

The Hypocretin/Orexin Story

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Newly described peptides, produced in neurons in the lateral hypothalamic area, have been shown to stimulate appetite and stereotypic behaviors associated with feeding. Discovered independently by two groups, the hypocretins/orexins stimulate autonomic function and have been shown to be physiological regulators of the arousal state. Neuroendocrine and metabolic effects of these peptides, some related to sleep-wakefulness and arousal state, are just now being discovered.

Subtractive cDNA cloning and orphan receptor technologies are revolutionizing neuropeptide discovery and the basic science of endocrinology. Both methodologies have greatly enhanced the process of discovery, and in the past few years have yielded a wealth of information about novel neuropeptides and biologically relevant receptors. Novel, site-specifically expressed mRNAs have been identified by tag PCR subtraction cloning, a process that allows the discovery of previously unrecognized peptides in endocrine/neuroendocrine tissues. Tools developed from the subtraction cloning strategy allow localization, in discrete tissue sites, of the predicted mRNAs (that is, sites of expression of mature peptide) and the elucidation of the amino acid sequence of the predicted neuropeptide. With the structure in hand, synthetic peptide can be produced for bioassay (behavioral, neurophysiological or neuroendocrine paradigms) and antibodies generated for peptide localization studies. The

second approach, orphan receptor cloning, has provided a novel strategy for the identification of biological ligands. The once laborious task of establishing a bioassay with which to screen tissue extracts for novel bioactive substances has been replaced by the use of receptor expression systems (transfected cells) as reporter assays for the isolation and purification of novel biological ligands. Perhaps no better example exists of the power of these novel approaches than in the saga of the hypocretins/orexins, peptides discovered simultaneously by two independent laboratories at the time of transition from the more classic peptide purification approaches to the subtraction cloning and orphan receptor methodologies.

• The Discovery of the Hypocretins: Subtraction Cloning Reveals a Novel Family of Neuropeptides

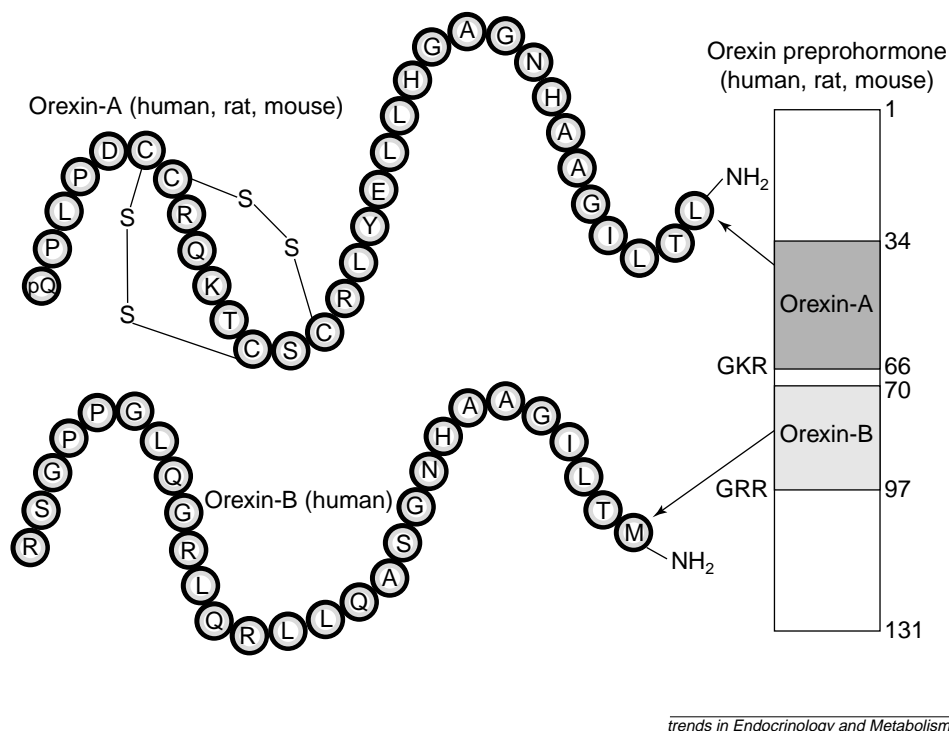
Using the subtraction cloning method¹, Gautvik and colleagues² constructed a rat cDNA library enhanced for hypothalamic-specific clones. From this library, they identified 38 distinct mRNAs that were expressed selectively in the hypothalamus, including some encoding well-characterized hypothalamic peptides such as oxytocin, vasopressin and proopiomelanocortin (POMC). Northern

