, such as cataplexy, HH, or SP. MRI revealed lesions in the hypothalamus, walls of the third ventricle, corona radiata, floor of the aqueduct, and raphe nuclei. She was positive for DR2/DQB1*0602. Her hypocretin-1 level was 87 pg/mL. She was treated with a high-dose steroid, and subsequent MRI showed smaller and fewer lesions. Six months later, her subjective sleepiness was partially improved. Her mean sleep latency by MSLT (four naps) was 4.4 min with four SOREMPs, and her hypocretin-1 level was 148 pg/mL. One year after initial examination, sleepiness persisted and the results of MSLT were almost unchanged.

Yoshikawa et al. *(129)* reported on a 7-yr-old girl with ADEM, visual symptoms, and hypersomnia. MRI revealed bilateral lesions in the white matter, basal ganglia, and hypothalamus. Her CSF hypocretin-1 levels were intermediate (146 pg/mL) on admission and gradually recovered to the normal range (263 pg/mL) within 47 days, and her excessive sleepiness was reduced. Decreased hypothalamic hypocretin neurotransmission may be involved in this symptomatic case of hypersomnia associated with the clinical course of ADEM. Interestingly, double vision was also noted.

Yano et al. *(130)* reported on an 11-mo-old girl with ADEM and hypersomnia. High-intensity T2 MRI showed multiple lesions in the white matter, brainstem, and bilateral hypothalamus. CSF hypocretin-1 was at undetectable levels. Hypersomnia and MRI lesions were improved by intravenous steroid administration. Changes in CSF hypocretin levels were not monitored.

EDS can be associated with immunological or postinfectious brain pathology, such as ADEM and other kinds of encephalitis. Von Ecomono's reports *(75)* suggested that the hypothalamus is a target site for immune-mediated postinfectious disease; an immunologic reaction to hypothalamic antigens including the hypocretin system may also be involved in immune-mediated encephalitis. However, after von Economo's series was published, relatively few cases of secondary EDS associated with postviral infection were reported. In recent years, four ADEM cases with hypocretin measurements were reported. Although a decreased level of consciousness is frequently found in ADEM, ADEM cases with well-defined hypersomnia are relatively rare *(132,133)*. These patients have both extended nocturnal sleep time and daytime hypersomnia, and only one patient has SOREMPs, indicating that sleep abnormalities may be distinct from those seen in symptomatic narcolepsy and idiopathic narcolepsy. Early diagnosis and treatment in recent patients may also have reduced the severity of sleep symptoms.

5.2.2. Guillain-Barré Syndrome (GBS)

Undetectably low CSF hypocretin-1 levels were found in seven cases of GBS in the Japanese population *(13,134,135)*. Reduced CSF hypocretin-1 levels in GBS are probably not caused by an increase in protein in the CSF or by the secondary effects of treatment or associated health conditions, but they might be under influence of a region/ethnicity-specific form of GBS *(136)*. This finding was rather unexpected, since GBS is a presumed autoimmune disorder of peripheral polyradiculoneuropathy. However, additional CNS involvement (i.e., in the hypothalamus), such as SIADH or diabetes insipidus, has also been suggested in severe cases. Interestingly, all these GBS subjects with low hypocretin-1 had severe disease, with tetraplegia, bulbar symptoms, and/or respiratory failure.

Since the clinical picture of these subjects is quite different from that of narcolepsy, it is unlikely that there CSF hypocretin-1 levels will cause diagnostic confusion. The occurrence of sleep abnormalities in GBS, especially in severe cases, has received little attention. Sleep latencies for two CSF hypocretin-deficient GBS subjects who complained of sleepiness after the recovery from GBS neurological symptoms were significantly shortened (<1 min) in both cases *(135)*.

5.2.3. Paraneoplastic Syndrome

A recent report described four patients with paraneoplastic anti-Ma2 antibodies who had hypothalamic inflammation, EDS, cataplexy and undetectable hypocretin-1 levels *(137)*. MRI showed abnormalities involving the medial temporal lobes, hypothalamus, basal ganglia, or upper brainstem. In addition, one patient also had DI and hypothyroidism. The authors claimed that anti-Ma antibodies indicate the immune-mediated disorder of the CNS in encephaletis that may result in low hypocretin-1 levels.

In contrast to MS and ADEM, distinct CNS lesions are not observed in GBS and neoplastic syndromes. Nevertheless, a significant degree (undetectable level) of hypocretin deficiency was observed in both conditions. This suggests that a hypocretin deficiency in these conditions may occur at the neuron or ligand levels. In view of the facts that an autoimmune hypothesis is the most popular theory for hypocretin cell death in narcolepsy *(138,139)*, but that no gross inflammation has been observed in the hypothalamus *(81)*, a subset of GBS and Ma2 antibody-positive paraneoplastic syndromes (the two neuroimmune conditions associated with hypocretin deficiency) may be important models for studying possible autoimmune cell damage in narcolepsy.

6. CONCLUSIONS

Symptoms of narcolepsy can occur during the course of neurological conditions. Although it is difficult to rule out the possibility of comorbid idiopathic narcolepsy in some cases, a review of the literature reveals numerous cases with unquestionable symptomatic narcolepsy. These include cases with HLA-negative patients and/or late onset and cases in which the narcoleptic symptoms occur in parallel with the rise and fall of the causative disease.

Symptomatic cases of narcolepsy are most often associated with brain tumors and inherited diseases, followed by head trauma. Cases associated with vascular diseases, degeneration, and autoimmune/immune-mediated diseases have also been reported. A review of these cases, especially those involving brain tumors, clearly shows that the hypothalamus is often involved. Several cases of symptomatic cataplexy (without EDS) have also been reported. In contrast, symptomatic cataplexy often appears to be associated with nonhypothalamic structures.

It was recently shown that the pathophysiology of idiopathic narcolepsy is linked to hypocretin ligand deficiency. CSF hypocretin-1 levels were also measured in a limited number of symptomatic cases of narcolepsy/EDS. Reduced CSF hypocretin-1 levels were seen in most symptomatic cases of EDS with various etiologies. EDS in these cases is sometimes reversible with improvement in the causative neurological disorder and improvement of hypocretin status. Some symptomatic EDS cases (with PD and thalamic infarction) do not appear to be associated with hypocretin ligand deficiency.

Since CSF hypocretin measurements are still experimental, cases with sleep abnormalities/ cataplexy are habitually selected. Therefore it is still not known whether all or a large majority of cases with low CSF hypocretin-1 levels and CNS involvement exhibit EDS/ cataplexy.

Occurrences of cataplexy in idiopathic narcolepsy cases are tightly associated with hypocretin ligand deficiency. However, this link is less clear in symptomatic cases. Since none of the acute and subacute symptomatic patients (such as those with MS, GBS, or ADEM) with undetectable CSF hypocretin-1 levels developed cataplexy, chronic hypocretin deficiency may therefore be required to express cataplexy.

Regarding hypocretin deficiency among immune-mediated neurological conditions, hypocretin deficiency with hypothalamic lesions was noted in some MS and ADEM patients. In contrast, no clear local lesions were noted when hypocretin deficiency was found in GBS and Ma2-positive paraneoplastic syndromes. It thus appears that hypocretin ligand deficiency in GBS and paraneoplastic syndrome may be more selective, possibly at the cellular level, and the mechanisms involved in these conditions should be studied further.

Finally, further studies of the involvement of the hypocretin system in symptomatic narcolepsy and EDS would be helpful in understanding the pathophysiological mechanisms of EDS and cataplexy. CSF hypocretin-1 measurements may also be useful for elucidating treatment options such as wake-promoting compounds, anticataplectic medications, and ultimately hypocretin agonists when they become available.

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22
Hypocretin Measures in Psychiatric Disorders

Ronald M. Salomon, MD

1. INTRODUCTION

Hypocretins participate in the regulation of sleep and wakefulness in narcolepsy and play an unclear role in other disorders. Properties of hypocretins (orexins) and their interactions will provide promising methods for studies of other systems and circadian rhythm regulation, for which sleep is the quintessential marker. Findings of diminished hypocretin function in narcolepsy will help explain the neurochemistry of daytime somnolence, sleep paralysis, hypnogogic hallucinations, and cataplexy. Studies of hypocretin interactions with other transmitter systems will allow an extension of the narcolepsy findings to our understanding of the neurochemistry of other disorders that cause similar symptoms. This will require the mapping of specific hypocretin-related symptoms to interactions with other homeostatic transmitter systems in the brainstem and basal ganglia, interactions that will be analyzable in a time-sensitive context since hypocretin is released in a circadian rhythm from a small cluster of neurons. Hypocretins are synthesized in a circumscribed set of neuronal cell somas in the posterior hypothalamus, transported along the axon, and released under central control across widely distributed terminals. This contrasts with most other central nervous system (CNS) transmitter substances, which are either synthesized in the terminals or in multiregional, dispersed cell somas, and whose release cannot be synchronized or coordinated by activity of a central nucleus.

The importance of this hyprocretin property extends beyond its direct effects, since circadian regulation of hypocretin release into cerebrospinal fluid (CSF) can be used as an outcome marker, not only as an independent variable affecting other systems. By contrast, corticotrophin-releasing factor (CRF) is synthesized in many different brain regions, making it difficult to study the CRF functions of a particular region. Regional rhythms of CRF function will be more difficult to detect in CSF. Hypocretin neuron clustering is an important property for parsing clinical effects because the entire cluster can be investigated *en masse*, acting in a temporally coordinated pattern. This is essential to the study of behaviors that follow circadian rhythms, including sleep, appetite, and normal daily activity patterns. This property will affect study designs and interpretations for other disorders and hypotheses of network interactions. This chapter discusses core features of several prevalent mental disorders, their overlap with narcolepsy symptoms, and then the relevance of specific hypocretin findings. The potential importance of nonclassical, pharmaco-chronobiologic mechanisms is discussed.

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2. HOW WILL HYPOCRETIN ROLES AND INTERACTIONS IN PSYCHIATRIC DISORDERS BE UNDERSTOOD?

Classical neurochemical methods associate momentary neurotransmitter levels or functional states with various mental symptoms or states, which are themselves potentially momentary events in the lifelong course of any given mental disorder. Outcome measures can be transmitter or metabolite levels, receptor densities, binding or displacement, or effects on hormone release. These can be determined in tissues or various fluids as average levels over time, peak levels, or simple statistical variance. The latter will be influenced by basal firing rates, densities of peak firing periods, and predictability of rhythmic functional bursts. Second-messenger events for a given transmitter will vary among cells and receptor subtypes. Additionally, all these factors may vary with dynamic patterns, circadian or ultradian cycles (perhaps "pharmadian") rhythms, reflecting temporal fluctuations in transmitter release, receptor binding, terminal density, and effective linkages. This becomes a little more accessible if the transmitter is synthesized and released in a coordinated, rhythmic pattern from a single brain region so that pulsating characteristics can be measured in CSF. Hypocretin interactions provide a unique opportunity for study, arising from a circumscribed hypothalamic region and producing behavioral symptoms.

As is discussed below, losses of hypocretin neurons affect other nearby hypothalamic cell clusters, thalamic regulatory nuclei, and brainstem monoaminergic nuclei (cholinergic, histaminergic, noradrenergic, serotonergic, and dopaminergic centers), in addition to affecting hippocampal, cortical, and spinal regions. Primitive homeostatic attention and decision processes, (functions that require vigilance and regulate food intake) are controlled by hypocretins through interactions with other systems. Nearby circuits in the hypothalamus and thalamus filter inputs among various sensory stimuli (representing the external environment) or internal stimuli (homeostatic, memory) while controlling output via motor expression and hormone secretion. These areas also ensure sleep as a cover during circuitry-maintenance downtime (associative processing) and provide wakefulness during interactions with the environment. It is no coincidence that we find hypocretin cells, which are associated with sleep, energy, and nutrition, at the anatomical crossroads of this information exchange. But how do hypocretin interactions behave in the context of psychopathology?

The Diagnostic and Statistical Manual (DSM-IV, 1994) *(1)* organizes contemporary definitions of clinical syndromes. It is operationalized for clinical diagnosis but is not always the most useful conceptualization for associating momentary glimpses of symptoms with even briefer neurochemical functions. There are several problems with efforts to relate individual DSM diagnoses with single neurochemical systems. First, symptoms overlap frequently among theoretically disparate clinical syndromes, and many "separate" syndromes can be, and frequently are, comorbid. Second, altered neurochemical systems are more likely tied to a symptom rather than a syndrome, as shown by effects of agonists and antagonists. Third, individual neurochemical phenomena generally have not correlated consistently with syndromes or even individual symptoms, but rather have to be factored with the momentary status of other neurochemical phenomena to understand behavioral expression. For example (discussed further below in Subheading 4.2.), effects of intracerebroventricular (icv) hypocretin differ depending on the time of administration in the circadian cycle *(2)*. Finally, key diagnostic symptoms may be hidden among generalized malaise. Indeed, the nearly pathognomic symptoms of narcolepsy are often either not elicited on exam or are mistakenly attributed to another disorder. The differentiation of narcolepsy from other clinical disorders

sheds light on the above arguments regarding the impact of hypocretin interactions with other transmitter systems.

3. MAJOR DSM SYNDROMES AND THEIR CLINICAL DIFFERENTIATION FROM NARCOLEPSY

3.1. Schizophrenia

A salient feature of the schizophrenias is a prominent deficit in higher thought processing. All four of Bleuler's classically described core symptoms (loosening of associations, affective flattening, autistic features, and ambivalence) can be observed in each of the five subtypes of schizophrenia. Extreme loosening of associations is among the most valuable criteria but must be differentiated from similar symptoms in mania, impaired consciousness, fatigue, or distraction. Bizarre behaviors (posturing, mirror gazing, grimacing) are another key symptom, suggestive of a disturbed integration of reality, and can include true catalepsy *(not cataplexy)*. However, schizophrenia has no pathognomic single symptom, and all symptoms may be observed in at least one other disorder. Dominant distinguishing symptoms in terms of frequency include lack of insight, auditory and verbal hallucinations, ideas of reference, suspiciousness, flat affect, voices addressing the patient, delusions, poor verbalization of problems, and thought alienation, but many of these more specific symptoms will dominate in only one of the subtypes and are not as descriptive of other subtypes.

The differential diagnosis of paranoid schizophrenia from narcolepsy *(3)* can be missed given the similarity between hypnogogic hallucinations and psychotic symptoms, sometimes resulting in the incorrect assignation of a psychotic disorder. A mixture of dreamlike/nightmarish distortions and cognitive deficits can be present in both disorders, and although, in narcolepsy, insight should remain intact, it is sometimes difficult to establish in younger patients. Additionally, sleep disruptions *(4)* and social withdrawal are common to both disorders. Heuristically, one could describe psychosis as an overpowering of the filters that normally discern reality by screening out random cortical inputs. There is a loss of "insight" (a diminished ability to differentiate illusion from delusion) and an absence of the restorative sleep that normally shields these perceptions from appearing at all, let alone during consciousness. In one interpretation, the schizophrenic experiences wakefulness during oneroid (dreamlike) states and assigns "reality" to the distortions. As the symptoms worsen in severity, the dreamlike quality becomes nightmarish and bizarre. When narcoleptic patients are given antipsychotics, the resulting sedation and near delirium can appear even more psychotoform, sometimes perpetuating the misimpression.

3.2. Depression

Salient features of the depressions, as chronicled in the DSM-IV, include subjective reports and clinical observations of prevalent sadness with diminished motivation and interest in pleasurable activities *(1)*. Altered sleep, derisive self-percepts (guilt, hopelessness, and worthlessness), decreased energy and complaints of fatigue, poor attention and concentration, altered nutritional and sexual appetites, motor symptoms, and intense self-destructive urges (fiscal, social, or bodily nihilism) are also frequently observed. Depressive affect—the expression of underlying emotion—is blunted and diminished, with poor vitality, a limited range, and slow changes. Failures of emotional flexibility, sometimes in the face of stress, sometimes unprovoked, can be characterized as loss of healthy emotional resilience. Unipolar patients describe a sense of feeling "stuck" in one mood, being unable to experience normal

changes in mood. Bipolar patients often describe uncontrollable swings from one "stuck" position to another. Individuals with more rigidly controlled daily routines may be vulnerable to depression when the routines are disrupted. Normally, rapid revisions of percepts aid in the recovery from singular interpretations of the self and of the outside world, but in depression these revisions are not forthcoming—the percepts become frozen. The pronounced behavioral responses in depression (changes in appetite, sleep, and the other factors just noted) may be viewed as diminished healthy variability and feedback response in dynamic homeostasis.

Depressive episodes present a challenge in the differential diagnosis with narcolepsy, since both often present clinically as pervasive fatigue, and patients rarely complain spontaneously of the sleep paralysis, hypnogogic or hypnopompic hallucinations, or even infrequent attacks of cataplexy that would raise suspicion of a primary sleep disorder. Depression is frequently comorbid with narcolepsy, both often produce weight gain, and both may improve with tricyclic antidepressants. The antidepressant effect of sleep deprivation, during which hypocretin release is enhanced, also suggests a link between depression and the low hypocretin states of narcolepsy *(5)*. However, this argument has a caveat: improved mood in sleep-deprived narcoleptics may be difficult to appreciate. Sedating antidepressants may worsen depressive symptoms and overall function in narcolepsy, whereas activation, possibly via hypocretin, by dopamine reuptake inhibitors or psychostimulants may be beneficial.

3.3. Substance Abuse

Substance use disorders encompass a very wide range of preferred substances, outcomes, and motivational sets. Their salient features include a voluntary or compulsive use of substances to induce ill-fated outcomes, often distorting perceptions and/or leading to compulsive unhealthy behaviors. This can also occur, paradoxically, in schizophrenic patients since additional distortions are not always worse than the original state, and some patients report temporary relief from their tormenting illness by overriding with another distortion. "Reward mechanisms" have been linked to dopaminergic mechanisms. Like hypocretins, drugs of abuse alter the depth of sleep or arousal states. The abused substance makes fluctuations in mood and cognitive states an elective event under the control or compulsive dyscontrol of the abuser. Abused substances often have profound effects on sleep and appetite.

The differential diagnosis with narcolepsy may be masked by effects of the substances, limiting the possibility of recognizing underlying narcolepsy. Stimulant abuse (amphetamines, methylphenidate, crystal meth, or cocaine) may be especially attractive to individuals with narcolepsy. Sedative abuse may mask narcolepsy by giving the clinician a parsimonious but only partial explanation for the somnolence. Even if narcolepsy (or the lesser, but in some ways similar, syndrome, Attention Deficit Disorder) is suspected, most clinicians avoid treating historically abusing individuals with stimulants because of a concern that these individuals may be more prone to abuse of prescribed drugs. The current availability of biological assays for narcolepsy may allow controlled studies to examine this belief, since it is possible that adequately medicated narcoleptic patients will not be prone to abuse their medications. Further study of this relationship is needed.

3.4. Other Disorders

Salient features of the impulsive, obsessive, eating disorder spectra of illnesses bind together a group of compulsive behaviors that are associated with uncontrolled anxiety, fear, or dread, or with undirected energies. Clinical differential diagnosis should not usually be

challenging, other than a relatively easily made distinction between the weight gain in some normally active bingeing or bulimic patients and the weight gain observed owing to low activity levels of narcoleptics. Problems in the differential may arise when advanced eating disorder patients present with complex secondary symptoms. Roles of the hypocretin system in the symptoms of these disorders also merit further study.

4. RELEVANT FINDINGS OF PRECLINICAL AND CLINICAL STUDIES

The hypocretin neurons are at a crossroad, interacting with numerous systems that have already been implicated in the physiology of mental illness. Evidence suggests that bidirectional hypocretin interactions exist with virtually all known neurochemical determinants of mental illnesses, including all of the centrally active monoamine pathways (dopamine, norepinephrine, and serotonin), histamine and acetylcholine pathways, and also peptide transmitters. The monoamine pathways interact closely with hypocretin neurons. Hypocretins excite serotonergic neurons in the dorsal raphe *(6)*, noradrenergic neurons in the locus coeruleus *(7)*, and dopaminergic neurons in the ventral tegmental area *(8)*. Central hypocretin administration suppresses prolactin (PRL) and growth hormone (GH) release *(4,9)*, suggesting that, if it is acting alone, a deficit in hypocretin could be permissive for release. However, secretion of both PRL and GH may be altered in narcolepsy. In a comparison of four drug-free males with narcolepsy with four healthy controls, plasma drawn every 20 min through a 24-h fasting period showed a markedly diminished GH peak at sleep onset in narcolepsy, diminished PRL in three of the four narcoleptic patients, and normal circadian periodicity of cortisol secretion in all four patients *(10)*.

Plasma drawn every 10 min from seven hypocretin-deficient narcoleptic patients and seven healthy controls (matched for age, sex, and weight) showed a loss of normal rhythms of GH release with less regular rhythms, despite normal basal and pulsatile GH secretion rates and a normal release response to the GH-releasing secretagogue, in the narcoleptics. Individuals with narcolepsy secreted twice the daily proportion of GH released during the daytime as their counterparts, with 25% daytime release in controls, but 50% daytime release in narcoleptic patients *(11)*. Hypocretins also stimulate sympathetic outflow and cortisol release, but the normal cortisol levels in narcolepsy suggest that at some level the hypothalamic-pituitary-adrenal (HPA) axis is not affected by hypocretin deficits *(7)*. Further research will be needed to assess the normalcy of CRF and adrenocorticotropic hormone (ACTH) release patterns in narcolepsy. In any case, future studies of neuroendocrine release will form a basis for understanding relationships between hypothalamic neuropeptides and the monoamine transmitter systems rising from the brainstem.

4.1. Symptoms of Schizophrenia

Symptoms of schizophrenia may be related to hypocretin effects, as suggested by preclinical findings of bidirectional interactions with dopaminergic neurons *(8)* and by observations that both dopamine (DA) and hypocretin enhance attention and wakefulness. Generally DA activates hypocretin, and probably vice versa as well *(12)*. A synergism with DA is evidenced by centrally administered hypocretin, which increases locomotion, stereotypy, and grooming, and is blocked by D_2 (haloperidol) and D_1 antagonists (SCH23390) *(12)*. The tendency toward weight gain in schizophrenia, as seen also in narcolepsy, is consistent with a deficit hypothesis regarding behavioral activation by hypocretin. However, elevated hypocretin might equally cause excessive behavioral activation and could just as easily be interpreted as agitation causing increased feeding behaviors. Overall, however, schizophrenic patients show

normal levels of hypocretin *(4,13)*. Among 13 schizophrenic patients, but not among 12 controls, higher hypocretin was associated with longer wakefulness *(10),* implying that schizophrenic patients may be more dependent on hypocretin for, or more likely to stimulate hypocretin in the context of, prolonged wakefulness than their healthy counterparts. Cerebrospinal fluid (CSF) hypocretin increases were also associated with longer sleep onset latency in untreated schizophrenia *(10)*.

Antipsychotic drugs, producing similar results to the amphetamine findings cited above, increased c-fos expression in hypocretin neurons *(14),* especially chlorpromazine, clozapine, olanzapine, and risperidone, but not haloperidol. Haloperidol-treated patients show 23% lower levels of CSF hypocretin compared with untreated schizophrenic patients *(15)*. In contrast, clozapine- or olanzapine-treated patients showed a bimodal clustering, with most subjects in the more elevated range of the "untreated" patients *(15)*.

4.2. Depressive Symptoms

Depressive symptoms may be related to hypocretin effects on several fronts. Sleep disturbances, both hypersomnias and insomnias, are prominent in depression. Hypocretin promotes wakefulness and increases grooming and face washing in rodents, whereas it suppresses REM sleep. A deficit of hypocretin could contribute to fatigue and hypersomnia, and to the shortened delay in REM sleep onset in depression, but is not as readily reconciled with the frequent insomnia. An excess of hypocretin might explain REM disturbances, but the hypersomnia would seem improbable. Appetite in depression can go either way and frequently fluctuates within depressed individuals over time. Increased food intake is observed with icv injections of Hcrt *(16,17)* and may be related to increased wake time, but this has been argued to be a relatively weak effect, dependent on the circadian time of administration *(2)*. Hypocretin knockout mice have normal weight *(18)*, but ataxin-3 mutants lacking hypocretin cells have increased weight *(19),* and decreased hypocretin production is linked to obesity in narcolepsy *(20)*. Since hypocretin increases the metabolic rate, it may be that it is activated by starvation and inhibited by satiety *(21)*. Evidence of an interaction between antidepressants and the hypocretin system has been observed after neonatal exposure to clomipramine, which later resulted in decreased levels of hypocretin-2 *(22)*. Antidepressants are useful in the treatment of narcolepsy. Although these findings do not directly implicate hypocretin in depression, a potential for finding faulty interactions of other neurotransmitter systems with hypocretin neurons is invoked by the marked overlap in effects of hypocretin and the symptoms of depression.

Circadian neurochemistry has been studied in rodents but is difficult to achieve in humans. Serotonin has been strongly implicated in psychopathologies including depression and is released in circadian patterns. The raphe cell firing rates and serotonin release are greatest during wakefulness, lower during non-rapid eye movement (REM) sleep, and cease during REM sleep. Hypocretin levels increase in rats during the second half of the activity period *(23,24)*. Hypocretin-1 release is higher during REM than during slow-wave sleep (SWS), especially in the hypothalamus and basal forebrain *(25)*. In active waking periods (taken in 2-h blocks, using intersample intervals of 10 min) CSF levels of hypocretin-1 were 67% higher than in quiet waking periods *(25)*. It appears that in both sleep and waking states, hypocretin may be associated with greater activity. Further study of the relationship between circadian neurochemistry and hypocretin cells is needed.

From a neuroanatomical perspective, as stated earlier, hypocretin makes an ideal probe for circadian rhythm because peptide synthesis is somatic (not at terminals), transport is axonal,

Table 1
Hypocretin-1 Levels in Depression[a]

Group	Mean 24-h concentration (0800–0700 h)	Daytime concentration (1100–1800 h)	Nighttime concentration (2300–0600 h)	Day/night difference
Controls	264.99 ± 11.00	251.70 ± 11.54	273.98 ± 10.64	22.28 ± 5.03
All depressed				
Before treatment	281.22 ± 11.80	275.78 ± 11.45	280.91 ± 12.17	5.12 ± 4.95**
After treatment	252.45 ± 8.59†	245.04 ± 10.14†	253.04 ± 7.94†	8.00 ± 6.52
Responders				
Before treatment	289.66 ± 13.52†	283.44 ± 14.16	288.34 ± 13.96†	4.90 ± 6.82
After treatment	254.33 ± 11.26	246.27 ± 14.58	255.64 ± 10.50	9.37 ± 10.33
Nonresponders				
Before treatment	268.57 ± 21.96	264.30 ± 19.82	269.76 ± 22.96	5.46 ± 7.70
After treatment	249.63 ± 14.47	243.19 ± 14.44	249.14 ± 13.10	5.96 ± 6.35
Sertraline				
Before treatment	290.86 ± 12.53*	282.62 ± 13.40*	290.76 ± 12.57*	8.15 ± 6.36
After treatment	251.08 ± 10.33	242.39 ± 12.69	249.75 ± 94.5	7.36 ± 9.67
Bupropion				
Before treatment	261.94 ± 24.81	262.11 ± 22.31	261.19 ± 26.42	–0.92 ± 7.81
After treatment	255.18 ± 17.08	250.32 ± 18.56	259.61 ± 15.62	9.29 ± 5.02

[a]Average 24-h levels from high-frequency sampling revealed slight, nonsignificantly elevated hypocretin-1 level in the depressed group and a significant decline in hypocretin-1 after 5 wk of sertraline ($n = 10$) treatment but not following bupropion ($n = 5$). Data are mean ± SD.

*Significant ($p < 0.05$, paired t-test) drug effects before and after treatment. †Significant ($p < 0.05$, paired t-test) effects from before and after treatment. **Different from controls ($p < 0.05$, t-test).

and release is coordinated by firing of the hypothalamic cell. This differs from findings from corticotropin-releasing hormone (CRH), providing a negative control for the rhythms found in the hypocretin levels. CRH neurons are not geographically circumscribed, and no significant ultradian synchronization and coordination of release was observed in serial CSF sampling (Loosen, Kennedy, Johnson, and Salomon, 2002, unpublished data). Even if we cannot show regional circadian variability for CRH because of the pooling of diverse regions' outputs in CSF, the effects of diminished circadian changes would be expected to be sizable for CRF.

For this reason, and to evaluate further the general properties of hypocretin function in depression, we assayed hypocretin-1 in samples from 24 h of CSF production in 10-min aliquots from 15 depressed patients, with a comparison posttreatment sampling after 5 wk of randomly assigned sertraline or bupropion *(26)*. A group of 14 control subjects was also studied in similar fashion, but only once and without medication. The 24-h levels achieved from averaging the high-frequency samples revealed a slight, nonsignificant elevation of hypocretin-1 levels in the depressed group (Table 1) and a significant decline in hypocretin-1 after sertraline treatment ($n = 10$) but not following bupropion ($n = 5$).

More interestingly, compared with controls, a significant loss of circadian amplitude was observed in depression, and this amplitude was enhanced by treatment (Fig. 1). Peak CSF concentrations of hypocretin-1 were observed shortly after midnight, an unexpected timing for the peak since the hypocretins were expected to be elevated during active times. In rodents, the peaks in cisternal or microdialysis samples were observed at the end of the active

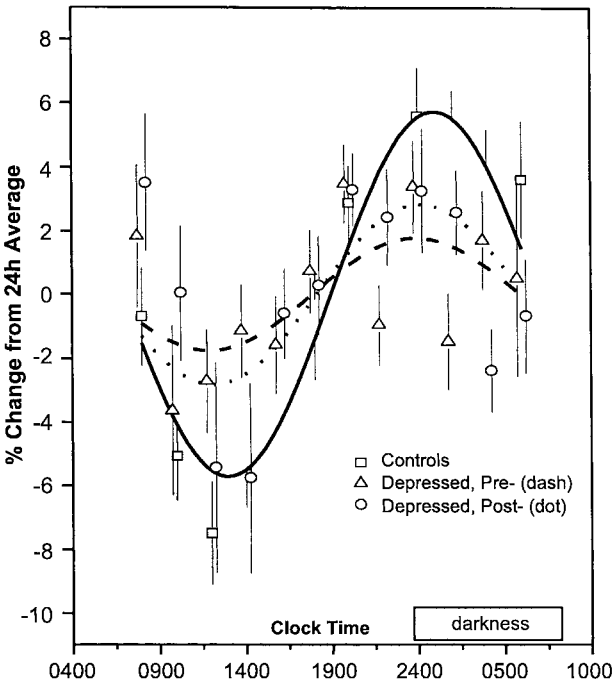

Fig. 1. Lumbar CSF hypocretin-1 levels measured every 2 h with fitted sinusoids, for control volunteers (square/solid line), depressed patients (triangles/dashed line), and the same patients after 5 wk of bupropion or sertraline treatment (circles/dotted line). Mean with SEM bars.

phase *(23,24)*. Squirrel monkeys also peak in cisterna magna CSF at the end of the active phase, just before dark *(27)*.

Contributing factors to explain this finding may include the sampling procedure and environment, the rate of CSF flow and distance to the lumbar collection site, and sleep disruptions in the course of the collection procedure. Subjects in our study were confined to bed rest, but were not in absolute darkness (for safety reasons) and were awakened briefly at 2 AM for temperature monitoring. Hand held lighting was allowed for sample collection every 30 min (capping samples and moving them to the dry ice chest) and safety monitoring once during the night (temperature checks). Most patients slept fairly well, but a few had uninterrupted sleep (especially during their first studies, while depressed) so the late activity period surge of hypocretin, hypothetically opposing sleep debt, may have been delayed. No association between hypocretin levels and individual sleep reports was observed.

Additionally, CSF passage along the spinal canal delayed collection from the lumbar sac, consistent with transit time estimates of about 2 h (based on observations of CSF 5-hydroxyindoleacetic acid changes during tryptophan depletion; Salomon, unpublished data). Spinal hypocretin terminals might support appreciable local release but would be unlikely to explain the delay in the acrophase since release is likely to be synchronous with firing activity in the hypothalamic hypocretin neuron soma. Axonal transit time for hypocretin should only be a factor if synthesis is blocked (depleting the terminal) and then released.

Finally, CSF laminar flow and mixing was expected to diminish the measured peak amplitude by dilution. Greater amplitudes, as well as higher peak levels, were observed in the fluctuations of squirrel monkeys sampled from the cisterna magna (*see* ref. *26* for further discussion).

The hypocretin circadian findings may be representative of diminished overall neuronal rhythmicities in depression. This is quite clinically expectable, given the blunted circadian behaviors (sleep and feeding) that are clinically observed in depression. It may imply, further, that the neuroendocrine findings of blunted GH variability in narcolepsy (discussed above in Subheading 4.1.) are not solely owing to diminished absolute levels but may also be related to an absence of variability in any persisting release of hypocretin and subsequent pulsatile receptor activation.

4.3. Substance Abuse Symptoms

Substance abuse symptoms and hypocretin effects share common ground in motivated behaviors and reward pathways of the brain. Hypocretin receptors in the dopaminergic ventral tegmental area, the noradrenergic locus coeruleus *(28)*, and the nucleus accumbens *(29)* ensure an influence of hypocretins on neurochemical responses to abused substances. Human opiate addicts show erratic circadian rhythms in their sleep and eating, with poor motivation for feeding. Chronic amphetamine administration increases intracellular cAMP in hypocretin neurons, which activates a response element binding (CREB) protein *(30)* and, in a subpopulation of hypocretin cells, cFos *(14)*.

The relationship between substance abuse and circadian rhythms is interesting, and the following speculations are based on preclinical data. Circadian clock afferents from the heterosynchronous subsets of suprachiasmatic nucleus (SCN) cells *(31)* project to *(32)*, and receive feedback from, hypocretin neurons. This lessens any expectation that the posterolateral hypothalamic populations should be perfectly synchronous on a circadian cycle: mapping efferents from individual cells in the SCN might differentially control hypocretin cells. Desynchonizations of circadian phase functions among SCN-connected neuronal populations may account for an appearance of cell-type heterogeneity among hypocretin neurons, but it may be pseudo-heterogeneity since the representative functions may simply be out of phase.

Systemic administration of exogenous opiates might artificially desynchronize circadian systems, causing abnormal behavioral responses. For example, this mechanism might explain the finding that hypocretin knockout mice show attenuated withdrawal from morphine *(30)*. An absence of hypocretin could impede circadian synchronicity. In the knockouts, an abnormal lack of distinct synchronized subpopulations might allow a more temporally homogeneous and less pronounced symptom set with fewer severe periods during the circadian cycle. Further study will be needed to ascertain whether hypocretin functional deficits may be expressed behaviorally as a disturbance of circadian synchronicity, an effect that could be additive with substance abuse in this model, given the substance-induced CREB and cFos responses in hypocretin cells.

In others words, the exogenous substances might disrupt normally phasic, distributed clusters of cell subpopulations, leading to a temporal homogeneity in which time and circadian events become bland and indistinct, lacking the dominance of an SCN-provided (or exogenous redosing) stimulus. If the hypocretin deficit interferes with loops conveying SCN rhythms *(33)*, the withdrawal phenomena could be distributed more evenly over time and could appear to produce less severe withdrawal symptoms. Numerous scenarios will need study before we conclude that these peptides, which have marked “pharmadian” characteristics, are simply driving a downstream system in one single direction or another at all times.

5. CONCLUSIONS

Disrupted circadian behaviors are prominent in many preclinical studies of hypocretin effects, and also in psychiatric patients, as well as patients with narcolepsy. The commonalities suggest that interactions among homeostatic systems lose circadian patterns in some disorders. Circadian patterns may be easier to study with hypocretins, which show a strong circadian variability in healthy subjects that appears to be blunted in depression. The ability to identify changes in neurochemical rhythms will offer a potentially important mechanism for explaining dysfunction in psychopathology and for seeking novel treatment approaches.

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Neuroendocrinology of Human Narcolepsy

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1. INTRODUCTION

Neuroendocrine and metabolic disturbances have been postulated to accompany or even cause narcolepsy ever since the first part of the 20th century. In 1924 Redlich *(1)* hypothesized that pituitary function is disturbed in patients with narcolepsy, in 1934 Daniels *(2)* described an association with obesity, and in 1957 Yoss and Daly *(3)* discussed (and questioned) the fact that hypothyroidism was frequently diagnosed in narcoleptic humans. However, these early observations were made at a time when narcolepsy was not clearly defined. Sleep apnea, for example, was not recognized as a separate disease entity. Moreover, determination of the plasma concentration of many hormones was impossible. The first (neuro) endocrine studies in narcoleptic humans were carried out in the second part of the 20th century, focusing on circulating levels of prolactin growth hormone (GH), and cortisol *(4–6)*. Unfortunately, these studies were all hampered by methodological shortcomings and/or immaturity of techniques. In the course of time, analytical techniques have greatly improved, and only recently mathematical methods were developed that allow quantitative appraisal of hormone secretion rates and mapping of pulsatile hormone release patterns *(7)*.

After the discovery of the hypocretin/orexin system, with its dense projections to nuclei involved in the control of neuroendocrine networks, numerous studies have addressed the metabolic and endocrine effects of hypocretin peptides in laboratory animals. It is now recognized that hypocretins increase arousal and stimulate feeding behavior, locomotor activity, the sympathetic nervous system, and energy expenditure *(8–11)*. The subsequent discovery that narcolepsy is caused by hypocretin deficiency revived interest in the quest for associated endocrine and metabolic anomalies *(12–14)*. Surprisingly, only a limited number of studies in animal models of narcolepsy address this issue *(15,16)*.

Various clinical studies evaluated the metabolic and endocrine status of narcoleptic humans. Body mass index (BMI) and leptin, a hormone known to be important in the regulation of feeding behavior and energy expenditure, are the focus of attention in a number of papers. The association between narcolepsy and obesity alluded to earlier was confirmed, and a specific role for hypocretin deficiency in the pathogenesis of body weight gain was suggested. This issue is discussed in the first part of this chapter.

To date, only one series of neuroendocrine studies has been performed in a well-defined group of hypocretin-deficient narcoleptic humans and matched controls. In these studies, the circadian plasma concentration profiles and secretion rates of leptin, GH, adrenocorticotropic

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hormone (ACTH), cortisol, thyroid-stimulating hormone (TSH), T3/T4, and luteinizing hormone (LH) were mapped. The findings and interpretations from these neuroendocrine studies are described in the second part of this chapter. The data not only provide insight in to the endocrine features of narcolepsy but also unveil neuroendocrine actions of hypocretin peptides.

2. BODY WEIGHT AND FOOD INTAKE

In 1934, Daniels *(2)* was the first to suggest that narcolepsy is associated with obesity. He observed that narcoleptic patients with or without cataplexy were more frequently obese than was expected in the general population. He proposed that a reduced basal metabolic rate was involved in the pathogenesis, based on metabolic studies in several of these patients *(2)*. During the next decades interest in this aspect declined. It was generally believed that daytime naps reduce physical activity and energy expenditure in narcoleptic patients. This point of view was challenged by the finding in the late 1980s that most narcoleptic patients do not spend more hours asleep than healthy controls and that their physical activity over 24 h is merely distributed otherwise rather than less *(17)*.

Only a few studies focusing on food intake, body weight, body composition, or metabolism were performed before we entered the "hypocretin era," and the interpretation was often unclear because of methodological shortcomings. In 1976, Bell *(18)* used a food questionnaire to conclude that narcoleptic patients eat more than normal and even crave food. However, the diagnostic criteria for narcolepsy were not clearly defined, controls were not matched for age or sex, and the use of medication was allowed but not specified. In 1990, Pollak and Green *(19)* studied the relationship between feeding and subjective alertness in drug-free narcoleptics and controls living without temporal clues. They incidentally touched on their finding that the narcoleptics ate less than the controls, even though they had a higher BMI. In 1996, we took a cross-check dietary history of 12 drug-free, nondepressed narcoleptic patients with clear-cut cataplexy and 12 matched controls *(20)*. In spite of the fact that they were more obese, the patients consumed fewer calories, in particular fewer carbohydrates.

As dietary studies are notoriously prone to bias when food intake is not quantified directly, these data were interpreted with caution. If they are true, however, the only way to explain the findings is to assume that narcoleptic patients have a lower metabolic rate. Interestingly, shortly after publication of our paper, the data were corroborated by animal studies. Ventricular hypocretin-1 injections during the light phase acutely increased oxygen consumption and the respiratory quotient through an apparent increase in carbohydrate metabolism in rats *(9)*. Furthermore, the orexin/ataxin-3 mouse, a narcoleptic animal model marked by progressive loss of hypocretin neurons, is hypophagic and obese *(16)*.

A number of recent studies carefully evaluated body weight of narcoleptic patients *(21,22)*. The association between narcolepsy and obesity was clearly confirmed in all these studies. In addition, circulating leptin levels appeared to be relatively low in narcoleptic patients, despite their increased body weight *(23)*. As leptin is critically involved in the control of energy balance and leptin deficiency leads to severe obesity in mice and humans *(24,25)*, it was suggested that relative hypoleptinemia may be involved in the pathogenesis of obesity in narcoleptic humans. It has also been proposed that hypocretin deficiency plays a role. A first hint came from the observation that narcoleptic patients who still have measurable hypocretin levels in their spinal fluid (a small minority of the total patient population) tended to be less obese than patients with explicit hypocretin deficiency *(26)*. A subsequent study in a large group of typical narcoleptic patients, known to have undetectable hypocretin peptides in their cerebrospinal fluid (CSF) in more than 90% of cases, and in equally sleepy controls with

normal hypocretin levels, corroborates this finding and suggests that hypocretin deficiency *per se* promotes body weight gain *(27)*. Hypocretin-deficient narcoleptics were significantly heavier than age- and sex-matched controls, and the percentage of obese patients (BMI > 30 kg/m^2) was significantly higher in the narcoleptic group. The differences were not explained by differences in medication use. Although the study was cross-sectional, the data suggest that weight gain mainly occurs during the early phase of the disease. A new and striking finding was that the waist circumference of the narcoleptic patients was within the range that is clearly associated with increased risk of cardiovascular disease and diabetes mellitus.

3. NEUROENDOCRINE STUDIES

We describe here the principal results of a comprehensive study of the activity of various neuroendocrine ensembles in hypocretin-deficient patients *(28–31)*. Seven male narcoleptic patients with typical cataplectic attacks, typical multi sleep latency test (MSLT) findings, and undetectable hypocretin levels in the CSF were studied. They were compared with seven controls who were carefully matched for age, sex, BMI, and body composition. The participants were medication free. On average the subjects were overweight (BMI 28.3 kg/m^2), and the percentage of total body fat ranged from 12.9 to 30.4%. Plasma concentrations of leptin, GH, TSH, T3/T4, ACTH, and cortisol were measured in blood samples that were collected every 10 min for 24-h Circadian plasma profiles and hormone secretion rates were quantified using sensitive assays, pulse detection algorithms, deconvolution techniques, and cosinor fitting. Subjects remained sedentary during the studies, and standardized meals were served. Additional stimulation tests for GH and TSH were performed on a separate occasion. During the 24-h sampling procedure, sleep was recorded polygraphically, using an ambulant electroencephalographic (EEG) recording system.

3.1. Leptin

Figure 1 shows the 24-h plasma leptin concentration of all individual patients and their matched controls. Leptin levels in narcoleptic subjects were about half of those in normal controls during the full 24-h period. In all but one of the control subjects the serum leptin concentration displayed a diurnal rhythm, which was mathematically characterized by a cosine wave with an acrophase around 01:00–02:00 AM. In contrast, the normal nocturnal rise in leptin concentrations was absent in narcoleptic subjects.

These findings are in line with earlier papers reporting that leptin levels are low in narcoleptic humans *(23)*. However, the interpretation of the findings is difficult. As alluded to above, leptin is critically involved in the control of feeding and energy expenditure. Low levels are associated with food craving and reduced basal metabolic rate *(24,25)*. Thus, low circulating leptin levels should increase food intake rather than reduce it in narcoleptics. However, hypocretin peptides are part of the complex neural network that conveys leptin signals to the brain *(15,32)*. In particular, hypocretin levels in the brain rise in response to a reduction of circulating leptin levels, which supposedly stimulates food intake in this physiological context. Therefore, it is tempting to speculate that hypocretin deficiency attenuates the effect of hypoleptinemia on food intake.