analysis then confirmed the highly selective nature of the hypothalamic localization of two of the cDNA clones, one encoding oxytocin and the other, clone 35, predicting a yet uncharacterized peptide. *In situ* hybridization histochemistry localized expression of the mRNA corresponding to clone 35 bilaterally in the posterior/lateral hypothalamic region. No signal was detected elsewhere in the brain.

In January 1998, these investigators³ reported the identification of the prohormone predicted from the nucleotide sequence of clone 35 and predicted the post-translational modification of that prohormone to produce two peptides, one of 39 and another of 29 amino acids. Both peptides possessed C-terminal glycine residues, substrates for amidation by peptidylglycine α -amidating monooxygenase, predicting C-terminal amidation of the endogenous product.

Because of the predominant hypothalamic localization of the peptides and their sequence homology to secretin, the peptides were named hypocretin-1 and hypocretin-2. This turned out to be a fortunate choice of names, because it avoided identification of the peptides on the basis of only one biological activity (*vide infra*). Importantly, these authors³ detailed the unique localization of hypocretin (Hcr) positive neuronal cell bodies in the dorsolateral hypothalamus, and immunoreactive fibers projecting intrahypothalamically and to the septal nuclei, the preoptic area, the paraventricular thalamus, locus coeruleus and midbrain central gray. Less prominent fiber pathways project to the colliculi, laterodorsal tegmental nucleus and the nucleus of the solitary tract, these last two areas being important autonomic centers in the hindbrain. Electron microscopy localized peptide immunoreactivity in synaptic vesicles, predicting a role for the peptides in

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Figure 1. The amino acid sequence of rat and human hypocretin-1/orexin-A and hypocretin-2/orexin B.

cell-cell communication, which was supported by the observation that Hcrt-2 increased postsynaptic currents in cultures of synaptically coupled hypothalamic neurons. This proposed synaptic activity, together with the reported distribution of Hcrt-positive cell bodies and axonal projections, led these authors to speculate that the hypocretins might be involved in such diverse, but not necessarily independent, central nervous system (CNS)-regulated functions as feeding behavior and blood pressure regulation.

**• Discovery of the Orexins:
an Orphan Receptor Provides
the Tool for Peptide Discovery**

One month after the publication of the discovery of the hypocretins, Yanagisawa and colleagues⁴ independently published the discovery of novel peptide ligands for a previously identified, G protein-coupled orphan receptor. Because most previously characterized peptide receptors are G protein linked, they reasoned that many of the 'orphan' receptors identified by cDNA homology cloning strategies might in fact have yet to be characterized peptides as their cognate

ligands. Thus, they created a screening assay for novel neuropeptides using a battery of orphan G protein-coupled receptor-expressing cells as reporter assays. Cell lines stably transfected with only one orphan receptor were used, and care was taken to avoid false positives generated by the fortuitous expression of endogenous receptors in those cells. This battery of cell lines then was used to screen high-resolution, high-performance liquid chromatography (HPLC) fractions of rat brain extracts. They identified peptide fractions that stimulated significant rises in cytoplasmic Ca^{2+} levels in a human embryonic kidney (HEK293) cell line expressing the orphan receptor previously designated HFGAN72. When the major peak of bioactivity was purified to homogeneity and sequenced, a novel peptide of 33 amino acids in length with an N-terminal pyroglutamyl residue and an amidated C-terminus was discovered. The peptide was found to contain two intrachain disulfide bonds, and sequencing of similar extracts from bovine brain revealed exact interspecies homology. A second peptide characterized from the rat brain extracts was 28 amino acids in