The loss of circadian rhythmicity of plasma leptin concentrations may relate to the disruption of the diurnal pattern of sympathetic tone that has been observed in narcoleptic humans *(33)*. A recent study in rats has demonstrated that the diurnal rhythm in leptin secretion is generated by the suprachiasmatic nucleus *(34)*. The autonomic nervous system is probably part of the efferent pathway mediating central nervous systems effects on leptin secretion by

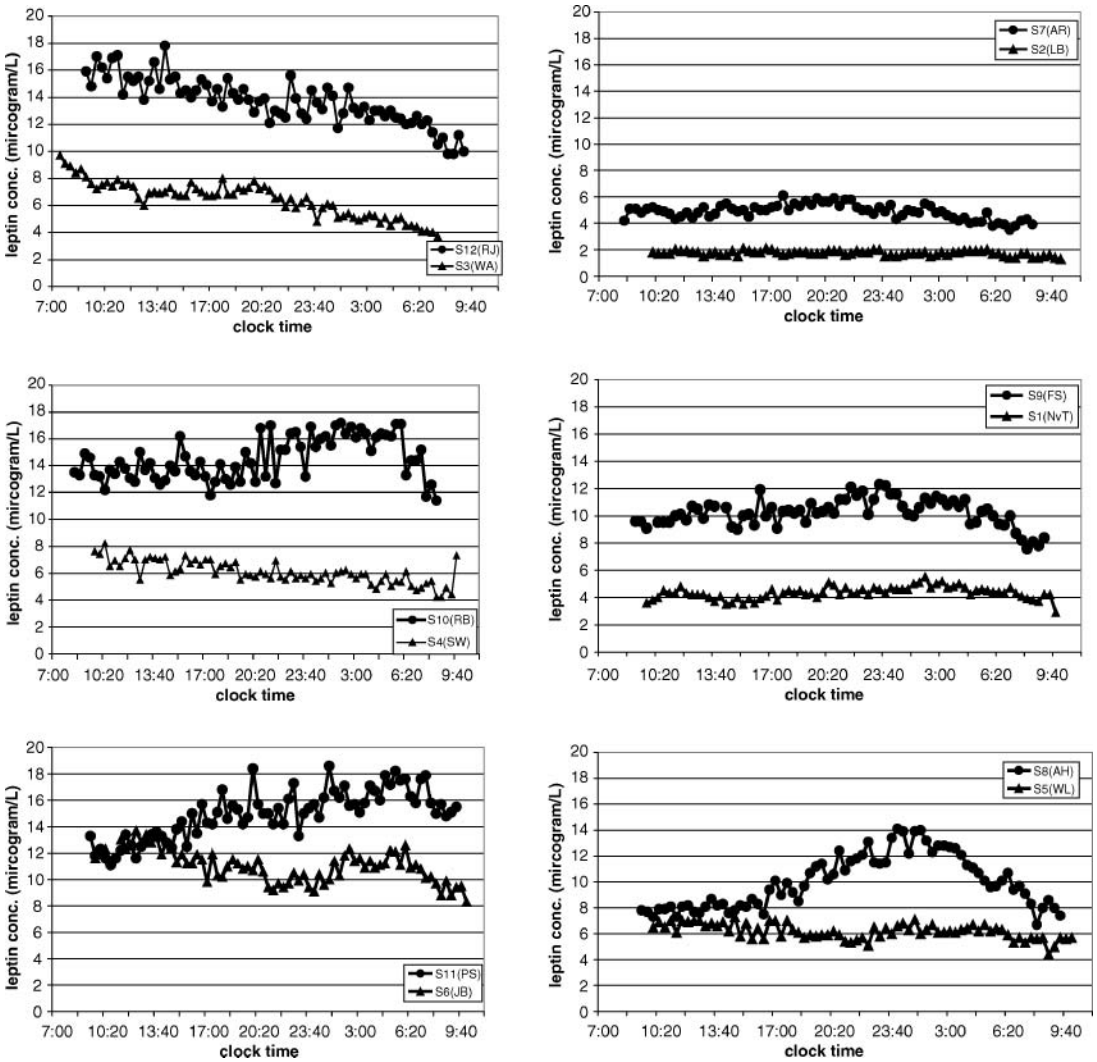


Fig. 1. Circadian rhythm of 24-h leptin plasma concentration in individual patients (triangles) and their matched controls (circles).

adipose tissue *(34)*. Anatomical and electrophysiological evidence supports a role of the suprachiasmatic neuron (SCN) in the regulation of sympathetic tone *(35)*. Since the SCN projects to hypocretin neurons *(36)*, and hypocretins clearly activate the sympathetic nervous system, we speculate that hypocretin neurons mediate part of the influence of the SCN on the circadian distribution of autonomic activity. If true, hypocretin deficiency would be predicted to disrupt the circadian rhythm of autonomic function, which may impact on leptin rhythmicity.

Alternatively, the narcoleptic pattern of sleep-wakefulness may be responsible for the disruption of the circadian rhythm of plasma leptin levels, perhaps through the absence of the normal sleep-mediated nocturnal reduction in sympathetic tone *(33)*. Recent data implicate sleep as an important regulator of plasma leptin levels, as evidenced by a considerable reduction in leptin concentration in response to sleep deprivation in healthy adults *(37)*. Also, the diurnal rhythm of leptin is sensitive to the phase shifting of sleep *(38)*. Sympathetic nerve activity normally decreases during the first hours of sleep in non-REM sleep stage IV, which

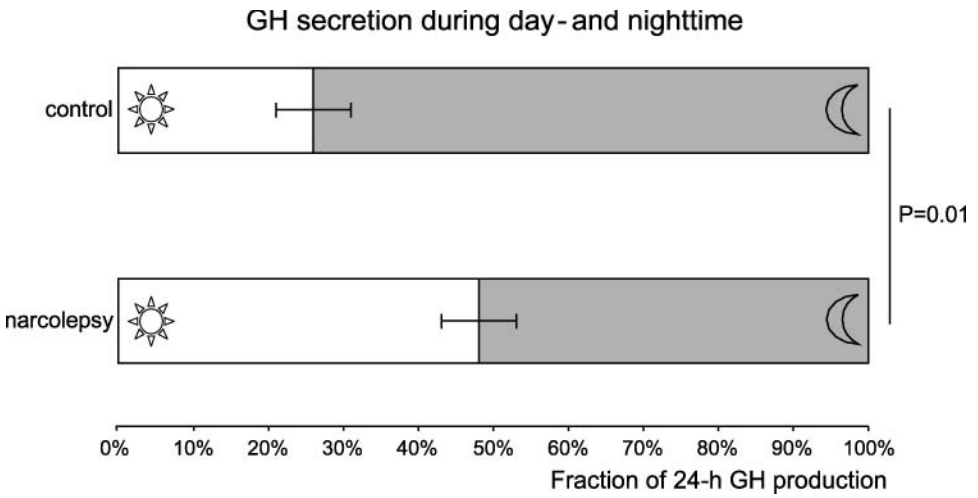


Fig. 2. Day- and nighttime growth hormone (GH) secretion expressed as a fraction of total 24-h secretion. Upper bar: controls. Lower bar: patients.

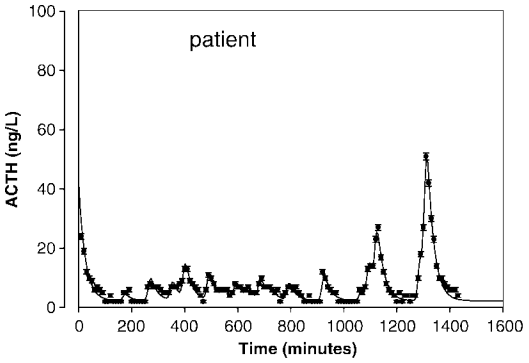
agrees with the timing of the nocturnal leptin peak *(39)*. Narcoleptic patients display a disruption of the circadian distribution of distinct sleep stages and fragmentation of sleep owing to failure to sustain steady levels of consciousness *(17)*. These alterations of sleep-stage distribution may contribute to the disruption of the circadian rhythmicity of plasma leptin levels, perhaps through their effects on sympathetic nervous system activity.

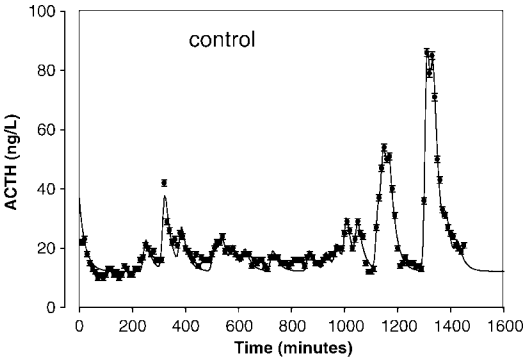
3.2. Somatotropic Axis

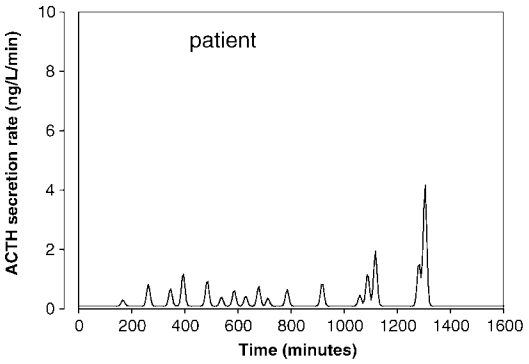
The 24-h mean serum concentration of GH was not significantly different in narcoleptic patients and controls. Patients with narcolepsy did not differ from controls with respect to basal secretion rate, total number of GH peaks, peak amplitude, or area. Consequently, the total production of GH was not different in both groups. However, the distribution of GH secretory events over 24 h differed considerably between groups. Narcoleptics secreted almost half of their total pulsatile production during the day, whereas controls produced only one-fourth during the daytime (Fig. 2). The total amount of GH released per day was strongly correlated with the total (24 h) amount of slow-wave sleep (SWS) in both controls and narcoleptics. GH release by the pituitary is primarily controlled by stimulatory impact of hypothalamic GH-releasing hormone (GHRH), which is counterbalanced by the inhibitory effects of somatostatin.

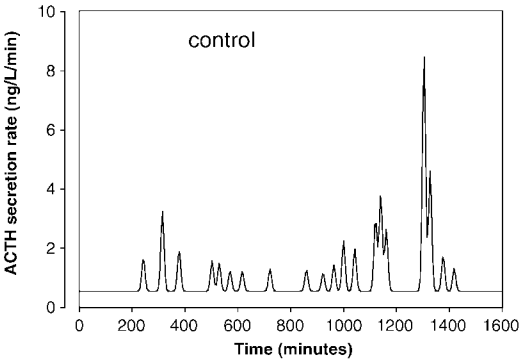
To evaluate the sensitivity of the pituitary somatotrophs to GHRH, we studied the GH response to iv GHRH injection. The narcoleptic patients secreted a similar amount of GH as the control subjects, suggesting that the somatotrope cells in the pituitary are normally sensitive to GHRH stimulation in these patients. Thus, the onset of nocturnal sleep remains an important correlate of GH release, and the relationship between SWS and GH secretion remains robust in narcoleptic humans. The dispersion of GH secretory events and sleep epochs over 24 h appears to be altered analogously in narcoleptic patients: they clearly had more (slow wave) sleep time and secreted more GH during the day.

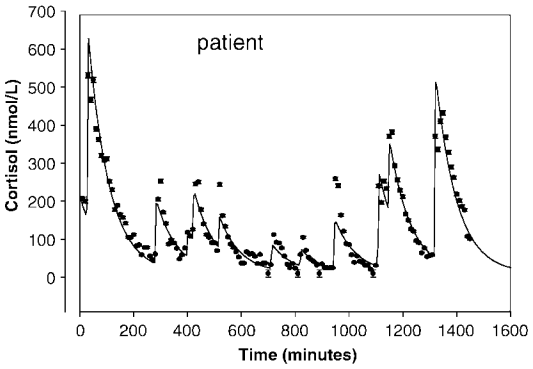
The most reproducible GH secretory event in humans occurs around nocturnal sleep onset, in close temporal association with the first epoch of SWS *(40)*. To explain this consistent finding, it has been suggested that GHRH simultaneously promotes sleep and GH release

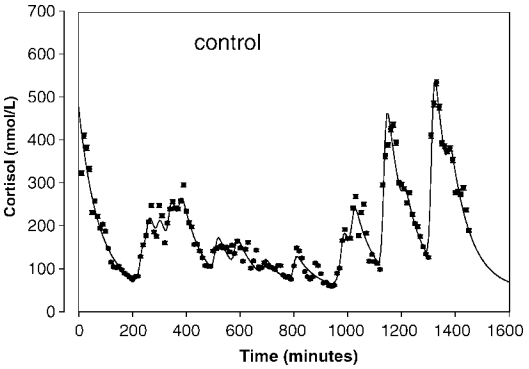

patient
ACTH (ng/L)
Time (minutes)

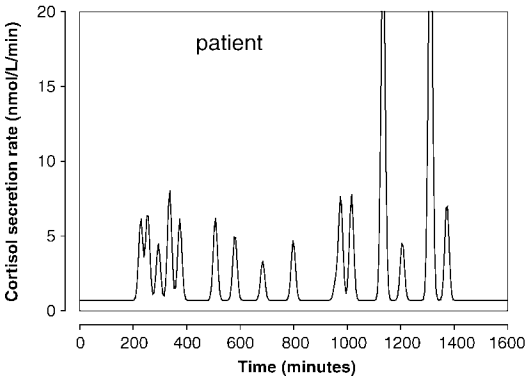

control
ACTH (ng/L)
Time (minutes)

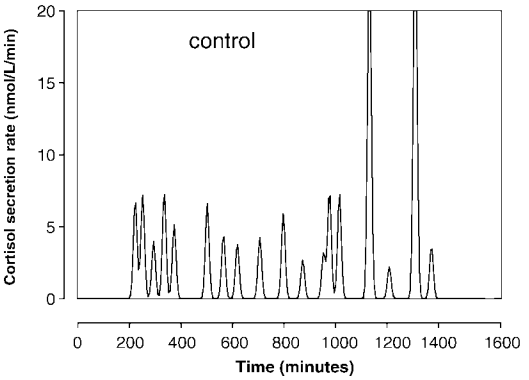

patient
ACTH secretion rate (ng/L/min)
Time (minutes)


control
ACTH secretion rate (ng/L/min)
Time (minutes)


patient
Cortisol (nmol/L)
Time (minutes)


control
Cortisol (nmol/L)
Time (minutes)


patient
Cortisol secretion rate (nmol/L/min)
Time (minutes)


control
Cortisol secretion rate (nmol/L/min)
Time (minutes)

(*see* ref. *41* and references herein). This dual regulatory effect of GHRH is probably intact in narcoleptics, as SWS and GH release appear to remain closely associated in these patients. Thus, the circadian distribution of hypothalamic GHRH release may be disrupted in narcolepsy to simultaneously cause diurnal GH secretion and enhance daytime sleepiness. It is tempting to speculate that hypocretin deficiency is involved in the pathogenesis of this neuroendocrine anomaly. Intracerebroventricular administration of hypocretin-1 causes a dose-dependent decrease of plasma GH in rats *(10)*. Hypocretin peptides, which are primarily released during the day *(42)*, may normally inhibit hypothalamic GHRH secretion to promote arousal and reduce pituitary GH output. Conversely, diminution of hypocretin tonus at night *(42)* may relax the inhibitory restraint of GHRH release to dampen arousal and increase GH output. In this scenario, hypocretin deficiency particularly enhances GHRH release during the day (when the system normally has its peak activity) to concurrently promote daytime GH secretion and compromise daytime wakefulness in narcoleptic patients.

3.3. Thyroid Axis

The mean 24-h serum TSH concentration vs time was almost 50% lower in narcoleptic patients (1.58 ± 0.27 [N] vs 2.88 ± 0.36 [C] mU/L, $p = 0.014$), which was primarily brought about by a reduction in TSH pulse area. The number of pulses was similar in patients and controls. The absolute and relative amplitudes of the cosine wave reflecting the circadian variation of the 24-h TSH concentration did not differ between groups, but the acrophase occurred 2 h earlier in patients than in controls. Average plasma levels of total T4, free T4, total T3, and free T3 were not different in patients and controls.

Since TSH release is regulated by TRH feedforward input on the hypothalamic level, we additionally studied the TSH response to thyroid-releasing hormone (TRH) injection. The narcoleptic patients responded to TRH injection by secreting similar amounts of TSH as the control subjects, suggesting that their pituitary is normally sensitive to TRH stimulation.

There are numerous possible explanations for the mechanism by which hypocretin deficiency induces the low TSH levels, in the face of normal thyroid hormone concentrations. The two most plausible, in our opinion, are a direct influence of hypocretin on TRH release, or an indirect effect mediated by hypoleptinemia. TRH is synthesized in the thyrotrophic area of the paraventricular nucleus (PVN) and in the anterior pituitary. Hypocretin neuronal cell bodies project heavily to the PVN *(43)*. However, it is currently unknown whether hypocretin axons actually synapse with TRH neurons. Therefore, it is uncertain whether hypocretin deficiency can directly modulate TRH release. Interestingly, administration of a TRH analog in narcoleptic dogs with a genetic defect of the hypocretin-2 receptor increased wakefulness and ameliorated cataplexy *(44)*. This finding suggests that TRH deficiency is involved in the pathophysiology of the sleep-related symptoms of narcolepsy. Obviously, TRH deficiency can also explain the fact that narcoleptic humans have reduced circulating TSH levels.

Alternatively, relative hypoleptinemia may blunt TSH release in narcoleptic patients. This notion is supported by the finding that leptin can directly stimulate prepro-TRH mRNA

Fig. 3. (*opposite page*) Examples of 24-h profiles with curves fitted by deconvolution analysis of adrenocorticotropic hormone (ACTH; four upper panels) and cortisol (four lower panels) in a narcolepsy patient (left) and matched control (right). The second and fourth rows represent the accompanying deconvolution-estimated secretory profiles. Note the decreased basal ACTH secretion in the narcoleptic patient. Time is depicted in minutes elapsed after taking the first sample. (From ref. *28*.)

synthesis and can promote TRH release via activation of leptin receptors on TRH neurons *(45)*. Moreover, leptin may have a direct effect on TSH release since leptin receptors are expressed in rodent anterior pituitary cells *(46)*. Furthermore, in vivo studies in humans showed that diurnal leptin and TSH release exhibit strong pattern synchronicity, whereas TSH release becomes disorganized and its circadian rhythm phase-shifted in leptin-deficient subjects *(47)*. Despite the reduced TSH concentration, circulating T3 and T4 levels appear to be normal in narcoleptics.

3.4. Hypothalamic-Pituitary-Adrenal (HPA) Axis

Figure 3 shows an average 24-h plasma ACTH (upper) and cortisol (lower) concentration vs time series of narcoleptic patients and controls. Total secretion of ACTH was lower in narcoleptic subjects (310 ± 86 vs 760 ± 160 ng/L/24 h, $p = 0.02$; and 920 ± 147 vs 1460 ± 220 ng/L/24 h, $p = 0.04$, respectively), which was fully attributable to a reduction in basal ACTH release. All features of pulsatile ACTH secretion were similar in narcoleptics and controls (i.e., the number, the duration and height of bursts, and the mass of ACTH secreted per burst). In contrast, basal, pulsatile, and total cortisol secretion levels did not differ between narcoleptic and control subjects. The diurnal rhythm of plasma ACTH and cortisol concentrations could be mathematically characterized by a cosine function in both narcoleptics and controls. The acrophase of the cosine fit occurred at similar clock times in the early morning.

These findings suggest that hypocretin peptides are involved in the control of basal pituitary ACTH release. ACTH synthesis and release are primarily governed by stimulatory inputs of hypothalamic corticotrophin-releasing hormone (CRH) and arginine-vasopressin (AVP). CRH neurons are located in the PVN of the hypothalamus, which is among the various brain sites that receive hypocretin inputs *(43)*. Intracerebroventricular injection of hypocretin-1 enhances the activity of this neuroendocrine ensemble through activation of both CRH and AVP neurons in rats *(48)*. Thus, hypocretin deficiency may blunt hypothalamic CRH tonus and thereby reduce pituitary ACTH release. Interestingly, CRH administration stimulates arousal in humans as well as in experimental animals *(49–51)*, whereas CRH deficiency in rats increases total sleeping time *(50)*. Thus, a putative reduction in central CRH tonus induced by hypocretin deficiency could simultaneously contribute to the sleep phenotype and the reduction of pituitary ACTH release in narcoleptic patients.

4. GENERAL INTERPRETATION AND DISCUSSION

The novel data on various neuroendocrine axes and body weight of narcoleptic humans presented in this chapter extend our knowledge of narcolepsy and may affect our thinking about the pathophysiology of the disease.

The data on the HPA axis strongly suggest that the master pacemaker dictating circadian timing is intact in narcoleptic patients. The circadian timing of HPA output is generally recognized to be a close reflection of pacemaker activity *(52)*. The timing and amplitude of the acrophase of ACTH and cortisol in plasma were similar in patients and controls. The circadian distribution of body temperature fluctuations, another reliable measure of endogenous time keeping by the biological clock *(53)*, is also normal in narcoleptic humans *(54)*, which supports the notion that the master pacemaker is unaffected by the disease. However, the distribution of sleep and wakefulness, which is primarily determined by clock timing, is severely disturbed in narcoleptics. To reconcile these facts, it has been proposed that inputs from the SCN into hypocretin neurons drive clock-dependent alertness in healthy humans and that destruction of these neurons therefore abrogates the impact of an intact master pacemaker on the circadian sleep-wakefulness cycle in narcoleptics *(55)*.

The data summarized in this chapter suggest that hypocretin neurons also link SCN activity with adipocyte leptin secretion, probably via the autonomic nervous system. The impact of hypocretin neurons on the autonomic nervous system may be either direct or indirect through modulation of sleep and wakefulness.

Reduced circulating leptin levels may be involved in the pathogenesis of obesity in narcoleptic patients. The fact that narcoleptic patients are obese in the face of hypophagia suggests that they spend less energy, which is supported by early observations *(2)*. Leptin is critically involved in the control of energy expenditure, and hypoleptinemia is associated with a lower metabolic rate in obese animal models. Alternatively, hypocretin deficiency may reduce basal metabolism directly via its inhibitory impact on the sympathetic nervous system.

In concert with reports alluded to earlier, the present data are in keeping with the postulate that hypocretin neurons play a role in the control of hypothalamic CRH and GHRH release. Hypocretin deficiency may blunt CRH secretion, which could compromise wakefulness and reduce pituitary ACTH release; disrupted hypocretin control of GHRH release may contribute to the pathogenesis of daytime naps and increased diurnal GH secretion in narcoleptic humans.

It remains to be established whether the neuroendocrine anomalies described here are of clinical significance. At first sight, it is hard to believe that reduced ACTH release in the face of normal circulating cortisol levels, disrupted circadian distribution of GH secretory events that produce normal amounts of GH, reduced circulating TSH levels in the face of normal thyroid hormone concentrations, and diminished circulating leptin concentrations have clinically relevant effects in narcoleptic patients. Also, narcolepsy is not known to be associated with any clinically relevant endocrine anomaly. However, it is important to realize that the hormonal ensembles were not explored in the studies reported to date.

In conclusion, hypocretin neurons may be involved in control of the autonomic nervous system, leptin secretion by adipocytes (via autonomic neural inputs), and hypothalamic CRH and GHRH release, which would explain the various neuroendocrine and metabolic anomalies in narcoleptic humans that are described here.

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Narcolepsy and Autoimmunity

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1. INTRODUCTION

Until recently, the pathological basis of narcolepsy was unknown. We now know that abnormalities in the posterior/lateral hypothalamic hypocretin (orexin) system result in human narcolepsy and in narcolepsy phenotypes in dogs and rodents *(1,2)*. Most cases of narcolepsy that are definitely associated with cataplexy have low or undectable levels of hypocretin-1 (orexin-A) in their cerebrospinal fluid (CSF) *(3,4)*. Compared with controls, the few postmortem brains examined from narcolepsy patients have been shown to have absent or a very reduced number of hypocretin neurons by *in situ* hybridization and immunocytochemistry *(5,6)*. In the lateral hypothalamus, hypocretin neurons are intermingled with other neurons such as melanin-concentrating hormone (MCH) neurons. Although hypocretin neurons are profoundly affected in narcolepsy, MCH neurons appear to be well preserved *(5)*. There is also minimal gliosis in the lateral hypothalamus of narcolepsy patients. These results should be interpreted with caution, since only a few brains have been examined to date; however, the intriguing possiblity remains that some process specifically afflicts hypocretin neurons in narcolepsy, without any damage to the surrounding brain tissue.

Human narcolepsy is not a simple mendelian genetic disorder. At best, there is only 30% concordance in monozygotic twins, and so far, only one case has been described that was associated with a mutation in the prepro-orexin (hypocretin) gene *(5,7–9)*. Unlike canine narcolepsy, a fully penetrant autosomal recessive disorder owing to mutations in the hypocretin-2 receptor gene, no human cases associated with hypocretin receptor gene mutations have been described *(5,10,11)*. Therefore, it may be said that narcolepsy is an environmentally determined disorder specifically associated with hypocretin neuron dysfuction (or destruction).