length, also possessing an amidated C-terminus. This peptide shared 46% homology with the larger 33 amino acid peptide. A third peptide was identified to be an N-terminally truncated, 26 amino acid form of the 28 amino acid peptide. Figure 1 shows the amino acid sequences of the rat and human peptides.

At the time of their experiments, it was impossible for Yanagisawa and colleagues to know that de Lecea and co-workers were working with a similar family of peptides, and indeed, the structure of Yanagisawa's 28 amino acid peptide was identical to that being independently named hypocretin-2. The larger 33 amino acid peptide was similar to that designated hypocretin-1 by de Lecea *et al.*; however, the N-terminal sequence designation of hypocretin-1 might have been somewhat in error⁵.

Just as de Lecea and colleagues³ sought to characterize the tissue distribution and initial synaptic activity of the hypocretins, Yanagisawa and co-workers⁴ did not stop merely at the identification of these two novel peptides. In their remarkable manuscript, they reported not only the structure of the novel orphan receptor ligands, but also the structure of the prohormone (deduced from the full-length cDNA), the identification of the amino acid sequences of the receptors for the two peptides, the tissue distribution of the peptides and their receptors in rat brain and an important biological (behavioral) activity of the peptides, which was predicted on the basis of those mapping studies.

Northern blot analysis revealed the prohormone to be expressed preferentially in brain, and *in situ* hybridization histochemistry and immunohistochemistry identified neuronal cell bodies in the bilateral posterior hypothalamus (note the similarity to the distribution of the hypocretins). The lateral hypothalamic localization of peptide predicted a potential effect on feeding behaviors, because this area has been intimately tied to food intake and energy metabolism. Intracerebroventricular administration of synthetic peptides in non-fasted rats significantly stimulated food intake in a dose-related, time-dependent fashion. The appetite-stimulating activity of these

peptides led the investigators to designate them the orexins. The 33 amino acid peptide, which was the more bioactive of the two, was designated orexin-A (ORX-A) and the shorter peptide, which was devoid of intrachain disulfide bonds, was termed orexin-B (ORX-B).

Two receptors for the ORXs were identified⁴, one that preferentially bound ORX-A, now designated the OX₁R receptor, and a second, the OX₂R receptor, which binds both ORX-A and ORX-B, apparently with similar affinity. Because both peptides stimulated appetite in fed rats, it can be speculated that the OX₂R receptor transduces the orexigenic stimulus in the brain; however, some studies demonstrated enhanced potency of ORX-A compared with that of ORX-B, suggesting that the effect was OX₁R receptor mediated. The biological function of the OX₁R receptors remained at the time unknown. It was, however, a good bet that the endogenous orexins were physiologically relevant modulators of appetite because, like the appetite stimulant neuropeptide Y (NPY)⁶, orexin mRNA was raised in the fasted state. Thus, the Yanagisawa group focused their attention on the potential for the ORXs to be endogenous mediators of energy utilization and metabolism, even speculating that ORX-producing neurons might be glucose sensitive. It is important to note that Yanagisawa and colleagues acknowledged in this original report⁴ that the orexigenic activity of ORX-A was less impressive in magnitude than that of NPY, a known appetite stimulant. Not having identified the axonal projection fields of the ORX-positive neurons, they at the time could not have speculated, as did de Lecea and co-workers³, on a potential role for these peptides in the CNS regulation of autonomic or endocrine function.

• What Do the Hypocretins/Orexins Do?

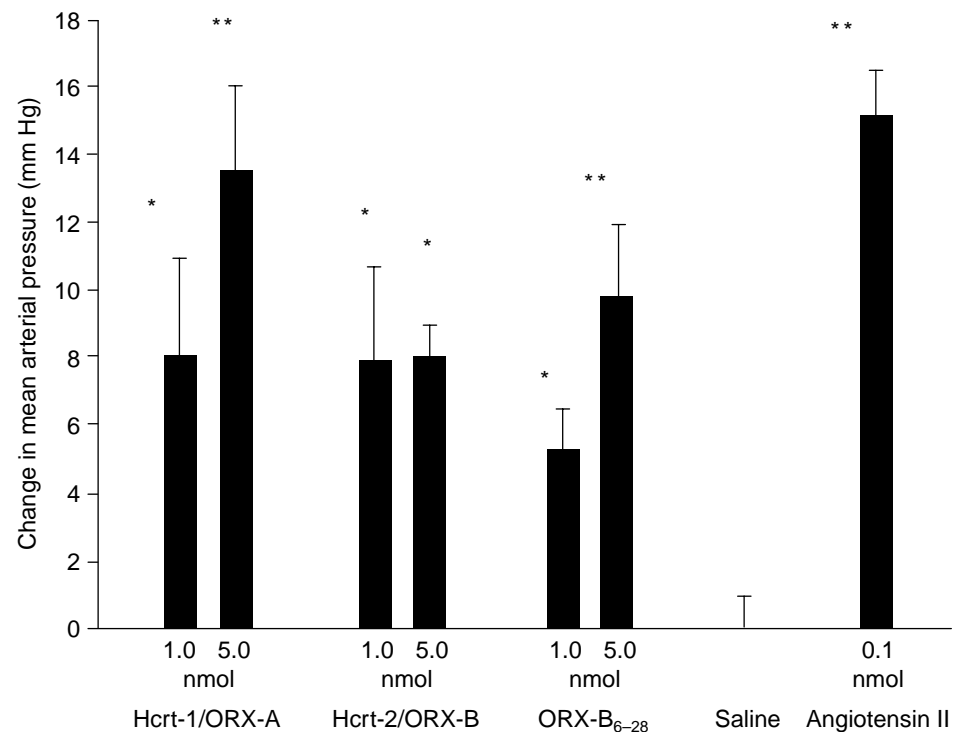
It is clear that the hypocretins/orexins (Hcrt/ORXs) can stimulate appetite; however, they are not as potent as other stimulatory neuropeptides. This does not negate the possibility that they play a physiologically relevant, modulatory role, and ultrastructural evidence for

the synaptic action of Hcrt/ORXs on NPY-producing neurons supports that hypothesis⁷. One group⁸ failed to observe a significant effect of fasting on hypothalamic Hcrt/ORX levels and, furthermore, no significant differences were observed in animals fed a high-fat diet or between obese Zucker rats and their lean controls. This led those investigators to hypothesize effects of the peptides on non-appetitive functions. However, at least two other groups^{9,10} have demonstrated significant stimulatory effects of hypoglycemia on peptide content and/or message. In fact, the CNS actions of the Hcrt/ORXs might be more related to the control of energy metabolism¹¹ than to appetite *per se*.

We were drawn to the distribution of Hcrt/ORX-immunopositive axon terminals in brain regions more related to autonomic function than to metabolic regulation and examined possible actions of the peptides unrelated to appetite. Already by the Autumn of 1998, we reported that both Hcrt-1 and Hcrt-2, when injected into the lateral cerebro-

ventricle of conscious, unrestrained animals, significantly raised mean arterial blood pressure and heart rate, suggesting a stimulation of sympathetic function¹². These effects were in fact predicted by the initial work of de Lecea and colleagues³ and, subsequent to our first report of this novel CNS action of the peptides, several excellent mapping studies focused attention on hypothalamic and brainstem sites, where Hcrt/ORX might modulate autonomic function. Indeed, the dense innervation by Hcrt/ORX-positive fibers of the hypothalamic paraventricular and dorsomedial nuclei, the lateral posterior hypothalamus, the midbrain central gray and brain stem nuclei, including the locus coeruleus and nucleus of the solitary tract¹³, provided the anatomical framework for our pharmacological experiments.