What causes the hypocretin neurons to be either destroyed or dysfunctional? The role of the hypothalamus in sleep regulation was well documented by Van Economo's studies of the encephalitis lethargica epidemic *(12)*. This epidemic resulted from a mysterious infection for which both viral and bacterial causal agents have been proposed. Therefore, narcolepsy, albeit generally without cataplexy, can result from infection or a postinfectious state with posterior hypothamaic and upper brainstem impact. An important clue to the etiology of narcolepsy was obtained through the work of Honda, et al. *(13)*, who showed a strong association between narcolepsy and the human leukocyte antigen (HLA)-DR2. This has now been further refined to show that narcolepsy is associated with HLA-DQB1*0602 *(8,14)*. Associations between diseases and HLA markers generally occur in autoimmune disorders.

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This therefore raised the possibity that narcolepsy is an autoimmune disorder. We can now extend this hypothesis to describe narcolepsy as a probable autoimmune disorder associated with specific dysfunction or destruction of hypocretin neurons. The objective of this chapter is to discuss briefly our current knowledge regarding the putative autoimmune basis of narcolepsy.

2. THE HLA SYSTEM AND NARCOLEPSY

The immune system distinguishes self from foreign antigens through the cell surface expression of MHC gene glycoprotein antigen products. In humans, the highly polymorphic MHC system (called the HLA system), stretching over 4 Mb, maps to the short arm of chromosome 6 (6p21.31), This region contains over 200 genes (not all related to immune function) and is generically divided into three MHC subregions: classes I, II, and III. Class I and II genes encode glycoprotein products that are essential in antigen processing and presentation to T lymphocytes (the conductors of the immune response).

Near the centromere, class II genes encode the α- and β-polypeptide chains of HLA-DR, -DQ, and -DP. Class II genes are normally expressed by immune cells (B and T lymphocytes, macrophages and dendritic cells, and thymic epithelial cells), but their expression in other cells may be triggered by interferon. HLA class I genes (HLA-A, -B, and -C) are in a 2-Mb DNA stretch at the telomeric end of the HLA gene region. Class I genes encode the α-polypeptide chain that combines with β_2-microglobulin, whose nonpolymorphic gene is on chromosome 15. The two polypeptides form a cell surface molecule that is found on all nucleated cells. Class I and II molecules present processed foreign peptides to T cells by engaging the T-cell receptor. HLA class III genes are between class I and class II and encode the complement components C2, C4, and Bf, heat shock protein (HSP) 70, the enzyme 21-hydroxylase, and the proinflammatory cytokine tumor necrosis factor-α (TNF-α).

The association of autoimmune diseases with various MHC proteins, particularly HLA class II antigens, is well appreciated. There are strong associations in rheumatological diseases such as spondyloarthropathies (B27) and rheumatoid arthritis (DRB1*04), the endocrine/metabolic disorder type I diabetes mellitus (DRB1*0301, DRB1*04, DQB1*0302, DQB1*0201, and DQB1*0602), the skin condition pemphigus vulgaris (DRB1*0402), the gut disorder celiac disease (DQA1*05 and DQB1*02), and the neurological disorder, multiple sclerosis (DB1*15, DQB1*0602). In autoimmunity, susceptibility HLA polypeptides derived from particular alleles are believed to bind peptide motifs initially derived from the processing of a foreign antigen. A sustained immune response with HLA presentation of self-antigens then ensues, resulting in tissue damage.

Early studies, using the less sensitive serological HLA typing, reported an association between narcolepsy and HLA class I antigens: HLA class I Bw35 in Japanese patients and HLA-Bw7 (but not Bw35) in Caucasians *(15).* Class II antigens proved to be more interesting with, surprisingly, all Japanese narcoleptic patients sharing two serologically defined HLA class II antigens, DR2 and DQ1. This remarkable DR association was subsequently confirmed in other ethnic groups: Caucasians (as high as 90–95%) and African Americans (60%), High-resolution typing HLA-DRB1 and -DQB1 in African Americans indicated a tighter association with DQB1*0602 (a subtype of DQ1/DQ6) than with HLA-DRB1*15 (a subtype of DR15/DR2) *(8).* In African Americans, DQB1*0602 can be found in association with DR2, DR5, and DR6. About a third of African-American narcoleptic patients carry DQB1*0602 independently of DR2. In Caucasians and Japanese, DQB1*0602 is almost always associated with DR-B1*15 because of a linkage disequilibrium between these two alleles. In African

Table 1
Evidence For and Against the Autoimmune Etiology of Narcolepsy

For autoimmune hypothesis
Tight association with HLA—90% DQB1*0602
Peripubertal onset
Specific degeneration of hypocretin neurons in lateral hypothalamus
Against autoimmune hypothesis
Lack of systemic immune markers—no changes in CRP, ESR, cytokines, T-cell subset studies
No oligoclonal bands
Equal sex distribution
No antibodies to brainstem antigens by Western blot
No antibodies against commonly known autoantigens
No antibodies to hypothalamus on Western blot or immunocytochemistry

Abbreviations: CRP, C-reactive protein; ESR, erythrocyte sedimentation rate.

Americans, fewer patients are DQB1*0602 positive independently of DR2, because of the absence of linkage disequilibrium. To conclude, HLA-DQB1*0602 is the major HLA susceptibility allele for narcolepsy across all ethnic groups *(14)*. Most patients (88–98%) with cataplexy are HLA-DQB1*0602 positive, with corresponding values in control populations being much lower (12% in Japanese, 25% in Caucasian, and 38% in African Americans).

3. NARCOLEPSY AND AUTOIMMUNITY: THE EVIDENCE

Table 1 summarizes the evidence for and against an autoimmune basis for narcolepsy. The tight association between narcolepsy and HLA-DQB1*0602 is generally greater than HLA associations observed with known autoimmune disorders such as multiple sclerosis or type I diabetes mellitus (a notable exception may be ankylosing spondylitis and HLA-B27) Furthermore, like most autoimmune diseases, narcolepsy tends to affect younger individuals (peripubertal onset). Unlike most autoimmune diseases, which tend to affect females more, however, narcolepsy is seen equally in both sexes *(1)*. Also, there is no known clustering of narcolepsy with known autoimmune diseases. Levels of inflammatory markers such as C-reactive protein and erythrocyte sedimentation rates, as well as CD4/CD8 lymphocyte subsets, have all been reported to be within the normal range. *(1,16–19)*. This could be because by the time narcolepsy is clinically apparent, the initial inflammatory response may have disappeared or the immune response is highly specific against hypocretin neurons, such that no systemic effect is seen.

The interaction of particular HLA proteins with processed autoantigen determines whether tolerance or autoimmunity occurs. Since HLA-DQB1*602 confers disease susceptibility in narcolepsy, whereas the very similar DQB1*0601 is protective, minor variations in the peptide binding pockets of these molecules determine disease susceptibility *(20)*. How these minor changes result in damage to hypocretin neurons is unclear. Access to the highly privileged central nervous system by immune cells and/or antibodies is required. There does not appear to be any difference in blood-brain barrier permeability between narcolepsy patients and controls. One study measured CSF/serum albumin ratios in a small number of patients, and these were only rarely and only slightly elevated in patients. Blood-brain barrier breakdown may be missed because of a delay in the diagnosis of narcolepsy, or because it may occur episodically. In any event, we now know that, as far as immune cells are concerned, the brain is not as restricted an area as previously believed.

We (and others) have not found any evidence for humoral autoimmunity in narcolepsy. No CSF oligoclonal bands are reliably observed in narcolepsy. Using enzyme-linked immunosorbent assay ELISA, we have not been able to detect any immunoglobulin G autoantibodies (from serum or CSF) directed against hypocretin-1, hypocretin-2, or prepro-hypocretin overlapping peptides *(21)*. It may, however, be possible that there are antibodies directed to hypocretin peptides complexed to HLA *(20)*. Also, the absence of these peptide antibodies does not rule out the possibility of autoantibodies directed against other antigens expressed by hypocretin neurons. In myasthenia gravis, an autoimmune disease targeting the acetylcholine receptor in the neuromuscular junction, pathogenic antireceptor serum autoantibodies are detected in most patients and transfer the disease when injected into mice. However, 15% of myasthenia patients do not have detectable antiacetylcholine receptor autoantibodies in their blood, yet their serum can still transfer the disease to mice. This raised the possibility of the existence of autoantigens independent of the hypocretin molecule but expressed by hypocretine- producing cells.

We investigated this hypothesis in several ways. First, we looked at antibodies directed against hypothalamic and other brain region proteins by carrying out Western blots using serum, purified immunoglobulin G, and CSF from both patients and controls *(21)*. Patients included young and recent-onset HLA-positive patients. Central nervous system (CNS) samples used were from dogs, rats, and mice. We were particularly interested in reactions to canine brain samples, since narcolepsy can occur sporadically in dogs in a similar fashion to humans (also with hypocretin deficiency) *(22)*. Although some patients had distinct bands on the Western blot at high dilutions (1:500–1:5,000), no specific pattern was markedly different from that of control subjects. It should be noted, however, that protein denaturation may have compromised any existing target antigen. We also looked at the possibility of autoantibodies by immunocytochemistry using brains from all the above species and postmortem human brains. Again, we could not detect a consistent pattern of binding affecting hypocretin neuronal cell bodies or projection areas.

We next attempted to transfer the disease by injecting mice with purified immunoglobulin G from patients and controls (with and without concomitant treatment with pertussis toxin, to open the blood-brain barrier). Similar experiments have shown passive transfer of myasthenia gravis or Lambert-Eaton syndrome in mice using similar protocols. Again, we could not detect any changes in brain hypocretin-1 levels or obvious narcolepsy between mice treated with patient immunoglobulin G and the control group. This may be because of several possibilities. For example, we may not have successfully opened up the blood-brain barrier for the passage of the immunoglobulin G, the dosage of antibody we used may not have been sufficient or the treatment long enough, or an additional insult such as fever (with the release of various cytokines) may be necessary.

Evidence for cellular autoimmunity in narcolepsy is also extremely weak. Blood levels and monolymphocyte secretion of TNF-α and other proinflammatory cytokines like interleukin (IL)1β, IL-1ra, IL-2, and TNF-β are not different between narcoleptic patients and controls. Only IL-6 secretion has been reported to be higher in narcoleptic patients *(23)*. T-cell subsets and natural killer activity were identical in both populations. Although these observations refute the hypothesis of a major peripheral proinflammatory cellular activation in narcolepsy, they do not exclude local CNS activation. Studies of hypocretin neurons in brains of narcoleptic patients have not detected any inflammatory infiltrate in the hypothalamus of patients, but nonspecific gliosis was observed in one study *(5,6)*. Also, CNS microglial class II expression is not significantly different between control and narcoleptic subjects *(24)*.

These studies may have missed an inflammatory infiltrate that may have occurred at disease onset. Based on our measurements of hypocretin-1 in the CSF, the disease appears to be rapidly progressive in many cases. Even in cases that are clinically detected early, hypocretin levels are low or absent in the CSF. We recently reported the case of a young boy within a few months of disease onset who had undetectable hypocretin-1 in his CSF at presentation but failed to respond to immunosuppression with corticosteroids *(25)*. Another group has reported success with high-dose immunoglobulin and corticosteroids, but the role of these treatments, if any, is at present unclear *(26)*.

4. CONCLUSIONS

Despite the finding that hypocretin neurons are important targets in narcolepsy and the fact that there is a high HLA association, there is currently no evidence that narcolepsy is the result of an antigen-specific autoimmune humoral or cellular process. The preliminary observation of a possible therapeutic response to intravenous immunoglobulin may rekindle interest in the area of autoantibody mediation in narcolepsy. There are several hurdles that may have prevented the direct demonstration of an autoimmune process in narcolepsy. First and foremost, narcolepsy is typically diagnosed years after onset. At this time, the pathological process of possible autoimmune origin may have ended, and hypocretin neurotransmission may already be almost entirely absent. The fact that hypocretin neurons are few in number may also make the identification of any factor specific for this cell population difficult, not to mention the fact that the putative autoantigen(s) may be human specific.

Other immune-related mechanisms of non-autoimmune origin may also be involved. For example, it is possible that in narcolepsy, the HLA association confers selective sensitivity to a particular infection or toxin that targets hypocretin neurons or that HLA has a neurodevelopmental role. Interestingly, nonnarcoleptic patients who are HLA-DQB1*0602 positive tend to have a slightly shorter REM latency than HLA-negative individuals. An additional insult, however, is necessary for narcolepsy to occur in a particular subset *(27)*. An infectious etiology is suggested by the distribution of patient birth months *(28)*. What is clear is that hypocretin neurons are highly susceptible to various insults, from trauma to encephalitis *(29–31)*. The mystery of narcolepsy continues.

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V

Pathophysiology: Narcolepsy and Orexin/Hypocretin Deficiency

C. Treatment/Pharmacology

25

Pharmacology of Hypocretin/Orexin Peptides and Small Molecules

Stephen J. Perry, PhD, David A. Schwarz, PhD, and Richard A. Maki, PhD

1. INTRODUCTION

Despite their relatively recent discovery in 1998, the hypocretins (also known as orexins) and their receptors are already the focus of several investigations as sites for therapeutic intervention in a number of endocrinological and neurological disorders. The rapidity with which the hypocretin system has been adopted as a high-interest target is mainly the result of an accumulation of compelling evidence from in vivo studies showing that the hypocretins regulate a number of aspects of physiology and behavior, especially those involved in sleep, arousal, and energy homeostasis. High-throughput screening efforts by a number of pharmaceutical companies have now identified novel small molecules that interact potently and specifically with the hypocretin receptors. Despite these considerable efforts, very little is known regarding the structures of the receptors, their endogenous ligands, the molecular basis of their interactions, or the signaling pathways they use. For example, no attempt has been made through receptor mutagenesis, or by any other means, to define the key interactions that occur between the receptors and the endogenous peptide ligands. Although such studies are likely to be ongoing within pharmaceutical companies with active hypocretin receptor drug discovery programs, the information available to the general scientific community remains very limited. This chapter reviews what is currently known about the molecular pharmacology of this system, focusing on the structures and activities of the peptides and some of the small molecules for which published biological data exist.

2. PEPTIDES

Both hypocretin-1 and hypocretin-2 peptides were originally isolated from rat brain extracts by virtue of their biological activity on a cell line expressing the then orphan G-protein-coupled receptor (GPCR) HFGAN72, which was subsequently renamed the hypocretin type 1 receptor (HcrtR1) *(1)*. Biochemical purification and sequencing of endogenous hypocretin-1 determined that it was a 33-residue peptide with an amidated carboxyl terminus, and a cyclized pyroglutaminyl residue at the amino terminus and that it contained two intramolecular disulfide bridges between Cys^{6}-Cys^{12} and Cys^{7}-Cys^{14} (Fig. 1). Purification and sequencing of endogenous hypocretin-2 determined that it was a 28-residue peptide amidated at its carboxyl terminus and sharing 46% amino acid identity with hypocretin-1. No similarity exists between the hypocretin peptides and all other known neuropeptides. A third peptide isolated by virtue

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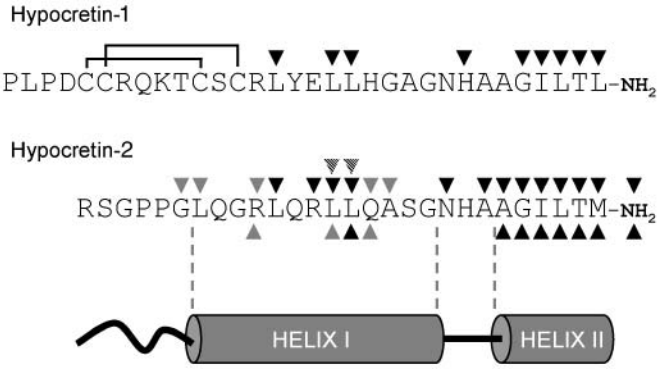


Fig. 1. Summary of the structure-activity relationships of hypocretin-1 and hypocretin-2 peptides. Residues identified by single amino acid substitutions that partially (gray arrowheads) or strongly (black arrowheads) affect activity at HcrtR1 are shown above the peptide sequence, and at HcrtR2 are shown below the peptide sequence. Hatched arrowheads indicate residues in hypocretin-2 that greatly increase selectivity for HcrtR2 when substituted with their D enantiomers. The relative positions of the amino acids within the secondary structure of [Phe^1]hypocretin-2 *(13)* are also shown. (Data from refs. *15–17*.)

of biological activity on the HcrtR1-expressing cell line was identified as the amino terminally clipped [des Arg-Pro] hypocretin-2. It is currently unknown whether this truncated peptide represents the product of a physiologically relevant processing event or is the result of artifactual proteolysis of hypocretin-2 occurring during the extraction procedure.

Both the hypocretin peptides are predicted to be derived from the same prepropeptide encoded by a single gene located on human chromosome 17q21 *(1,2)*. Within the prepropeptide, the sequence of the mature hypocretin-1 peptide follows directly after the predicted signal peptide cleavage site and is followed by a glycine residue and a dibasic prohormone convertase cleavage site at its carboxyl terminus. The glycine residue presumably serves as an NH_2 donor for enzymatic carboxyl terminal amidation. The sequence of the mature hypocretin-2 peptide is also followed by a glycine residue, thus predicting its amidation, and is flanked at both ends by dibasic convertase cleavage sites. Hypocretin prepropeptide cDNAs identified from several species reveal that whereas the signal sequence, mature peptide sequences, and sites of post-translational processing are highly conserved, the remaining portions of the prepropeptide are not and thus are unlikely to encode other biologically active peptides *(1–4)*.

The affinities of hypocretin-1 and hypocretin-2 at HcrtR1 have been determined by measuring displacement of [^{125}I-Tyr^{17}]hypocretin-1 (inhibitory concentration of 50% [IC_{50}]values of 20 and 420 n*M*, respectively). A second hypocretin receptor (HcrtR2), which was cloned based on its high sequence identity to HcrtR1 (63%), binds both hypocretin-1 and hypocretin-2 with equal affinities (IC_{50} = 30–40 n*M*). Both peptides stimulate calcium ion mobilization in cells expressing either of the receptor subtypes, and the potencies observed in these functional assays correspond closely to their affinities *(1)*. It has been noted that the affinities of the peptides are relatively low compared with those of other peptides and their receptors *(5)*, which may indicate that iodination of the ligand alters its binding characteristics, preventing accurate determination of its affinity. This is supported by other studies in which the unlabeled peptides stimulate calcium release at much lower concentrations *(6)*.

Both hypocretin receptors are believed to couple primarily to Gq proteins, since they have been shown to utilize a pertussis toxin-insensitive G protein (non-Gi/o family) to mobilize calcium ions in several cell types, including those in neurons *(7)*. Coupling to alternative

G proteins has also been reported for both receptors. In dispersed primary rat zona fasciculate reticularis cells, hypocretins stimulate synthesis of corticosterone through a Gs and cyclic AMP-dependent mechanism, probably through HcrtR1 *(8,9)*. Coupling of HcrtR2 to Gi/o in neuronal and Gi in adrenal cell types is also reported *(7,10–12)*.

The 3D solution structure of human [Phe^{1}]hypocretin-2 has been determined by 2D two-dimensional ^{1}H-nuclear magnetic resonance (NMR) spectroscopy *(13)*. The peptide consists of an amino-terminal random coil region (residues 1–6) followed by two α-helices connected by a short flexible linker. The helical axis of helix I (which spans amino acids 7–19) is oriented 60–80° relative to helix II (amino acids 23–28). It is interesting to note that although hypocretin-2 shares no primary sequence homology with other neuropeptides, its structure is similar to that of neuropeptide Y *(14)*. Furthermore, the neuropeptide Y type 2 receptor shares significant identity (28–29%) and similarity (44–47%) with the hypocretin receptors, suggesting that a common mechanism of interaction may exist between these receptors and their peptides.

To date three studies have been published that address the structure-activity relationship of the hypocretin peptides regarding their agonist activity *(15–17)*. Whereas the principal goal of two of these studies was to identify key residues within the peptides involved in receptor activation *(15,17)*, an equally important discovery was the existence of potent peptide agonists that display a high degree of selectivity for HcrtR2. These peptide analogs should greatly accelerate future efforts to define distinct roles for the two receptors in in vivo studies. A major drawback of all three studies is their failure to correlate binding affinity of the peptide analogs with their apparent activity, since two of the studies relied solely on functional assays measuring calcium mobilization *(16,17)* and the third measured affinity for just two of the 24 analogs investigated *(15)*. Thus, although many peptide analogs were synthesized that lacked functional activity, it is not possible to determine whether these represent potentially useful high-affinity antagonists or are simply of too low affinity to activate the receptors.

The first of these studies focused almost entirely on defining residues within hypocretin-1 that are critical for activation of HcrtR1 *(15)*. By truncating hypocretin-1 from the amino terminus, it was shown that removal of the first 14 residues ([15–33] hypocretin-1) produces a peptide with 170-fold reduced potency and 20-fold lower affinity for HcrtR1, but that still possesses full agonist activity at both receptor subtypes. Alanine scanning and limited D-amino acid substitution within this minimum sequence were then used to define further the functionally critical residues of hypocretin-1 as Leu^{16}, Leu^{19}, Leu^{20}, His^{26}, and each of the last five residues at the carboxyl terminus (Fig. 1). Furthermore, substitution of D-Thr at position 32 ablates all biological activity, whereas substitution with D-Leu at 33 greatly reduces activity. Thus, it was postulated that (1) the amino-terminal 14 residues of hypocretin-1 are required for full agonist potency (and binding affinity), (2) full biological efficacy is retained within the last 19 residues, and (3) the side chains and orientation of the last five residues are critical for this activity.

The second study set out to develop HcrtR2-selective analogs of hypocretin-2 through sequential alanine scanning and D-amino acid substitution *(16)*. An initial series of single alanine substitutions at every position within the peptide (or glycines at positions occupied by alanines in the native peptide) identified residues that either do not significantly affect activity at either HcrtR1 or HcrtR2 (positions 1–9, 12–14, 16 and 17) or affect activity at both receptors (positions 10, 15, and 18–28). Only substitution of Leu^{11} for alanine shows selectivity for HcrtR2, specifically reducing activity at HcrtR1 alone by 10-fold and obtaining 120-fold selectivity for HcrtR2 over HcrtR1. Further substitutions of natural and unusual amino acids at this position identified tryptophan and serine as also emphasizing HcrtR2

selectivity, although both these residues reduce HcrtR2 activity to some extent. In addition, D-amino acid substitutions at every position (or D-alanine for native glycines) identified D-leucines at positions 14 and 15 as significantly improving HcrtR2 selectivity (by fivefold and eightfold, respectively) while having little or no adverse effect on biological activity at HcrtR2. In agreement with the findings of Darker et al. *(15)* in hypocretin-1, D-amino acid substitutions at any of the last five positions of hypocretin-2 eliminate or greatly reduce biological activity at both receptors. Combining Ala^{11} with either D-Leu^{14} or D-Leu^{15} further increases selectivity for HcrtR2 to 140-fold and 400-fold, respectively, without significantly affecting activity at HcrtR2. Comparison with the solution structure of [Phe^{1}] hypocretin-2 *(13)* shows that all three of these positions reside in helix I, implicating this portion of the peptide in determining selectivity of hypocretin analogs, whereas the carboxy-terminal residues in helix II are the primary determinants of biological activity.