Blood pressure rose upon central injections of both Hcrt-1 and Hcrt-2 (Fig. 2), suggesting an action on the OX₂R receptor, which was consistent with the demonstration of mRNA for the non-selective



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Figure 2. Hypocretin/orexin (Hcrt/ORX) administration into the lateral cerebroventricle significantly increases mean arterial blood pressure in conscious, unrestrained rats. The dose-related ability of the ORX-B fragment (ORX-B₆₋₂₈) is demonstrated. Comparison with the blood pressure-increasing effect of angiotensin II. Results are mean \pm SEM. Adapted, with permission from Elsevier Science, from Ref. 12. Key: * $p < 0.05$, ** $p < 0.01$, versus pre-injection baseline.

receptor in the hypothalamic paraventricular nucleus¹⁴, a site that would be perfused by peptide injected into the lateral cerebroventricle. However, the magnitude of the rise seen after injection of Hcrt-1 exceeded that of Hcrt-2 (Fig. 2), leaving open the possibility that the relatively selective OX₁R, which has been localized to brain stem sites known to be important in CNS–cardiovascular regulation¹⁵, transduced these effects. This greater potency of Hcrt-1 in terms of blood pressure regulation was not apparent in our feeding model, because Hcrt-1 and Hcrt-2 were equipotent stimulators of food intake in fed rats¹². Thus, we hypothesize that the metabolic and the autonomic actions of the Hcrt/ORX peptides might be expressed through differing receptor populations and isoforms.

In our conscious animal studies, we observed a general arousal and the apparent stimulation of stereotypic locomotor activities in response to centrally administered Hcrt/ORX. Two excellent studies established the existence of those pharmacological effects^{16,17}. Ida and colleagues¹⁶ reported that while both ORX-A and ORX-B stimulated the behaviors associated with feeding, as did NPY, only NPY at the doses tested stimulated significant food consumption over the 3-h period of the trial. ORX-A, but not ORX-B, did stimulate a transient increase in feeding (at 10 min) but this increase was not sustained. Both ORX-A and ORX-B stimulated face washing (ORX-A being more potent). Grooming and burrowing were increased by ORX-A and burrowing and searching behavior by ORX-B. Face washing, burrowing and grooming behaviors were not altered by the appetite-stimulatory dose of NPY but, like the Hcrt/ORXs, NPY stimulated searching behavior. Thus, there might be a component of the stimulatory effects of the Hcrt/ORXs in feeding that is more related to arousal and exploratory behavior than that observed with NPY.

The localization of Hcrt/ORX peptide and receptors in locus coeruleus, a site known to be important in the determination of the state of arousal, predicted such an action. Hagan and colleagues¹⁷ demonstrated the ability of ORX-A to

stimulate intrinsic noradrenergic neurons in the nucleus and to alter the sleep–wake cycle of experimental animals. ORX-A, at a dose similar to those used by us to stimulate autonomic function, increased the proportion of time spent in arousal and decreased the proportion of time spent in paradoxical sleep¹⁷. Light and deep slow-wave sleep intervals were not affected, and sleep stage distribution normalized within 4 h. These authors also observed an increase in stereotypic grooming behaviors. No effects on X-maze behavior or startle response were seen, supporting the hypothesis that the effects on arousal state were specific and not anxiogenic in origin, distinguishing these CNS effects from peptides like corticotropin-releasing factor (CRF). Apparently, these effects were also not caused by generalized stress, because plasma prolactin levels actually declined after CNS administration of ORX-A. However, in those same animals corticosterone levels increased at the higher doses of peptide administered, which might reflect the sympatho-stimulatory effects of the peptide. Growth hormone levels, on the other hand, declined significantly, which might reflect the general arousal state.