The third study addressed a number of structural requirements for biological activity of both hypocretin-1 and hypocretin-2 at HcrtR1 and HcrtR2, as well as determinants of selectivity for HcrtR2 over HcrtR1 *(17)*. Of particular interest is the demonstration that the disulfide bridges within the amino terminus of hypocretin-1 are not required for its activity, since both the oxidized and reduced forms of the peptide exhibit similar activities. These data conflict with a previous report in which analogs of hypocretin-1 that contain L-alanines substituted for each disulfide bonding pair of cysteines ([$Ala^{6,12}$] hypocretin-1 and [$Ala^{7,14}$] hypocretin-1), or all four cysteines ([$Ala^{6,7,12,14}$] hypocretin-1) display significantly reduced activity at HcrtR1 and HcrtR2 *(6)*. However, it is the difference in the size and electronic properties between the native cysteines and the substituted alanines that appears to be responsible for the loss of activity rather than elimination of the disulfide linkages, since two hypocretin-1 analogs in which the cysteines are replaced with the structurally more similar amino acid 2-aminobutyric acid (Abu) ([$Abu^{6,12}$] hypocretin-1 and [$Abu^{7,14}$] hypocretin-1) show 10-fold reductions in potency but still retain full biological activity.

Deletion of or substitutions made at any of the last five residues in either hypocretin-1 or -2 ablate or greatly reduce their biological activity, in agreement with the previous two studies *(15,16)* (Fig. 1). In addition, removal of the amide moiety from hypocretin-2 ablates biological activity at both receptor subtypes, reiterating the critical role played by the carboxyl terminal portion of the peptide in conferring biological activity. Sequential deletions from the amino termini of both hypocretins were used to define [15–33] hypocretin-1 and [8–28] hypocretin-2 as the minimum lengths of the peptides to retain biological activity at HcrtR1, and [15–33] hypocretin-1 and [10–28] hypocretin-2 to retain activity at HcrtR2. Although the potency of the truncated peptides is reduced in all cases, the effect is greater at HcrtR1 (20-fold and 220-fold reductions in the median effective concentration [EC_{50}]) than at HcrtR2 (7-fold and 9.5-fold reductions), supporting the hypothesis proposed by Asahi et al. *(16)* in which helix I of hypocretin-2 (residues 7–19) plays a more important role in recognition and binding to HcrtR1 than to HcrtR2.

This hypothesis is further supported by the results from comprehensive alanine and proline scans made through [6–28] hypocretin-2, the shortest portion of hypocretin-2 to contain both the α-helical regions and to retain reasonable potency and full activity at both the receptors. Substitutions made at 8 of the 13 residues within helix I (residues 7–19) produce significant reductions in potency at HcrtR1, whereas only substitutions at Leu^{14}, Leu^{15}, and Gln^{16} affect potency at HcrtR2. Changes made at any of the residues within helix II (residues 23–28) significantly reduce potency at both receptors. Furthermore, introduction of L-proline but not L-alanine into several positions in helix I cause a profound reduction in activity at

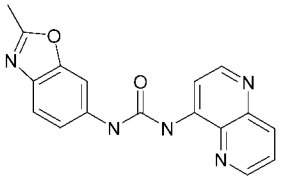
HcrtR1, suggesting that it is the disruption of the α-helicity of helix I by prolines, and not alteration in the side chains of these individual amino acids, that is responsible for loss of activity at HcrtR1. This is also the case for Ala^{23}, the first residue of helix II, where L-proline substitution reduces activity, whereas L-alanine does not. The remaining residues in helix II are sensitive to substitution with either L-proline or L-alanine, indicating that the side chains of these residues are important for conferring activity. Both proline and alanine scanning also identified a number of analogs with marked selectivity for HcrtR2 over HcrtR1, including [Pro^{11}, 6–28] hypocretin-2, and [Ala^{27}, 6–28] hypocretin-2, which show at least 1000-fold higher activity at HcrtR2. However, unlike the HcrtR2-selective analogs identified by Asahi et al. *(16)* that retained high potency for HcrtR2, both these analogs exhibit significant reductions in potency at HcrtR2, making them of questionable value as tools for studying HcrtR2-specific functions.

3. SMALL-MOLECULE ANTAGONISTS

The high-throughput screening efforts of a number of pharmaceutical companies have yielded many small-molecule compounds with antagonist activity at either one or both hypocretin receptors. Only one of these compounds has been studied in vivo to ascertain its bioavailability and physiological effects, and most of the others have only been reported within patent applications with little or no in vitro data to support claims of biological activity. This section reviews what is currently known regarding the structures and activities of these compounds, focusing mostly on those molecules for which published in vivo and/or in vitro data exist.

The first HcrtR1 antagonist reported was 1-(2-methylbenzoxanzol-6-yl)-3-[1,5]naphthyridin-4-yl urea hydrochloride (SB-334867) *(18,19)* (Fig. 2). It has 50-fold selectivity for HcrtR1 over HcrtR2 and at least 100-fold selectivity over a range of 50 other GPCRs and ion channels on which it was tested. SB-334867 inhibits hypocretin-1 and hypocretin-2 induced calcium responses in cells expressing HcrtR1 with apparent pK_b values of 7.27 and 7.23, respectively. In rat brain slices, SB-334867 inhibits hypocretin-1 excitation of locus coeruleus neuronal activity *(20)*. Following ip dosing of rats, SB-334867 is found in the brain but has a relatively short half-life of 0.4 h *(18)*. Even so, SB-334867 inhibits both natural food intake and feeding induced by central administration of hypocretin-1 *(21–23)*, accelerates the transition between feeding and resting, and reduces the occurrence of several activity-related behaviors *(22–24)*. Intracerebroventricular administration of SB-334867 to rats also increases barbiturate-induced anesthesia times and reverses the decrease produced by hypocretin-1 treatment *(25)*. Furthermore, SB-334867 reduces feeding, body weight, and fat mass and induces thermogenesis in *ob/ob* obese mice *(26)*. All these effects are arguably consistent with the specific blockade of hypocretin receptors expressed in brain regions involved in energy homeostasis and arousal and represent the first evidence that a small-molecule antagonist of the hypocretin system could be used to treat disorders that involve these systems. This compound has also been reported to reverse other physiological effects of hypocretin-1 administration, including analgesia *(27)*, hypertension, and central release of norepinephrine *(28)*.

A further three antagonist compounds with selectivity for HcrtR1 are described by Langmead et al., *(29)*: 1-(5-(2-fluoro-phenyl)-2-methyl-thiazol-4-yl)-1-((*S*)-2-(5-phenyl-(1,3,4) oxadiazol-2-ylmethyl)-pyrrolidin-1-yl)-methanone (SB-674042), 1-(6,8-difluoro-2-methyl-quinolin-4-yl)-3-(4-dimethylamino-phenyl)-urea (SB-408124), and 1-(5,8-difluoro-quinolin-4-yl)-3- (4-dimethylamino-phenyl)-urea (SB-410220). Tritiated SB-674042 has a pK_d of 8.29 for HcrtR1s and displays more than 100-fold selectivity for inhibiting calcium



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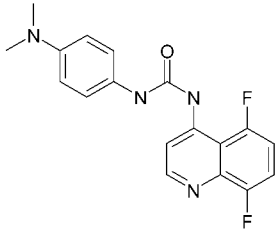

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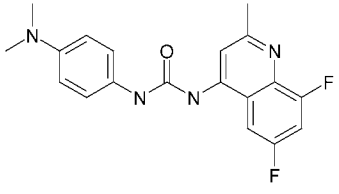

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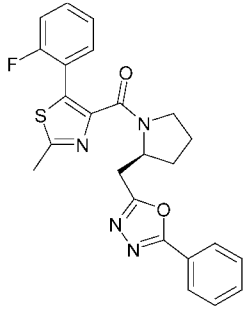

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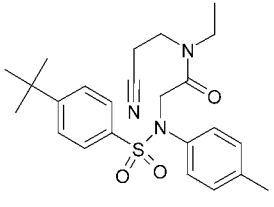

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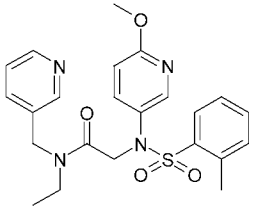

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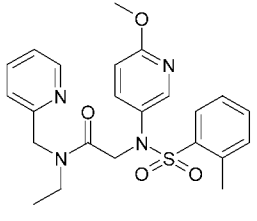

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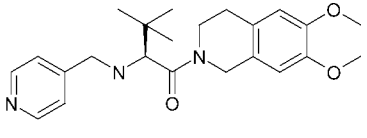
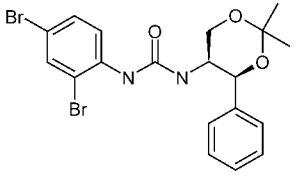


Hirose *et al.* (Ref.30)



McAtee *et al.* (Ref.31)

Fig. 2. Structures of small-molecule hypocretin receptor antagonists (described in refs. *18* and *29–32*.).

-mobilization by HcrtR1s over HcrtR2s. SB-408124 and SB-410220 bind HcrtR1s with pK_i values of 7.57 and 8.35, respectively (measured by displacement of [^{3}H]SB-674042) and show more than 50-fold selectivity for HcrtR1s in functional assays. No in vivo pharmacokinetics or physiological effects have been reported for these compounds.

Recently, three series of compounds with HcrtR2 selectivity have been reported. The first is a series of *N*-acyl 6, 7-dimethoxy-1,2,3,4-tetrahydroisoquinoline derivatives *(30)*, the most selective of which, *N*-methyl-4-pyridyl (*S*)-*tert*-leucyl 6,7-dimethoxy-1,2,3,4-tetrahydroisoquinoline, shows more than 250-fold selectivity for human HcrtR2 (IC_{50} = 40 n*M*) compared with human HcrtR1 (IC_{50} > 10,000 n*M*) and displays less than 30% antagonist activity at 10 μ*M* within a panel of 50 other GPCRs and ion channels. The solubility in water of this compound is high (0.81 mg/mL at pH 7.0), indicating that this molecule may be suitable for further in vivo characterization.

The second series comprises substituted 4-phenyl-[1,3]dioxanes, the most potent of which is 1-(2,4-dibromo-phenyl)-3-((4*S*,5*S*)-2,2-dimethyl-4-phenyl-[1,3]dioxin-5-yl)-urea, which has a pK_i value of 8.3 for HcrtR2 and a pK_b of 7.9 in a calcium mobilization assay *(31)*. The compound binds the HcrtR2 with 600-fold selectivity over HcrtR1 and at 10 μ*M* inhibits less than 40% of binding to κ-opioid, neuropeptide Y1, and neurokinin 3 receptors and less than 20% of binding to 47 other GPCRs and ion channels on which it was tested.

A third series of antagonists described in a patent filed by Actelion Pharmaceuticals Ltd. is worthy of mention for their high selectivity for HcrtR2 *(32)*. The compounds are sulfonyl-amino-acetic acid derivatives, the three most selective of which are 2-[(4-tert-butyl-benzene-sulfonyl)-*p*-tolyl-amino]-*N*-(2-cyano-ethyl)-*N*-ethyl-acetamide; *N*-ethyl-2-[(6-methoxy-pyridin- 3-yl)-(toluene-2-sulfonyl)-amino]-*N*-pyridin-3-yl-methyl-acetamide; and *N*-ethyl-2-[(6-methoxy-pyridin-3-yl)-(toluene-2-sulfonyl)-amino]-*N*-pyridin-2-yl-methyl-acetamide. All three compounds inhibit HcrtR2-mediated calcium mobilization with low nanomolar IC_{50} values and are more than 1000-fold more potent at HcrtR2 than at HcrtR1. To date, there are no reports in the peer-reviewed literature describing the properties of these compounds.

The biological data from the analysis of the hypocretin system has provided an exciting starting point for a better understanding of the role of hypocretins in normal biology as well as disease. In this review we have focused on a description of the basic pharmacology of the hypocretin system and described the available peptides and small molecules that interact with the hypocretin receptors. The work on the peptides has been instructive with regard to which amino acids are important for functional activity and defining peptides with selectivity to HcrtR2 vs HcrtR1. Additional work is needed to define specific interactions between amino acids in the peptide ligands and sites in the receptors. The development of small-molecule antagonists to the hypocretin receptors is just beginning. The small molecules developed so far will continue to be valuable tools to identify how the hypocretin system functions. Although the challenge of developing a small-molecule antagonist to HcrtR1 for the treatment of obesity or the development of a small-molecule agonist to HcrtR2 for the treatment of narcolepsy is significant, the results so far are encouraging.

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Rescue of Narcoleptic Orexin Neuron-Ablated Mice by Ectopic Overexpression of Orexin Peptides

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1. INTRODUCTION

Human narcolepsy-cataplexy is a debilitating neurological disease characterized by excessive daytime sleepiness, premature transitions to REM sleep (called sleep-onset REM periods), and cataplexy (sudden bilateral skeletal muscle weakness without impairment of consciousness). Narcolepsy-cataplexy affects males and females equally, with an estimated prevalence of 0.02–0.18% within white populations. Most cases of narcolepsy-cataplexy are idiopathic, and symptoms appear around adolescence and last throughout life. Several studies have reported a strong association between certain class II HLA haplotypes on human chromosome 6 and narcolepsy-cataplexy; HLA DQB1*0602 and DQA1*0102 are found in up to 90% of affected populations, compared with 12–38% in the general population, suggesting that autoimmunity plays a role in the disorder. Excessive sleepiness is treated using psychostimulants, such as amphetamines, and modafinil; cataplexy is treated with tricyclic antidepressants such as clomipramine. However, this therapeutic regimen is problematic owing to limited effectiveness, undesirable side effects such as insomnia or symptom rebounds, and the potential for abuse *(1,2)*.

Recent studies have concluded that narcolepsy-cataplexy in humans and animal models results from failure of signaling mediated by orexin (hypocretin) neuropeptides *(3–8)*. Orexin-A and orexin-B are cleaved from a single precursor polypeptide, prepro-orexin, which is expressed by a select population of neurons clustered around the perifornical lateral hypothalamus (LH) *(9)*. Orexin neurons robustly innervate specific nuclei in the basal forebrain, hypothalamus, and brainstem that are involved in sleep/wake regulation *(6,10)*. Intracerebroventricular (icv) injections of orexin peptides, administered acutely in mice and rats, have been shown to increase wakefulness and suppress both non-REM and REM sleep *(11,12)*.

Murine models of narcolepsy-cataplexy, generated by disrupting the *prepro-orexin* gene (*orexin* knockout mice) or inducing postnatal death of orexin-producing neurons (*orexin/ataxin-3* transgenic mice), exhibit a phenotype remarkably similar to that of human narcolepsy-cataplexy: difficulty maintaining waking periods (sleep/wake state fragmentation), intrusions of REM sleep into wakefulness resembling cataplexy, and increased amounts of REM sleep during the active phase *(6,8)*. Whereas mutations in one of two known genes encoding orexin receptors (orexin receptor type 2 [OX_2R]) are responsible for an autosomal recessive form of inherited narcolepsy-cataplexy in canines *(4)*, studies in mice suggest that loss of signaling

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through both orexin receptors is associated with a more severe form of the symptom complex analogous to that observed in humans *(13,14)*.

Indeed, orexin-A, a nonselective agonist for both orexin receptors *(9)*, is low or undetectable in the cerebrospinal fluid of up to 95% of human cases of narcolepsy-cataplexy examined *(1)*, and *orexin* mRNA and orexin-A immunoreactivity are drastically reduced in postmortem hypothalamic samples from narcoleptic-cataplectic humans *(5,7)*. Because of a strong association with certain human leukocyte antigen alleles, as described above, it has been speculated that narcolepsy-cataplexy may result from selective autoimmune degeneration of orexin neurons *(15)*. Consistent with this hypothesis, residual gliosis was also observed in the area of loss of orexin neurons in postmortem brains from narcoleptic-cataplectic humans *(7)*. These findings open up the possibility that replacement therapies based on administration of orexin receptor agonists or chronic ectopic expression of orexin peptides by some genetic methods might prove beneficial. Negative arguments against this idea are that release of orexin peptides might need to be tightly regulated temporally and spatially to execute their normal functions, and loss of orexin signaling for a long time might cause secondary degeneration of other brain areas that could take away the ability of narcolepsy-cataplexy patients to respond to orexins.

To test these arguments, we examined whether symptoms of murine narcolepsy-cataplexy could be reversed by constitutive ectopic production of orexin peptides from a *prepro-orexin* transgene in *orexin/ataxin-3* transgenic mice; these mice suffer from specific and postnatal degeneration of orexin neurons, as supposed in human narcolepsy-cataplexy patients, through the expression of a poly-Gln-containing fragment of the ataxin-3 polypeptide, the product of the Machado-Joseph disease gene *(8)*.

2. GENETIC RESCUE OF NARCOLEPSY IN OREXIN NEURON-ABLATED MICE

To examine whether the narcolepsy-cataplexy phenotype of orexin neuron-ablated mice could be rescued by ectopic production of orexin peptides, we produced transgenic mice that overexpress a *prepro-orexin* transgene under the control of a β-actin/cytomegalovirus hybrid promoter, which is an ubiquitous promoter (*CAG/orexin* transgenic mice) *(16,17)*. Several stable transgenic lines overexpressed orexin, as determined by Northern blots, radioimmunoassays, and anti-orexin-A immunohistochemistry. In this study, we utilized one line in which the whole-brain levels of orexin-A and -B peptides were increased by nearly 30- and 80-fold, respectively. Mice of this line were crossed to *orexin/ataxin-3* transgenic mice to produce offspring that carry both transgenes (double hemizygous mice). Littermate pairs carrying either one transgene (*CAG/orexin* or *orexin/ataxin-3* transgenic mice), both transgenes (*orexin/ataxin-3; CAG/orexin* double transgenic mice), or neither transgene (wild-type mice) were selected for further experiments.

Anti-orexin-A immunohistochemistry of the brains of 15-wk-old wild-type mice produced a pattern of staining in which orexin-producing cells were clustered in the perifornical LH, as previously described (Fig. 1A and B). In contrast, we found no orexin staining in any part of the brain of *orexin/ataxin-3* transgenic littermates, consistent with destruction of endogenous orexinergic neurons by this age, as previously reported (Fig. 1C and D). Littermates carrying the *CAG/orexin* transgene alone exhibited a widespread, diffuse orexin-immunoreactive staining throughout most of the brain, with highest levels observed in the hypothalamus, amygdala, hippocampus, and brainstem (Fig. 1E and F). Notably, the native orexinergic neurons in *CAG/orexin* transgenic mice were still visible above background in sections containing the LH, owing to the extremely high levels of orexin immunoreactivity in endogenous

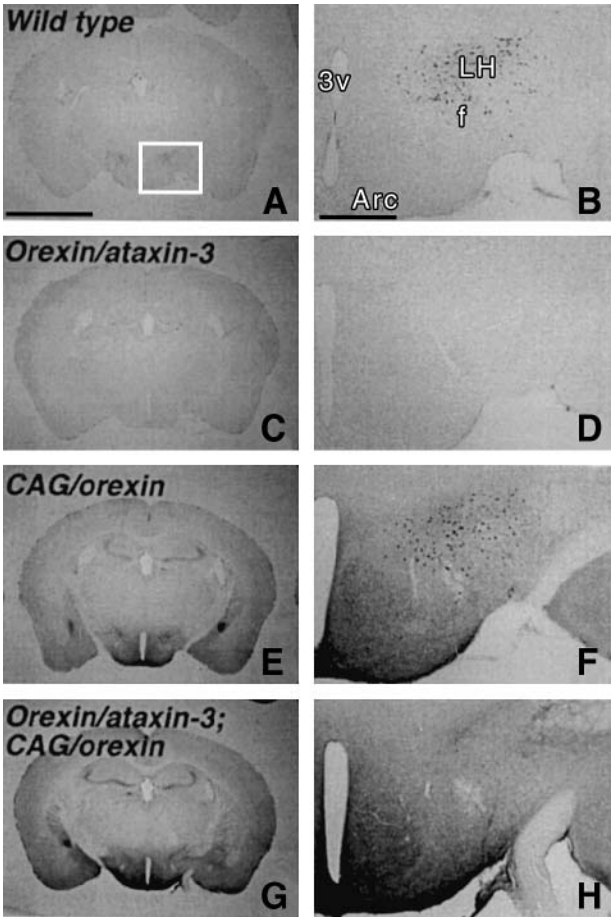

Fig. 1. Immunohistochemical analysis of brains from transgenic mice. Anti-orexin-A immunostaining of coronal sections of brain tissue from wild-type, *orexin/ataxin-3* hemizygous transgenic, *CAG/orexin* hemizygous transgenic, and double hemizygous transgenic mice was performed. Note the punctate staining of native orexin-A-immunoreactive neurons clustered in the perifornical lateral hypothalamus of wild-type mice **(A,B)**, and the absence of orexin-A in the brains of *orexin/ataxin-3* transgenic mice in which native orexinergic neurons have degenerated **(C,D)**. *CAG/orexin* transgenic mice have widespread, diffuse, ectopic production of orexin-A in addition to that observed in native neurons **(E,F)**. In contrast, *orexin/ataxin-3;CAG/orexin* double transgenic mice exhibit only the ectopic pattern of orexin-A; native neurons are absent **(G,H)**. The inset in (A) illustrates the location and size of magnified panels on the right. LH, lateral hypothalamus; f, fornix; Arc, arcuate nucleus of the hypothalamus; 3V, third ventricle scale bar = 3 mm (*A, C, E, G*) and 0.5 mm (*B, D, F, H*). (Modified from ref. *16*.)

neurons relative to those expressing the transgene ectopically. The o*rexin/ataxin-3; CAG/orexin* double transgenic mice exhibited a similar pattern of widespread orexin signal throughout the hypothalamus and remaining brain. Notably absent, however, were endogenous orexinergic neurons (Fig. 1G and H).

We tested mice of all four genotypes for the presence of cataplectic arrests during the dark (active) phase using infrared videophotography. We adapted a behavioral paradigm in which narcoleptic mice are exposed to a novel environment that tends to increase the frequency of such arrests, a method that has proved effective in screening for pharmacological suppression by clomipramine of cataplexy in narcoleptic mice. As in wild-type mice, mice carrying the

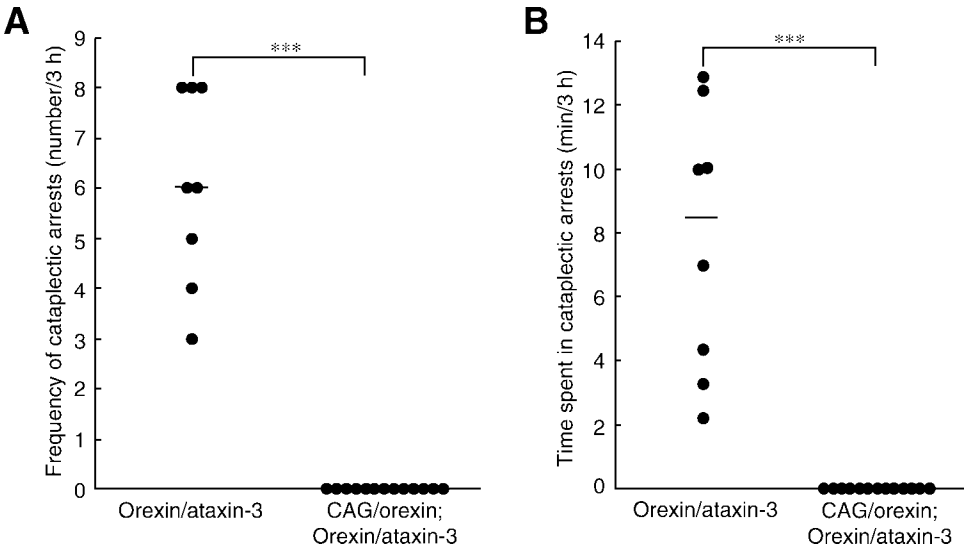

Fig. 2. The *CAG/orexin* transgene prevents cataplectic behavioral arrests in orexin neuron-ablated mice. Cataplectic arrests are absent in *orexin/ataxin-3;CAG/orexin* double hemizygous transgenic mice compared with *orexin/ataxin-3* hemizygous transgenic litter mates. Number of cataplectic arrests observed **(A)** and total time spent in cataplexy **(B)** in each mouse are shown for 3-h sessions. Bars represent medians. ***, $p < 0.0001$ by Mann-Whitney test. (Modified from ref. *16*.)

CAG/orexin transgene alone exhibited no behavioral arrests of any kind (data not shown). The frequencies of arrests in *orexin/ataxin-3* mice and *orexin/ataxin-3;CAG/orexin* double transgenic littermates were then compared (Fig. 2). All *orexin/ataxin-3* mice exhibited cataplexy-like arrests within the 3-h observation period, as previously described *(8)*. In contrast, no arrests were observed in any of the *orexin/ataxin-3;CAG/orexin* double transgenic littermates under these same conditions. Thus, ectopic transgenic expression of orexin peptides prevents cataplectic arrests in mice in which endogenous orexin neurons have been ablated.

We then examined sleep/wake patterns over the entire 24-h day in each group of mice, by carrying out electroencephalograph/electromyelograph (EEG/EMG) recordings. As expected, the dark-phase sleep/wake patterns of *orexin/ataxin-3* mice, summarized in hypnograms (Fig. 3), exhibited severe fragmentation of wakefulness and sleep with numerous brief non-REM sleep episodes and frequent transitions to REM sleep with little or no latency from preceding wakefulness. Such transitions were observed without exception in each mouse carrying the *orexin/ataxin-3* transgene alone. In contrast, *orexin/ataxin-3; CAG/orexin* double transgenic littermates exhibited longer, more consolidated bouts of wakefulness and normalized amounts of REM sleep during the dark phase. Most importantly, no direct or premature wake-REM sleep transitions were ever detected in any double transgenic mice. Hypnograms of wild-type mice from the same line were typical of the C57BL/6J strain, illustrating normal sleep/wake patterns during this period. Notably, compared with wild-type and narcoleptic mice, hypnograms of *orexin/ataxin-3;CAG/orexin* double transgenic mice exhibited increased levels of fragmentation of sleep specifically during the light (resting) phase, resulting possibly from chronic, abnormally high orexin tone owing to the *CAG/orexin* transgene (discussed below in Subheading 3.).

Sleep/wake states in these mice are also illustrated by hourly plots of amounts of time spent in wakefulness, non-REM sleep, and REM sleep over 24 h (Fig. 4). Consistent with a previous description *(8)*, *orexin/ataxin-3* mice showed selective increases in amounts of REM

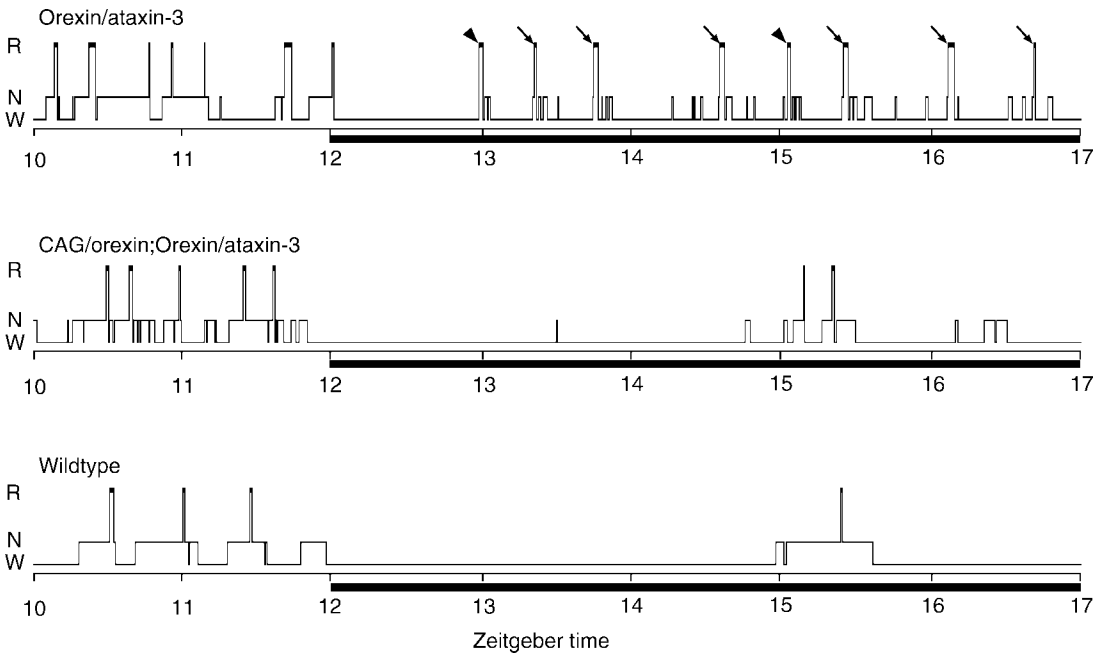

Fig. 3. Sleep/wake cycles of typical transgenic and wild-type mice. Hypnograms represent concatenated 20-s epochs of EEG/EMG activity, scored as either awake (W), non-REM sleep (N), or REM sleep (R). Seven hours per mouse, including transitions from the light phase to the dark phase (solid bar) are shown. The *orexin/ataxin-3* transgenic mouse exhibits fragmentation of wakefulness during the dark phase and frequent premature onsets of REM sleep that occur immediately after wakefulness (arrowheads) or after less than 1 min of preceding non-REM sleep (arrows). In contrast, the *orexin/ataxin-3;CAG/orexin* double transgenic mouse has more consolidated wakefulness during the dark phase. As in the wild-type mouse, no direct or premature transitions from wakefulness to REM sleep were ever observed in the double transgenic mouse. (Modified from ref. *16.*)

sleep during the dark phase compared with wild-type controls. Importantly, mice carrying both transgenes exhibited a normalization of REM sleep amounts during the dark phase with an additional tendency toward reduced amounts of REM sleep during the light phase as well. Overall, ectopic expression of the *CAG/orexin* transgene prevented the development of symptoms of narcolepsy-cataplexy despite the postnatal ablation of endogenous orexin neurons, supporting a specific role for orexin peptides in preventing narcolepsy-cataplexy.

From genetic studies alone, the formal possibility remains that the *prepro-orexin* transgene rescues the phenotype of *orexin/ataxin-3* mice by precluding any period of orexin deficiency following the degenerative loss of native orexin neurons in young *orexin/ataxin-3* mice. To exclude this explanation, we also demonstrated that central administration of orexin-A in *orexin/ataxin-3* mice acutely suppressed cataplectic behavioral arrests and increased wakefulness *(16)* (discussed in chap. 25). Thus, results of genetic and pharmacological rescue experiments indicate that even after substantial periods of complete orexin deficiency, the neural mechanisms required for orexin-mediated arousal and suppression of cataplexy, e.g., orexin receptors, intracellular signaling, postsynaptic neural networks, and other downstream neurotransmitter pathways, remain anatomically and functionally intact. Our pharmacological study suggested that acutely administered orexin-A has stronger arousal effects in *orexin/ataxin-3* transgenic mice than in wild-type controls. This conclusion provides a valid ground for pursuing replacement therapies of human narcolepsy-cataplexy

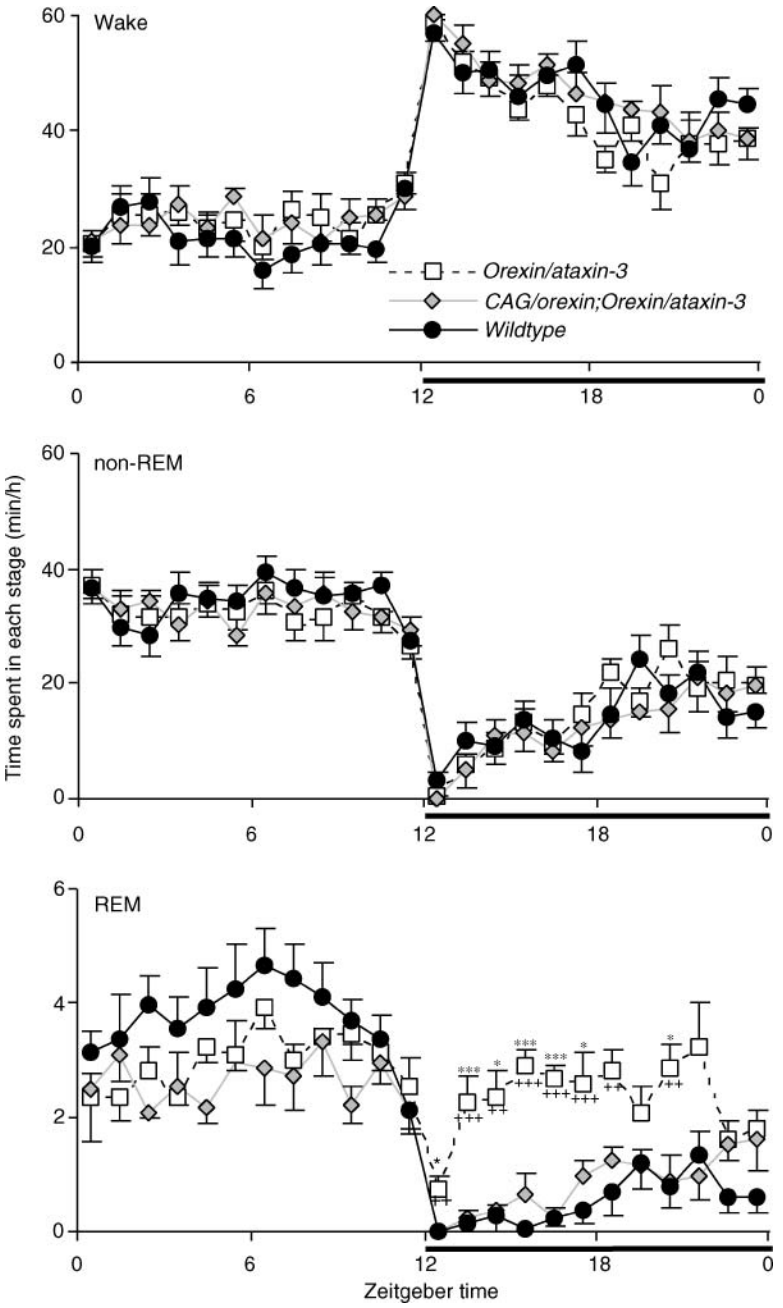

Fig. 4. Hourly plots of sleep/wake states in transgenic and wild-type mice. Narcoleptic *orexin/ataxin-3* transgenic mice as well as *orexin/ataxin-3;CAG/orexin* double transgenic mice exhibit hourly amounts of wakefulness and non-REM sleep similar to that of wild-type mice. In contrast, *orexin/ataxin-3* transgenic mice exhibit significantly increased amounts of REM sleep during the dark phase (solid horizontal bars). Double transgenic mice exhibit a specific rescue of this abnormality compared with wild-type mice. *, $p < 0.05$, **, $p < 0.005$, ***, $p < 0.0005$ compared with wild-type mice; †, $p < 0.05$, ††, $p < 0.005$, and †††, $p < 0.0005$ compared with *orexin/ataxin-3;CAG/orexin* double transgenic mice by ANOVA and Tukey's post hoc tests. Values are means ± SE ($n = 10$ for wild-type mice, $n = 6$ for *orexin/ataxin-3* mice, and $n = 5$ for *CAG/orexin;orexin/ataxin-3* double transgenic mice). (Modified from ref. *16*.)

based on administration of orexin receptor agonists or chronic ectopic expression of orexin peptides by some genetic methods.

3. DOES OVEREXPRESSION OF OREXIN PEPTIDES BRING ABOUT ONLY GOOD THINGS?

Now we know that spatially or temporally specific activation of orexin receptors is not necessary to prevent narcolepsy-cataplexy. The next important question is: does ectopic overexpression of orexin peptides only restore the narcoleptic phenotype of *orexin/ataxin-3* transgenic mice, or does it do anything more? To examine the effects of orexin overexpression itself on spontaneous sleep/wake patterns, we monitored the EEG/EMG of *CAG/orexin* transgenic mice and their wild-type littermates. Most notably, *CAG/orexin* mice have significant suppression of the amounts of REM sleep exhibited during the light phase. In addition to disturbance of REM sleep, the sleep/wake cycle was mildly fragmented in *CAG/orexin* transgenic mice, especially in the light phase, as already observed in *orexin/ataxin-3;CAG/orexin* double transgenic mice (Fig. 3, described above in Subheading 2). Primarily, orexin overexpression seemed to induce an inability to maintain sleep states (J.T.W., T.S., and M.Y., unpublished data). Thus, too many orexin peptides, as well as a lack of orexins, results in failure of proper regulation of the sleep/wake cycle.

4. FUTURE TREATMENT OF HUMAN NARCOLEPSY-CATAPLEXY

How can we apply orexin biology to the treatment of human narcolepsy-cataplexy? Since central orexin administration in mice *(16)* and systemic orexin administration in dogs *(18)* have antinarcoleptic effects in animal narcolepsy-cataplexy models, the most practical and straightforward way to treat human narcolepsy-cataplexy seems to be administration of orexin receptor agonists, as discussed in chapter 25. As we saw above, however, chronic overproduction of orexins in transgenic mice caused destabilization of sleep mainly in their inactive phase. In contrast to mice, normal humans maintain a very long awake episode and usually do not need to sleep during their active phase. Thus, in humans, therapy would be taking orexin receptor agonists, whose effects last for several hours, during the day (the active phase for humans) to maintain wakefulness but not during the night; improvement of daytime wakefulness might also consolidate nighttime sleep secondarily. The development of specific, and blood-brain barrier-permeable, agonists for orexin receptors is awaited, and further evaluation of the effects caused by long-term administration of such agonists would be necessary.

Our demonstration of a genetic rescue of narcolepsy-cataplexy may also have implications for future therapies of human narcolepsy-cataplexy, which might involve orexin gene therapy by using viral vectors, or transplantation of orexin neurons, stem cell precursors, or cells engineered to release orexin peptides. These strategies might allow more spatially restricted administration of orexin peptides than systemic administration of orexin receptor agonists, which could prevent undesirable side effects, if any, although temporal regulation of orexin release would be more difficult.

5. CONCLUSIONS

Owing to the discovery of orexin signaling deficiency in human narcolepsy-cataplexy, we now have a huge potential for understanding the precise etiology, determining a reliable diagnosis, and providing novel treatment of this life-long, debilitating neurological disease. Progress in these fields would be expected to improve the quality of life dramatically in patients with narcolepsy-cataplexy.

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Hypocretin/Orexin Replacement Therapy in Hypocretin/Orexin-Deficient Narcolepsy

An Overview

Nobuhiro Fujiki, MD, PhD and Seiji Nishino, MD, PhD

1. INTRODUCTION

Soon after the discovery of narcolepsy genes in dogs (*hypocretin receptor 2* [*hcrtr-2*]) *(1)* and mice (*preprohypocretin*) *(2)*, it was determined that most (about 90%) human narcolepsy is associated with hypocretin/orexin ligand deficiency *(3–5)*. The hypocretin/orexin ligand deficiency in humans is probably owing to the acquired cell death of hypocretin-containing neurons, which is triggered for several reasons: (1) the onset of narcolepsy in humans is in the late developmental years (i.e., puberty) *(6)*, whereas in narcoleptic animals with hypocretin-related gene mutations onset is in the much earlier, developmental years *(2,7)*; and (2) mice/rats with postnatally ablated hypocretin-containing neurons (*orexin/ataxin-3* transgenic) exhibit a narcolepsy phenotype (cataplexy, sleep fragmentation, and tendency to obesity) similar to that of human narcolepsy *(8)*.

The findings on hypocretin ligand deficiency in human narcolepsy led to two important clinical applications: (1) biological measurements of hypocretin levels to diagnose narcolepsy, and (2) hypocretin replacement therapy. The first application is now being established, and cerebrospinal fluid (CSF) hypocretin measures will be included in the second revision of the International Classification of Sleep Disorders (ISCD).

Hypocretin replacement therapy for deficiency is also straightforward, and it is likely that narcolepsy can be treated like other endocrine and neurological disorders, such as type 1 diabetes (deficiency or failure of the pancreas to secrete insulin, treated with insulin injections) *(9)* and Parkinson's disease (progressive degeneration of the substantia nigra pars compacta dopamine neurons treated with dopamine precursor) *(10)*.

However, several concerns need to be addressed to make this therapeutic option in human narcolepsy practical: (1) compounds administered should reach the CNS adequately, (2) the spatiotemporal issues of drug administration, must be addressed, and, most importantly, (3) it is still not known whether any secondary, irreversible changes owing to chronic hypocretin deficiency are involved in the symptoms of narcolepsy. If this is the case, hypocretin replacement therapy may only possess limited efficacy. In this chapter, we discuss the potential for hypocretin replacement therapy for human narcolepsy based on available data on the pharmacokinetics and pharmacodynamics of the hypocretin peptide as well as the results of hypocretin replacement obtained in animal models of narcolepsy.

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2. THE HYPOCRETIN/OREXIN SYSTEM AND NARCOLEPSY

Human narcolepsy is a chronic sleep disorder characterized by excessive daytime sleepiness (EDS), cataplexy, and other abnormal manifestations of REM sleep, such as hypnagogic hallucinations and sleep paralysis *(11)*. Narcolepsy is not a rare disease, affecting approximately 1 in 2000 in the general population, which is equivalent to Parkinson's disease *(12)* or multiple sclerosis *(13)*. EDS in narcolepsy is overwhelming and is characterized by recurring episodes of sleep during the daytime *(11)*. Patients also experience disturbed nocturnal sleep, although less frequently emphasized *(11)*. The total amount of sleep that narcoleptic subjects have over 24 h does not exceed that of normal subjects *(14)*. Therefore, narcolepsy is not an intrinsic hypersomnia but rather a pathological condition involving instability of the vigilance state (sleep/wake fragmentation or problems maintaining wake/sleep for longer period).

Cataplexy, another disabling symptom, is the sudden onset of muscle atonia triggered by emotional excitement, such as laughter, elation, or anger during wakefulness *(11)*. Cataplexy has been thought of as an abnormal intrusion of the physiological phenomenon of REM sleep into wakefulness. It should be noted, however, that other REM sleep phenomena (hypnagogic hallucinations, sleep paralysis, and sleep-onset REM periods) are often observed in normal subjects, especially when their sleep/wake pattern is disturbed *(15–17)*. In contrast, the occurrence of cataplexy is unique and pathognomonic to narcolepsy; thus cataplexy is more closely related to pathophysiological mechanisms of narcolepsy *(11)*.

It is now known that narcolepsy is caused by loss of the signal transduction of the hypocretin/orexin peptides in humans and animals *(1–4,18,19)*. Hypocretins (hypocretin-1 and hypocretin-2), also called orexins (orexin-A and orexin-B), are hypothalamic neuropeptides cleaved from a precursor preprohypocretin (prepro-orexin) peptide *(20,21)*. Hypocretin-1, with 33 residues, contains four cysteine residues forming two disulfide bonds. Hypocretin-2, consisting of 28 amino acids, shares a similar sequence homology, especially on the C-terminal side, but has no disulfide bonds (a linear peptide) *(21,22)* (Fig. 1). There are two G-protein-coupled hypocretin receptors, hypocretin receptor 1 (hcrtr-1) and hcrtr-2, also called orexin receptor 1 and 2 (OX_1R and OX_2R), and distinct distributions of these receptors in the brain are known *(21,22)* (Fig. 1).

In humans, mutations in hypocretin-related genes are extremely rare *(19)*, but hypocretin ligand production is absent or extremely low in most patients with narcolepsy-cataplexy *(3,4)*. On the other hand, familial narcolepsy in Dobermans and Labradors is caused by a mutation in *hcrtr-2/OX_2R* (Table 1) *(1)*. In these animals, the production of hypocretin/orexin ligands is preserved, suggesting the continuation of hypocretin receptor 1-mediated functions *(23)*. Thus, the loss of hypocretin signal transduction via hcrtr-2/OX_2R is the essential qualification for expression of the narcolepsy phenotype. This is consistent with a sequence of studies in mice models. Chemelli et al. *(2)* produced the *prepro-orexin* gene knockout mouse and found a phenotype (i.e., shorter REM sleep latency, sleep fragmentation, and cataplexy-like behavioral arrest) similar to that of human narcolepsy (Table 1). Hara et al. *(8)* subsequently produced hypocretin/orexin cell-ablated mice by transferring the human ataxin-3 cDNA with the human *prepro-orexin* promoter gene as a cell-specific enhancer. The phenotype of this cell-ablated model is almost identical as that of ligand gene knockout mice (Table 1). Willie et al. *(24)* further produced *hcrtr-2/OX_2R* gene knockout mice and reported that these mice apparently exhibited the narcoleptic phenotype, although the severity was less than that of ligand gene knockout mice. On the other hand, *hcrtr-1/OX_1R* gene knockout mice

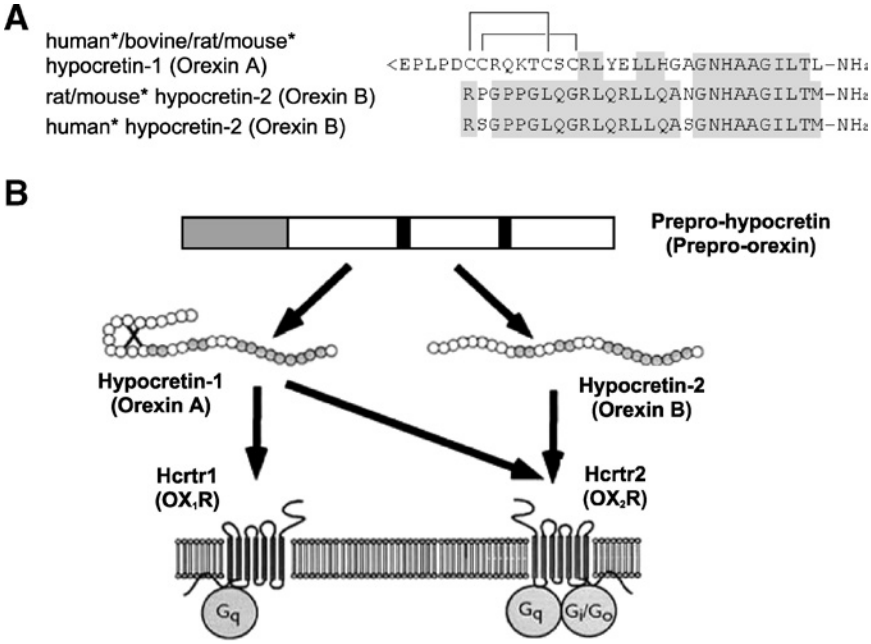

Fig. 1. (A) Structures of mature hypocretin-1 (orexin-A) and hypocretin-2 (orexin-B) peptides. The topology of the two intrachain disulfide bonds in orexin-A is indicated. Amino acid identities are indicated by shaded areas. Asterisks indicate that human and mouse sequences were deduced from the respective cDNA sequences and not from purified peptides. **(B)** Schematic representation of the hypocretin (orexin) system. Hypocretin-1 (orexin-A) and hypocretin-2 (orexin-B) are derived from a common precursor peptide, prepro-hypocretin (prepro-orexin). The actions of hypocretins are mediated via two G-protein-coupled receptors termed hypocretin receptor 1 (hcrtr-1) and hypocretin receptor 2 (hcrtr-2), also known as orexin-1 (OX_1R) and orexin-2 (OX_2R) receptors, respectively. Hcrtr-1 is selective for hypocretin-1, whereas hcrtr-2 is nonselective for both hypocretin-1 and hypocretin-2. Hcrtr-1 is coupled exclusively to the G_q subclass of heterotrimeric G proteins, whereas in vitro experiments suggest that hcrtr-2 couples with Gi/o, and/or Gq. (Adapted from ref. *22*.)