• Which Hypothesized Actions of the Hcrt/ORXs are Physiologically Relevant?

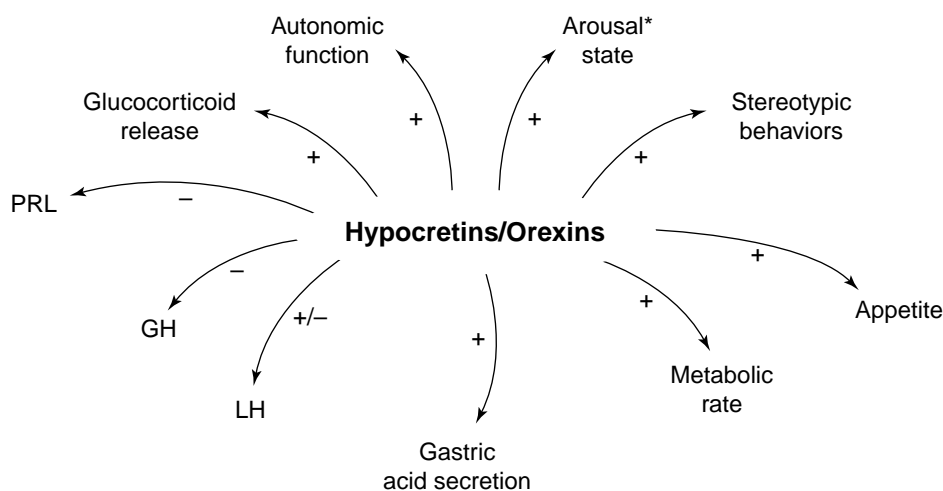
Although there is evidence for the physiological relevance of the orexigenic action of Hcrt/ORX, based on inhibitory effects of anti-ORXA antibody administration on feeding after 24-h food deprivation¹⁸, the identification of a naturally occurring mutation in the gene encoding the OX₂R receptor¹⁹ and the development of a knockout mouse lacking the gene for orexin²⁰ convincingly placed the Hcrt/ORXs in a physiologically relevant context.

Once again, modern methodologies brought two independent laboratories down converging paths to exactly the same discovery: the Hcrt/ORXs, acting via the Hcrt-2/OX₂R receptor, are physiologically relevant modulators of sleep–wakefulness. It had long been known that animal models mirrored human narcolepsy, a condition charac-

terized by daytime fatigue, cataplexy, sleep fragmentation and precipitous descent into rapid-eye-movement (REM) sleep, with associated atonia. In humans, the condition has been thought to have a genetic basis of predisposition and has been hypothesized to be autoimmune (HLA-associated) in nature²¹. Narcolepsy in dogs is an autosomal recessive trait, transmitted by the *canarc-1* gene²². Lin and co-workers¹⁹, using positional cloning strategies, identified the canine narcolepsy gene to be a mutation in the gene encoding the Hcrt-2/OX₂R receptor. The resultant protein encoded by this gene has amino acid deletions in one of the transmembrane-spanning domains, lacks another of the domains entirely and is truncated at the C-terminus. These investigators reasoned that the mutations result in altered membrane insertion of the receptor and a lack of function.

Simultaneously, Yanagisawa and colleagues²⁰ reported that ORX-knockout mice display a phenotype similar to human narcoleptics and *canarc-1*-mutant dogs. Using homologous recombination strategies, they targeted for deletion exon 1 of the gene encoding prepro-orexin⁴ and produced chimeric mice for germline transmission of the mutant allele. Mendelian transmission of the mutant gene resulted in homozygous, ORX-null mice with no lethality²⁰. ORX immunoreactivity was absent in the brain of the ORX-null mice and *in situ* hybridization histochemistry using a riboprobe directed against exon 2 failed to detect any ORX mRNA as well. To their surprise, no obvious behavioral deficits were seen in these mice in experiments conducted during the day, when the mice are normally inactive. On the other hand, infrared videophotography detected evidence of profound behavioral anomalies during the active phase at night. These included behaviors suggestive of the narcoleptic condition in human and animal models. These behaviors can be seen in video clips posted on the Internet by *Cell*, the journal that published the original manuscript (<http://www.cell.com/cgi/content/full/98/4/437/DC1>).