Table 1
Characterization of Narcolepsy in Humans and Experimental Animals[a]

Type	Abnormality in hypocretin system
Human	
Sporadic (95%)	Hypocretin ligand deficiency (~90%)
Familial (~5%)	Hypocretin ligand deficiency (~75%)
Dog	
Sporadic (17 breeds)	Hypocretin ligand deficiency
Familial (Dobermans, Labradors, Dachshunds)	Mutation in *hcrtr2* gene
Mouse	
Hypocretin knockout	Hypocretin ligand deficiency
orexin/ataxin-3 transgenic (also in rat)	Hypocretin cell death

[a]There are three other genetically engineered mouse models, the *hcrtr-1*, the *hcrtr-2*, and both genes (double knockout mouse). The double knockout mouse has a phenotype similar to that of hypocretin ligand knockout mice. The *hcrtr-1* knockout mouse does not show an overt narcoleptic phenotype. On the other hand, although the symptoms are less severe than in the ligand knockout mouse, the *hcrtr-2* knockout mouse has apparent symptoms.

have no overt behavior abnormalities and exhibit only mild fragmentation of the vigilance states *(25,26)*. These observations also confirm that hcrtr-2/OX_2R-mediated function plays a critical role in the pathophysiology of narcolepsy, even though hcrtr-1/OX_1R may have some additional effects on controlling the sleep wake states in mice.

3. CURRENT TREATMENT IN HUMAN NARCOLEPSY

For management of narcolepsy symptoms in humans, pharmacological treatment is usually employed *(11,27,28)*. For EDS, amphetamine-like central nervous system (CNS) stimulants or modafinil (a nonamphetamine stimulant with undetermined mechanisms of action) are most often used (Table 2). These compounds possess wake-promoting effects in narcoleptic subjects as well as in control populations, but very high doses are required to normalize the abnormal sleep tendency during the daytime *(29)*. For consolidating nighttime sleep, benzodiazepine hypnotics or γ-hydroxybutyrate (GHB) are occasionally used *(11,27,28)*. Since amphetamine-like stimulants and modafinil have little effect on cataplexy, tricyclic antidepressants, such as imipramine or clomipramine are used in addition to control cataplexy *(11,27,28)* (Table 2). However, these compounds can cause a number of side effects, such as dry mouth, constipation, or impotence. GHB is also used for the treatment of cataplexy; its mechanism of action remains unknown. The antidepressants and GHB are also effective for the other REM sleep phenomena.

Most of these compounds are known to act on the monoaminergic systems. The compounds effective for EDS mostly target the presynaptic enhancement of dopaminergic neurotransmission (dopamine [DA] release and DA uptake inhibition) *(30,31)*, whereas anticataplectics are mostly mediated by enhancement of noradrenergic neurotransmission *(30)*. Animal data suggest that these compounds are effective for EDS and cataplexy, regardless of hypocretin receptor dysfunction and ligand deficiency *(27,32,33)*, and are likely to act on downstream pathways of hypocretin neurotransmission. A series of anatomical and functional findings have suggested that these monoaminergic systems are likely to mediate the effects of hypocretin on vigilance and muscle tonus control *(34–41)*. In addition, the loss of hypocretin input could induce monoaminergic dysfunction *(41,42)*.

Treatment with these compounds controls symptoms and does not act at the level of primary abnormalities in narcolepsy. Unfortunately, these drugs have undesirable side effects and are not tolerance by many patients. Furthermore, most patients need to take two different classes of compounds to manage both EDS and cataplexy, creating a variety of complications *(11,27,28)*. For these reasons, an ideal treatment is awaited that is more directly pathophysiologically oriented. Hypocretin/orexin peptides or their mimetics are the most promising agents for this ligand-deficient condition.

4. HYPOCRETIN REPLACEMENT THERAPIES IN GENETICALLY ENGINEERED NARCOLEPTIC MICE

Using hypocretin neuron-ablated narcoleptic mice (*orexin/ataxin-3* transgenic mice) *(8)*, Mieda et al. *(43)* recently demonstrated that replacement of central hypocretin by either pharmacologic (icv injection of hypocretin-1) or genetic (ectopic expression of hypocretin in the brain) manipulations allowed the rescue of the narcolepsy-cataplexy phenotype in these mice *(43)*. These narcoleptic mice frequently exhibited behavioral arrests (i.e., cataplexy) during the active period *(8)*. ICV administration of 3 nmol of hypocretin-1 at Zeitgeber time 12 (ZT12) almost completely reduced these attacks (effects were estimated during a 3-h observation period), whereas saline injections did not modify the severity of attacks (Fig. 2A). The effects

Table 2
Commonly Prescribed Treatments for EDS and Cataplexy and Their Pharmacological Properties

Compound	Pharmacological properties
Stimulants	
Amphetamine	Increases monamine release (DA > NE >> 5-HT); blocks monoamine reuptake and MAO at high doses; the D-isomer is more specific for dopaminergic transmission and is a better stimulant compound
Methylphenidate	Slightly less effect on monoamine release; short half-life; blocks monoamine uptake at lower dose than amphetamine
Modafinil	Low binding affinity for DA transporter; mode of action debated
Anticataplectic compounds	
NE/5-HT selective	
Venlafaxine	Very effective
NE selective	
Atomoxetine	Very effective
5-HT selective (SSRI)	
Fluoxetine	High therapeutic doses are often needed; active metabolite norfluoxetine has more adrenergic effects
Trycyclics	
protriptyline, imipramine, desipramine, chlomipramine	Monomoaminergic uptake blockers, less selective for NE, DA, and 5-HT; anticholinergic effects; often have active metabolites (desipramine for imipramine, desmethyl-chlomipramine for chlomipramine)
Other	
γ-Hydroxybutyric acid (GHB, sodium oxybate)	May act via $GABA_B$ or via specific GHB receptors; reduces dopamine release

Abbreviations: DA, dopamine; NE, norepinephrine; 5-HT, serotonin; EDS, excessive daytime sleepiness; MAO, monoamine oxidase; SSRI, selective serotonin uptake inhibitor; GABA, γ-aminobutyric acid.

of icv administration of hypocretin-1 on sleep architecture was also assessed in these mice. Narcoleptic mice exhibited selective increases in the amount of REM sleep during the dark phase, compared with wild-type mice. Administration icv of hypocretin-1 (3 nmol) at ZT 12 h or ZT 15 h significantly increased wakefulness and suppressed non-REM and REM sleep in both wild-type and narcoleptic mice. Interestingly, the authors reported that narcoleptic mice showed greater responses to hypocretin-1 compared with wild-type animals and promoted more profound wakefulness and a reduction in non-REM and REM sleep were promoted. These results point to the feasibility of treating symptoms of narcolepsy-cataplexy by using agonists for hypocretin receptors.

Mieda et al. *(43)* further examined whether symptoms of hypocretin neuron-ablated narcolepsy mice could be reversed by ectopic production of hypocretins in the mouse brain. The *CAG/orexin* transgene was constructed so that the rat *prepro-orexin* gene would be expressed under control of the *β-actin/CAG* hybrid promoter, which drives widespread expression of hypocretin in the brain *(44)*. The authors found that ectopic expression of hypocretin completely prevented cataplectic arrests in orexin-ataxin-3 narcoleptic mice. Furthermore,

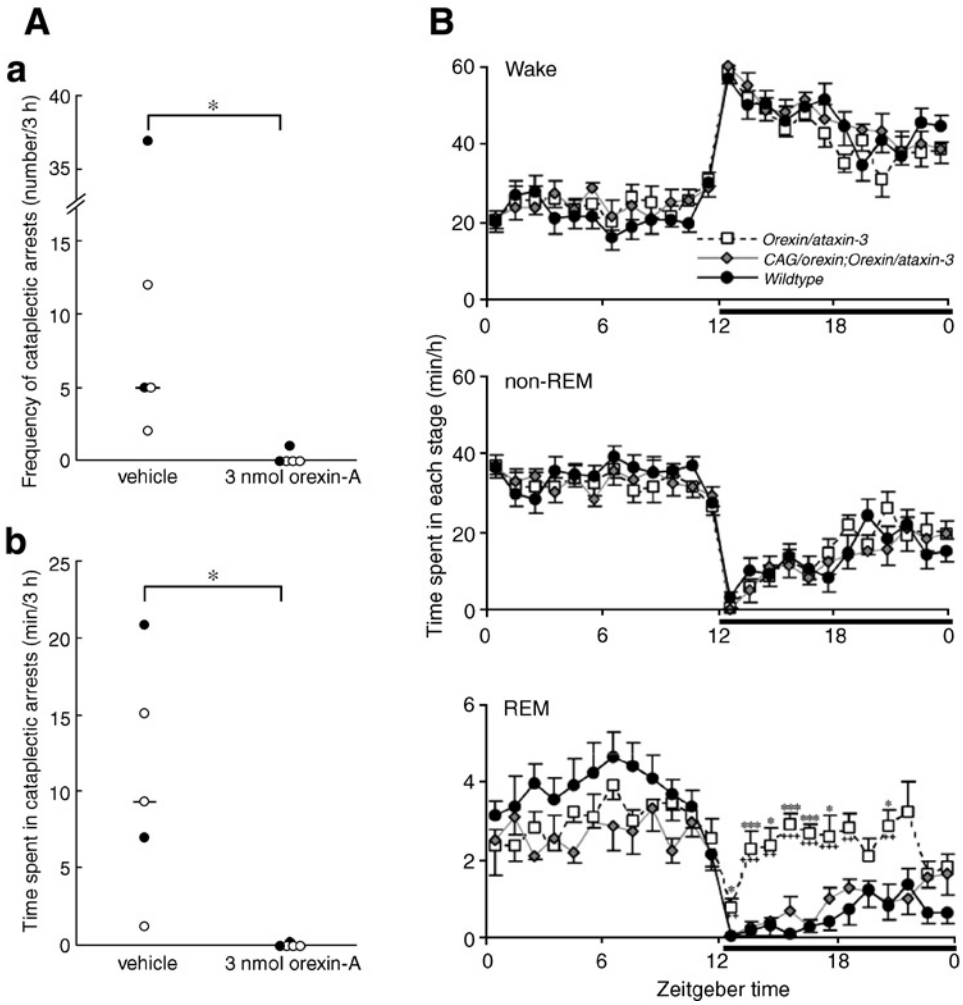

Fig. 2. (A) Pharmacological rescue of narcolepsy in hypocretin neuron-ablated mice. Vehicle (artificial cerebrospinal fluid) and hypocretin-1/orexin-A (3 nmol per mouse) were administered by bolus injections into the lateral ventricles of five narcoleptic *orexin/ataxin-3* transgenic mice before onset of the dark phase. Number of cataplectic arrests observed (a) and total time spent in cataplexy (b) in each mouse are shown for 3-h sessions. Bars represent medians. *, $p < 0.05$ by Wilcoxon's signed-rank test **(B)** Genetic rescue of narcolepsy in hypocretin neuron-ablated mice. Hourly plots of sleep-wake states in transgenic and wild-type mice. Narcoleptic *orexin/ataxin-3* transgenic mice as well as *orexin/ataxin-3*; *β-actin cytomegalovirus (CAG)/orexin* double transgenic mice exhibit hourly amounts of wakefulness and non-REM sleep that are similar to those of wild-type mice. In contrast, *orexin/ataxin-3* transgenic mice exhibit significantly increased amounts of REM sleep during the dark phase (solid horizontal bars). Double transgenic mice exhibit a specific rescue of this abnormality compared with wild-type mice. *, $p < 0.05$; **, $p < 0.005$; ***, $p < 0.0005$ compared with wild-type mice; and, †, $p < 0.05$; ††, $p < 0.005$; †††, $p < 0.0005$ compared with *orexin/ataxin-3*;*CAG/orexin* double transgenic mice by ANOVA and Tukey's post hoc tests. Values are mean ± SEM. ($n = 10$ for wild-type mice, $n = 6$ for *orexin/ataxin-3* mice, and $n = 5$ for *CAG/orexin*;*orexin/ataxin-3* double transgenic mice.) (Adapted from ref. *43*.)

orexin/ataxin-3; *CAG/orexin* double-transgenic litter mates exhibited longer, more consolidated bouts of wakefulness and normalized amounts of REM sleep during the dark phase, and no direct or premature wake-REM sleep transitions were ever detected in any double transgenic mice *(43)* (Fig. 2B).

These results indicate that hypocretin neuron-ablated mice retain the ability to respond to orexin neuropeptides and that hypocretin receptors, intracellular signaling, postsynaptic neural networks, and other downstream neurotransmitter pathways remain anatomically and functionally intact. Most importantly, a temporally regulated and spatially targeted secretion of hypocretin is not likely to be necessary to prevent narcoleptic symptoms *(43)*.

5. HYPOCRETIN REPLACEMENT IN CANINE NARCOLEPSY

In contrast to murine narcolepsy models, canine narcolepsy is a naturally occurring animal model. Although the mutation in the *hcrtr-2* gene was found in narcolepsy in Dobermans and Labradors *(1)*, to date seven of seven sporadic narcoleptic dogs tested were found to be ligand deficient, sharing a common pathology with most human narcoleptic subjects *(23,45–47)*. Both familial and sporadic narcoleptic dogs have been used for various pharmacological experiments including use of the compounds currently employed for narcolepsy treatment *(27,32,33,48–56)*, and results demonstrate that compounds effective with human narcolepsy are also effective in these animals.

Using hypocretin *hcrtr-2*-mutated narcoleptic Dobermans, John et al. *(57)* reported that a single iv administration of 3 μg/kg hypocretin-1 significantly reduced cataplexy, whereas 4 μg/kg significantly aggravated it. They also reported that iv hypocretin-1 at 3 μg/kg significantly reduced REM sleep and improved sleep fragmentation in narcoleptic Dobermans. The authors argued that some of these effects might have occurred because of hypocretin-1 acting at hcrtr-1, which is abundant in the pontine locus coeruleus (LC), or at other unidentified hypocretin receptors. However, we could not replicate these results, and the iv administration of hypocretin-1 at these dose ranges did not modify cataplexy (up to 2 μg/kg) in *hcrtr-2* mutated narcoleptic Dobermans or the sleep patterns (up to 6 μg/kg) in *hcrtr-2* mutated narcoleptic Dobermans, as well as in control Dobermans *(45)*. The reason for this discrepancy is not known, although it might be owing to the quality of the peptides used in both studies. However, we further confirmed that icv administration (up to 30 nmol for sleep study, and 120 nmol for cataplexy testing) of hypocretin-1 in *hcrtr-2*-mutated narcoleptic Dobermans had no effect on cataplexy and wake/non-REM sleep (only REM sleep was significantly reduced) (Fig. 3) *(45)*. In contrast, 30 nmol of icv hypocretin-1 potently reduced non-REM and REM sleep in control Dobermans (Fig. 3) *(45)*. Our results were also consistent with the results on the effects of hypocretin-1 on sleep in *hcrtr-1* and *hcrtr-2* knockout mice: non-REM sleep suppression by hypocretin-1 is mainly mediated by hcrtr-2, whereas REM suppression is mostly mediated by hcrtr-1 (Mieda et al., personal communication). Thus we concluded that iv administration of hypocretin-1 at this dose range (up to 4 μg/kg) is not likely to have any effects on either narcoleptic or control Dobermans *(45)*.

Kodama and Kimura *(58)* injected rats with hypocretin-1 iv at 1 or 10 nmol/animal (corresponding to 12 and 120 μg/kg) and observed increases in CSF hypocretin levels of 1.4-fold (at 1 nmol/animal) and 1.8-fold (at 10 nmol/animal). At the extracellular levels, they found twofold increases (at 1 nmol/animal) as well as increases in glutamate release from the LC and wake-promoting effects, suggesting that systemic administration of hypocretin-1 induces wake-promoting effects through central activations *(58)*.

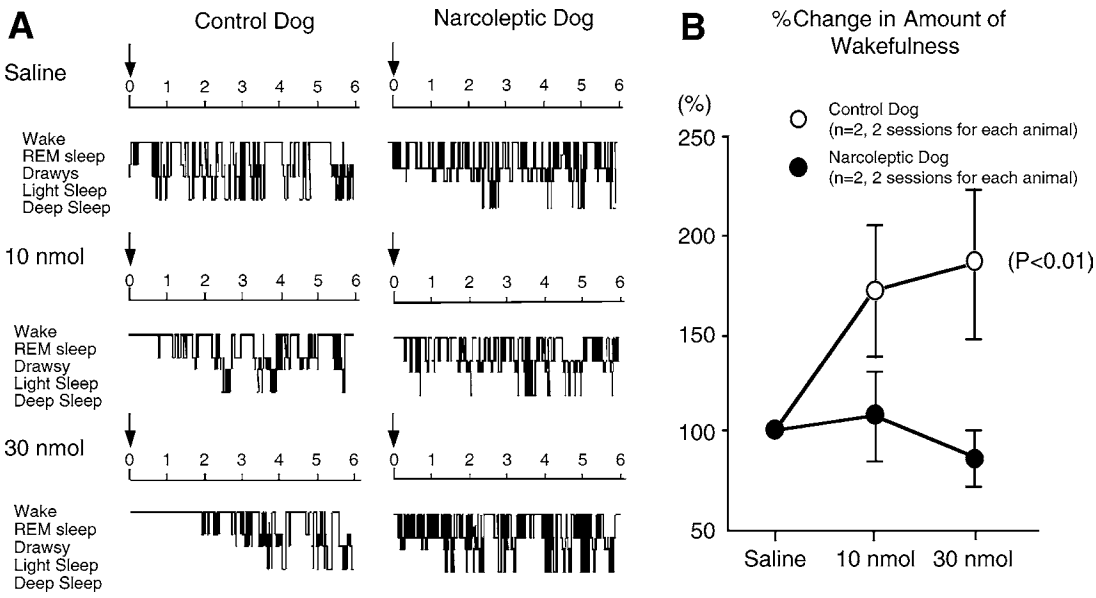

Fig. 3. Effects of intracerebroventricular (icv) injections of hypocretin-1 on vigilance states in control and genetically narcoleptic dogs. **(A)** Typical hypnograms obtained from 6-h polygraph recordings after icv administration of hypocretin-1 in control and narcoleptic dogs. In each hypnogram, the levels of vigilance (i.e., wake, rapid eye movement [REM] sleep, drowsy, light sleep, and deep sleep) are displayed from top to bottom. **(B)** Percentage changes in wake amounts after icv administration of hypocretin-1 in two control and two genetically narcoleptic dogs. Data are expressed as mean ± SEM. The icv administration of the hypocretin-1 dose dependently increased wakefulness in control dogs, but not in narcoleptic dogs (Adapted from ref. *45.*).

Our canine data also indicated that a small portion of iv hypocretin-1 can penetrate the brain *(45)*. Administration of higher doses (96–384 µg/kg) increased hypocretin-1 levels in the CSF by up to 2.4-fold in Dobermans, which was small compared with plasma levels (from undetectable, at <40 pg/mL, to 12×10^5pg/mL; Fig. 4).

An acute effect of iv hypocretin-1 on cataplexy (with a wide dose range) was therefore assessed in one sporadically affected narcoleptic dog (a Belgian Schipperke) *(45)*. Only one sporadically affected dog is currently available at the Stanford Center for narcolepsy research (by previous agreement with the animal donor, this dog was not to be used for invasive study). Similar to the results observed in *hcrtr-2*-mutated narcoleptic Dobermans, iv administration of hypocretin-1 at doses up to 24 µg/kg did not induce any change in cataplexy in the ligand-deficient sporadically affected dog (Fig. 5A) *(45)*. Interestingly, doses higher than 48 µg/kg of hypocretin-1 had a short-lived anticataplectic effect (<30 min; Fig. 5B) *(45)*. Suppression of cataplexy was dose dependent, and in two of three 384-µg/kg sessions, cataplexy was completely suppressed 5 min after injection (Fig. 5C). No changes in appetite, behavior, or heart rate were observed during and after drug injections.

Another hypocretin-deficient narcoleptic dog (a 3-yr-old Weimaraner) was identified at Cornell University, and the effects of intrathecal administration by implanting a Medtronic pump with catheterization of the cisternal magna were observed *(47)*. It was expected that at a high dose (up to 96 µg/kg), some reverse flow would occur back into deeper brain structures, providing therapeutic relief. However, no significant effect on cataplexy was observed *(47)*, possibly because the intracisternal hypocretin injections did not diffuse in upper ventricular

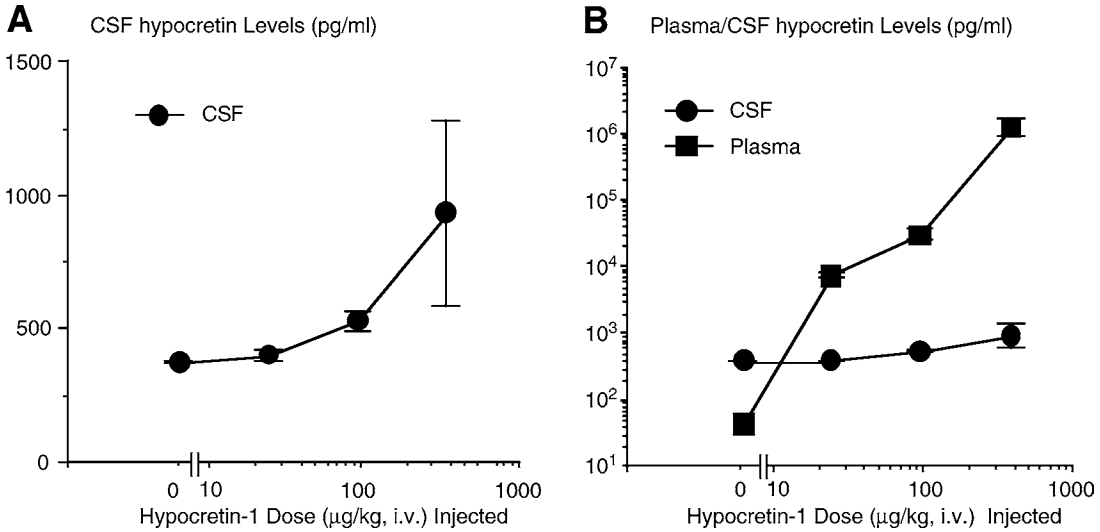

Fig. 4. Central penetration of hypocretin-1. Changes in hypocretin levels in the cerebrospinal fluid (CSF) **(A)** and blood (plasma) **(B)** of two narcoleptic Dobermans were measured after intravenonus administration of hypocretin-1. The dogs were injected with saline and 24, 96, and 386 µg/kg of hypocretin-1. Cisternal CSF taps and blood collections were carried out 30 min after injection. In (B), the CSF hypocretin levels are replotted together with plasma levels using a log scale. (Adapted from ref. *45*.)

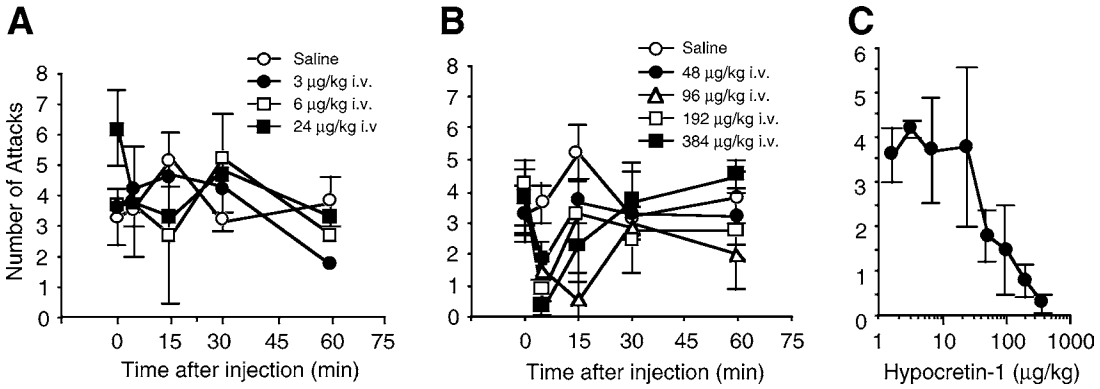

Fig. 5. Effect of intravenous administration of hypocretin-1 on cataplexy in a sporadic narcoleptic dog. After a baseline session of the food-elicited cataplexy test (FECT), saline or hypocretin-1 at doses of 3, 6, 24, 48, 96, 192, or 384 µg/kg was administered intravenously. Effects on cataplexy were evaluated at 5, 15, 30, and 60 min after injection. Experiments were repeated three times for each dose, and the mean results of the three sessions are displayed. Time-course effects on cataplexy are shown at low doses **(A)** and high doses **(B)** Intravenous administration of hypocretin-1 at high doses (over 24 µg/kg) induced a very short-lasting anticataplectic effect. **(C)** Numbers of attacks 5 min after injection of each dose were replotted. The suppression of cataplexy was dose dependent; in two out of three sessions involving 384 µg/kg, cataplexy was completely suppressed. (Adapted from ref. *45*.)

compartments. Thus, additional studies using intraventricular rather than intracisternal injections will be needed to verify that hypocretin-deficient narcoleptic canines are responsive to supplementation. Our results obtained from canine experiments, however, clearly suggest that more stable, centrally active hypocretin analogs need to be developed for hypocretin supplement therapy as a viable alternative in human narcolepsy.

6. SOME CONSIDERATIONS ON LIGAND REPLACEMENT THERAPY

6.1. The Primary Cause of Hypocretin Ligand Deficiency, Secondary Changes in Hypocretin Ligand Deficiency, and the Occurrence of EDS and Cataplexy

Although the study by Meida et al. *(43)* suggested that hypocretin neuron-ablated mice retain the ability to respond to hypocretin neuropeptides, it is not known whether human narcoleptic subjects react to the replacement of hypocretin peptides in a similar way as the genetically engineered murine model. The etiological mechanisms of hypocretin ligand deficiency in humans are not known. Thannickal et al. *(59)* recently reported that the loss of hypocretin cells and elevation of glial fibrillary acidic protein (GFAP) are strongly correlated with the regional density of hypocretin axons and the message density for hcrtr-2 in human narcoleptic postmortem brains. These results suggest that hypocretin function in human narcolepsy is lost because of cytotoxic or immunologically mediated attack focused on hcrtr-2 or an antigen anatomically linked to it *(59)*. The authors argued that it might be possible that hypocretin-receptive sites (in addition to impairment of hypocretin ligand production) are also damaged in human narcolepsy. However, detectable mRNA signals for both *hcrtr-1* and *-2* are observed in human narcoleptic postmortem brains (unpublished data) suggesting that no massive damage has occurred in hcrtr-2 and hypocretin-receptive neurons.

It is also possible that chronic hypocretin deficiency may cause a secondary deficit that is important for mediating hypocretin neurotransmission; such deficits may also be responsible for some of the narcolepsy phenotype. A series of recent human studies demonstrated that undetectable CSF hypocretin levels are likely to be measured at very early stages of the disease even before the onset of cataplexy and REM sleep abnormalities *(60)*. Various neurological conditions associated with significant hypocretin ligand deficiency, such as acute disseminated encephalomyelitis, Guillain-Barré syndrome, and multiple sclerosis have been reported *(5,60–65)*. In some of these cases, hypocretin deficiency occurred acutely, and the degree of hypocretin deficiency was as significant (undetectable levels in the CSF) as in most sporadic cases of narcolepsy-cataplexy *(3–5)*. Hypocretin deficiency is often associated with the occurrence of EDS, but the occurrence of cataplexy is rare, suggesting that a chronic and more selective loss of hypocretin neurons (rather than lesions in the lateral hypothalamus) may be required for cataplexy, to exist and that some additional factors, including secondary changes, may also be involved in cataplexy induction. To understand these results, more experiments must be undertaken.

6.2. Central Penetration and the Blood-Brain Barrier

The brain is probably one of the least accessible organs for the delivery of active pharmacological compounds. Despite its relatively high blood flow, two physiological barriers separate the brain from its blood supply. The first is the blood-brain barrier (BBB), which controls the entry and exit of endogenous and exogenous compounds. The BBB is defined by the microvasculature of the brain, which consists of a monolayer of polarized endothelial cells connected by complex tight junctions *(66–69)*. The second is the blood-CSF barrier (BCSFB), located at the choroid plexus, which is formed by tight junctions between the epithelial cells *(67,69)*. Since the surface area of the human BBB is estimated to be 5000 times greater than that of the BCSFB, the BBB is considered to be the main region controlling the uptake of drugs into the brain parenchyma and the target for delivering drugs to the brain *(70)*.

Because of the limiting effects of the BBB, many CNS diseases remain difficult to treat. Limited brain uptake also prevents numerous and otherwise promising agents from becoming

pharmaceutically useful entities. Neuropeptides, their analogs, and peptidomimetics are typical agents *(71,72)*.

Kastin and Akerstorm *(73)* assessed the in vivo ability of hypocretin-1 and hypocretin-2 to cross the BBB of mice. They reported that hypocretin-1 was highly lipophilic and an intravenous bolus of [^{125}I]hypocretin-1 rapidly entered the brain from the blood, with an influx rate ($K_i = 2.5 \pm 0.3 \times 10^{-4}$ mL/g/min) much faster than that of the 99mTc-albumin control in the nonsaturable simple diffusion process *(73)*. This suggests that no transport system exists across the BBB for the hypocretins: hypocretin-1 did not remain bound to the endothelial cells comprising the BBB but reached the brain parenchyma as intact peptides *(73)*.

In contrast, hypocretin-2 has a low lipophilicity, was rapidly degraded in the blood, and no [^{125}I]hypocretin-2 was detected in an intact form in the brain when it was injected peripherally *(73)*.

Structural differences between hypocretin-1 (with two intramolecular disulfide bridges) and hypocretin-2 (with no disulfide bridges—a linear peptide) probably contribute to make hypocretin-1 more resistant to catabolic peptidases. We have also shown that hypocretin-1, but not hypocretin-2, is highly stable in the CSF *(74)*. High levels of immunoreactive peptides and biological activity were observed for several hours after hypocretin-1 was administered exogenously in the cerebroventricle of rats *(74)*, suggesting that the stability of the peptide is another important issue for clinical applications.

Although rat experiments suggest that high-dose iv hypocretin administration may cause central effects by activating central hypocretin receptors, iv administration of high-dose hypocretin-1 in ligand-deficient narcoleptic dogs produced very short-lasting anticataplectic effects *(45)*. This, together with the fact that hypocretin cell-ablated mice respond to icv hypocretin-1 with a reduction in cataplexy, suggested that hypocretin analogs and hypocretin mimetics may be required for the clinical application of hypocretin-1 (Table 3).

6.3. Peptide Prodrug and Nonpeptide Agonints

In addition to the issue of BBB penetration, size, innate water solubility, and absence of specific transport systems also make it difficult to deliver peptides into the brain parenchyma. Also, some peptides, such as hypocretin-2, have short biological half-lives, with rapid metabolism and clearance *(73–75)*.

Because of these shortcomings, many specific delivery strategies using modification of the native peptide or its analog (i.e., prodrug) have been developed in an attempt to overcome hurdles to peptide pharmacotherapy; some of these methods may also be applied in hypocretin replacement therapy (Table 3).

Enhanced brain uptake and bioavailability of peptides may be achieved by reducing their size through the removal of amino acid residue(s) or subunits that are not essential for biological activity. Replacement of polar, ionizable amino acid resides with nonpolar ones (e.g., Leu) at the sites that do not interfere with binding to the target receptor could also enhance the lipid solubility essential for transport through the BBB *(71)*. Other molecular manipulations for increasing lipid solubility, which ideally also improve metabolic stability, include *N*-alkylation of one or more amide nitrogens in the backbone of the peptide *(76)*. Another possibility involves amino acid side chains (e.g., converting Tyr to *ortho*-alkyl- or *ortho, ortho*-dialkyl-Tyr *[77]*, halogenation *[78,79]*, various amide-bond surrogates *[80]* and the introduction of unnatural amino acids such as lipoamino acids *[81]*). Several studies have demonstrated that O-linked glycosylation of peptides (on Ser or Thr) can promote their penetration across the BBB via the glucose transporter system (GLUT-1) *(82)*.

Table 3
Possible Treatment Options for Hypocretin Replacement Therapy in Human Hypocretin Ligand-Deficient Narcolepsy

Treatment type	Limitations	Comments
Peptide agonists	Low CNS penetration, unstable	Intranasal administration; Prodrug, modification
Nonpeptide agonists	Not yet available	The first priority
Cell transplantation	Low survival rate and limited availability of donor cell	Embryonic stem cell, neural stem cell
Gene therapy	Requires appropriate vector selection	Promising in the future

Regarding the structure-activity relationship of hypocretin peptides, Asahi et al. *(83,84)*, Darker et al. *(85)*, and Lang et al. *(86)* tested binding and calcium mobilization using hypocretin receptor-expressing cell lines of various truncated hypocretins and found that the entire hypocretin sequence is not required for agonistic activity *(83–86)*. Furthermore, the C-terminal half of hypocretin-1 (15–33) and hypocretin-2 (10–28) is critical for their biological activity to take place *(83–86)*. Interestingly, the C-terminal halves of both peptides have similar sequences, but the respective truncated peptides still possess the selectivity for each receptor, suggesting that ligand/receptor selectivity is also related to the C-terminal-half sequences. Furthermore, Asahi et al. *(83)* also showed that three L-leucine residues at the 11, 14, and 15 positions of hypocretin-2 were important to show selectivity for hcrtr-2 over hcrtr-1. In addition, [Ala11], [Ser11], [Ala11, D-Leu15], and [Ala11, D-Leu15] hypocretin-2 significantly enhanced the selectivity for hcrtr-2 over hcrtr-1 at 120-, 120-, 140-, and 400-folds respectively. These results suggest that the possibility of developing small hypocretin peptide analogs with various degrees of selectivity, as well as certain other modifications, may improve the bioavailability/CNS uptake of the compounds.

As far as we know, no other publications are available on the structure-activity relationship of hypocretin peptides and their central penetration. The strategies of producing appropriate peptide analogs by irreversible alteration of the structure of the parent peptide generally exploits specific knowledge of structure-activity relationships and rely on a hypothetical "degree of freedom" related to ligand/substrate binding to the target CNS receptor *(87)*. This requires large-scale drug screening and thus depends greatly on efforts by the pharmaceutical industry.

Hypocretin and most peptide-activated receptors belong to the superfamily of seven transmembrane domain receptors coupled to G proteins (Fig. 1). Peptides of different sizes (from a few to more than 100 amino acids) activate peptidergic receptors by interacting at specific sites that often differ from those where antagonists bind *(88)*. Peptides are a rapidly growing class of mediators whose activity regulates homeostasis as well as pathological processes. The great impact of nonpeptide agonists/antagonists in current and future medicine reflects these large varieties of functions and modes of activation of peptidergic GPCRs. As we discussed earlier, the development of nonpeptide (small-molecule) hypocretin agonists is another critical step in replacement therapy for hypocretin-deficient human narcolepsy, but, again, this is mostly dependent on the efforts of the pharmaceutical industry.

Several nonpeptide hypocretin receptor antagonists, such as SB-284422, 334867-A, 408124, 410220, and 674042 (GlaxoSmithKline Beecham) *(89–91)*; NBI 36487, 36266 (Neurocrine) *(92)*; *N*-acyl 6,7-dimethoxy-1,2,3,4-tetrahydroisoquinoline (Banyu) *(93)*; and

4-phenyl-[1,3]dioxanes (Johnson and Johnson) *(94)* have been produced and tested in various in vitro and in vivo *(94–99)* experimental settings. However, no nonpeptide hypocretin agonists have yet been reported and tested in the animal model of narcolepsy. In general, agonist screening is more complex and difficult, because agonists need to induce conformational changes in the receptors and to stimulate the second-messenger system in addition to their binding properties. (Binding affinity alone is required for the antagonists.) However, a dozen nonpeptide agonists for GPCR peptide receptors, including urotensin-II receptor (GPR14) *(100)*, opioid receptor-like (ORL1) receptor *(101)*, thrombin receptor *(102)*, and galanin receptor *(103)* are currently under development. This suggests that it is quite possible to develop nonpeptide hypocretin agonists; such development would also mostly depend on the efforts of the pharmaceutical industry. Since narcolepsy is a life-long disease and medications are likely to be used over a lifetime, orally active nonpeptide agonists are most preferable. Although the prevalence of narcolepsy-cataplexy is not great, nonpeptide hypocretin agonists may also have therapeutic applications for other types of hypersomnia and sleep disorders associated with jet lag or shift work, as well as other neuroendocrine and feeding disorders. Thus the global need for nonpeptide hypocretin agonists may be unpredictably high.

6.4. Routes of Administration

Although intraparenchymal, icv, and intrathecal means of administration are often used to deliver central effects of compounds in animals, these methods are not preferable in humans except for life-threatening conditions.

Frey et al. *(104)* have recently proposed intranasal delivery as a novel route for bypassing the BBB to deliver therapeutic agents to the brain and spinal cord *(104)*. The olfactory and trigeminal nerves (involved in sensing odors and chemicals) provide a unique connection between the brain and the external environment *(105,106)*. Delivery occurs by an extracellular route and does not require that the drugs bind to any receptor or undergo axonal transport. This method allows drugs that do not cross the BBB sufficiently to be delivered rapidly to the CNS. In addition, intranasal drug administration may eliminate or minimize the need for systemic delivery and may reduce peripheral side effects.

A series of studies has also demonstrated the therapeutic efficacy of intranasal administration of peptides in animal models of disease. In a transgenic mouse model of Alzheimer's disease, intranasal nerve growth factor has been shown to reduce neurodegeneration *(107)*. Intranasal neurotrophins have also been reported to stimulate neurogenesis in normal adult mice *(108)*. In humans, drug delivery to the CSF after intranasal administration was also demonstrated for neuropeptides, including melanocyte-stimulating hormone, insulin, and vasopressin *(109)*. Thus, the intranasal route may be a useful method for introducing large-molecule peptides, including hypocretins, into the CNS.

Using γ-counting and autoradiography assessment of [^{125}I]hypocretin-1 in the brain, Hanson et al. *(110)* recently reported that intranasally delivered [^{125}I]hypocretin-1 to anesthetized (14–61 n*M*) and awake mice (6–13 n*M*) reached the brain directly. This distribution was not uniform and was high in the hypothalamus, cortex, and brainstem. This finding suggests that these peptides may bind to hypocretin receptors. However, whether the [^{125}I]hypocretin-1 detected is intact and has biological activity cannot be confirmed. Similarly, no behavioral assessments, such as locomotor or EEG measurements after intranasal administration of hypocretin-1 were carried out. Regardless of the shortage of critical information, it may be worthwhile to explore this therapeutic option further in an animal model of hypocretin-deficient narcolepsy, since this route may be acceptable in humans for chronic drug administration (Table 3).

7. CELL TRANSPLANTATION

Neural transplantation is one of the most promising approaches for the treatment of Parkinson's disease, a major neurodegenerative disorder with a prevalence as frequent as that of narcolepsy. Neural transplantation involves implantation of living neuronal tissue into a host system. Several studies using animal models have demonstrated that grafted tissue survives, integrates within the host brain, and provides functional recovery following brain inventions *(111–116)*. This type of study for Parkinson's disease began in the latter half of the 1970s. Initially, DA neurons from animal fetuses were used as donors, and then paraneurons such as chromaffin cells were used. Based on a large number of experimental animal studies, neural transplantation has been applied clinically *(117)*. Beneficial effects have been demonstrated, and autopsy cases have shown that many transplanted cells were able to survive in the human brain for long periods. These findings contributed a great deal to the research in regeneration of the central nervous system.

Thus it would be interesting to know whether a graft of hypocretin neurons into a host brain could survive (Table 3). Arias-Carrion et al. *(118)* recently examined survival of grafted hypocretin neurons (a suspension of cells from the posterior hypothalamus of 8–10-d-old rat pups) into the pons (a region of the brain that is innervated by hypocretin axons but where the hypocretin somata are not present) in adults rats. The authors found that well-defined hypocretin-immunoreactive somata with processes and varicosities were present in the graft zone 36 d after implantation of the cell suspension, suggesting that hypocretin neurons obtained from rat pups can be grafted into an adult host brain *(118)*.

However, these preliminary results have not been assessed quantitatively; neither the number of hypocretin cells injected nor the number of hypocretin cells that survived were quantified, and long-term effects (more than 36 d) were not assessed. The functional consequences of these grafted neurons are not yet known.

In the murine narcoleptic model of hypocretin ligand deficiency, heterozygous prepro-hypocretin knockout mice (exhibiting about 80% hypocretin ligand concentration) were symptom free *(2)*. In humans, less than 1/3 of the normal hypocretin ligand concentration in the CSF is defined as abnormally low for narcolepsy-cataplexy *(3,5,63)*. Thus, at least 50% of the ligand concentration in the brain is likely to be required for rescuing the narcolepsy phenotype. This estimate is under the condition that proper synaptic connection/regulation occurs in the hypocretin projection sites.

It has been suggested that the survival rate of DA neurons from the fetal mesencephalon in grafting is likely to be as low as 5–10% *(119)*. If only this amount of hypocretin cells injected survive, then this approach might not be practical. Furthermore, efficient sorting/selecting methods for hypocretin-containing cells need to be developed since hypocretin neurons are not clustered like nuclei. The possibility of immune reactions to the grafted hypocretin cells may be another concern, especially if a homologous graft is used and/or if an autoimmune process causes hypocretin-deficiencies in humans.

Because of these methodological limitations of transplantation using cells obtained from live animals, many researchers working on Parkinson's disease have been exploring methods to generate dopaminergic neurons from neural stem cells (NSCs) or embryonic stem (ES) cells *(117,120,121)*. Forced expression of Nurr1 in NSCs has been reported to promote their differentiation into dopaminergic neurons *(122)*. If researchers knew how stem cells differentiate into specific neurons under the influence of neurotrophic factors, they might be more interested in neurotrophic factor delivery using neural transplantation procedures and gene

therapy. Cell lines that produce neurotransmitters and neurotrophic factors also became available as molecular biology techniques were introduced, and these lines have been used as donor cells in recent studies *(121,123–126)*. Although the instability of the transgene in the transformed cells should be improved *(127)*, similar progress and transitions may be expected for hypocretin cell transplantation approaches for treatment of narcolepsy.

8. GENE THERAPY

Hypocretin gene therapy using various vectors may also be an option for future human therapy; the basic concept of gene therapy is similar, but this technique may have several advantages over cell transplantation (Table 3); *see* Subheading 7 above and Chap. 26 for details).

The strategy for hypocretin gene therapy will probably be focused on the local production of hypocretin peptides, since the mechanisms of loss of hypocretin production and differentiation of hypocretin neurons are not yet known.

For somatic gene transfer, several different viral vector systems are in use or under consideration, such as adenoviral *(128)*, adeno-associated virus (AAV) *(129–132)*, lentiviral (such as the human immunodeficiency virus) *(133)*, and retroviral vectors *(134)*. Among these, AAV and lentiviral vectors can transduce nondividing neurons, and long-term gene expression can be obtained *(132,133)*. Furthermore, AAV vectors, derived from a nonpathogenic virus, possess several unique properties and are potentially the most appropriate for gene therapy of neurological diseases *(132)*. Adenoviral vectors are able to transduce neurons efficiently, but transgene expression is transient and their cytotoxicity may be a problem *(128)*. An appropriate vector should be chosen depending on the type of target cell and the purpose of the gene transfer/gene therapy.

Recently, the study of NSCs as vectors for applying gene therapy (in addition to study of the control of proliferation and differentiation of NSCs by introducing genes) has also been promoted since it is expected that NSCs would have good adaptability to the CNS *(135–138)*. NSCs could thus be one of the most promising sources in developing new therapies for CNS disorders *(121,139)*.

Further technological advances are required to optimize gene repair and regulation of gene expression for treating human genetic disorders; such gene therapy could apply to various diseases including hypocretin-deficient narcolepsy.

9. CONCLUSIONS

Although major progress in understanding the pathophysiology of human narcolepsy (i.e., hypocretin ligand deficiency) has recently been made, this knowledge has not been used to develop new treatments. Considering that replacement therapy for missing substances has been successfully used for other neurologic and neuroendocrine disorders, hypocretin replacement therapy is the most promising approach we currently have for treating human narcolepsy.

Recent findings in animal studies suggest that hypocretin-deficient animals retain the ability to respond to hypocretin neuropeptides to rescue the narcolepsy phenotype. Furthermore, temporally regulated and spatially targeted secretion of hypocretin is not required to prevent narcoleptic symptoms. However, the results of the systemic hypocretin administration experiments in hypocretin ligand-deficient narcoleptic dogs suggest that the development of more centrally penetrable hypocretin agonists is required. Thus we extended our discussion of strategies to deliver hypocretin agonists to the CNS (by prodrug, nonpeptide agonists, intranasal administration, cell transplantation, and gene therapy).

Among these possibilities, the development of nonpeptide hypocretin agonists is the most urgent issue. However, all approaches to replacement therapy are tightly linked conceptually and practically; any information gained from one approach can be incorporated in others. It is not known whether systemic administration of effective doses of hypocretin agonists will produce peripheral side effects in the CNS. If this is the problem, intranasal administration, cell transplantation, and gene therapy may be effective.

We hope that major progress in hypocretin replacement therapy will occur in the next 10 yr and that many patients will benefit from this new therapeutic option.

Finally, it is also important to find the cause and mechanisms of hypocretin ligand deficiency, since this knowledge may enable us to prevent and reverse the disease process.

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