The observed behaviors were displayed by all of the $-/-$ mice, but not by the



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Figure 3. Multiple pharmacological effects of hypocretins/orexins. * indicates proven physiological relevance. Abbreviations: GH, growth hormone; LH, luteinizing hormone; PRL, prolactin.

+/- mice (heterozygotes) or the wild-type littermates. Motor disturbances ranged from sudden cessation of all activity with collapse to twitching and rocking. These behaviors were often socially initiated, sudden in onset and equally sudden in resolution. Using electroencephalograph (EEG) recordings, they were able to rule out seizures as a cause of collapse and rocking motor activity; they also ruled out a metabolic cause by observing normal plasma electrolytes and glucose levels in the -/- mice. Instead, these mice displayed EEG patterns consistent with a disruption of REM sleep. ORX-null mice entered into REM sleep more frequently and with shorter latency. In addition, they cycled between sleep and wakefulness more frequently than normal mice. Further evidence for a disruption in sleep-wakefulness came from dual EEG/electromyograph (EMG) recording, where pre-REM spindles were observed in the ORX-null mice, suggesting a disruption of sleep initiation.

Double labeling immunohistochemistry identified Hcrt/ORX-positive nerve terminals innervating brain nuclei known to be important in the control of sleep-wakefulness²⁰. These terminals were seen adjacent to histaminergic neurons in the tuberomammillary nucleus, noradrenergic neurons of the locus coeruleus, serotonergic neurons of the

dorsal and median raphe and cholinergic neurons in the pedunculopontine nucleus, lateral dorsal tegmental nuclei, diagonal band (Broca) and medial septal nuclei. Importantly, the anti-narcoleptic drug Modafinil, when administered intraperitoneally to wild-type mice, induced a ninefold increase in early gene expression (FOS immunoreactivity) in ORX-positive cells in the perifornical region of the lateral hypothalamus, suggesting a target site. Before this observation the site and mechanism of action of Modafinil remained unknown.

Although both groups of authors^{19,20} correctly acknowledged at the time of publication of the gene mutation model or the gene deletion study that no pharmacological evidence for an effect of Hcrt/ORX on arousal state had been published, just one month later that evidence was reported. Hagan and colleagues¹⁷ had pursued the initial localization of Hcrt/ORX immunoreactivity with studies aimed at uncovering the possible action of the peptides in the locus coeruleus. This area not only received the most dense innervation of Hcrt/ORX-positive axon terminals, but had been recognized for some time to be a major site involved in the maintenance of the arousal state. ORX-A stimulated the neuronal firing rate of locus coeruleus neurons in a brain slice preparation, with a threshold of

≈10–30 nM. When administered *in vivo* at the onset of a normal sleep period, ORX-A increased the amount of subsequent time spent in arousal and decreased the proportion of paradoxical sleep. No significant alterations in the proportion of time spent in light or deep slow-wave sleep was seen with the dose of ORX-A used. Stereotypic behaviors were also affected by ORX-A, with increased time spent grooming and an increased number of grooming events observed. No significant changes in body temperature, X-maze behavior or startle responses were observed. Changes in plasma hormone levels, particularly a significant depression of growth hormone concentrations, were also consistent with the Hcrt/ORXs being significant modulators of the neural and endocrine events in sleep-wakefulness. Figure 3 summarizes the proposed effects of Hcrt/ORXs.

• Quo vadis?

The convergence of modern molecular technologies and the more conventional physiological and behavioral methodologies has yielded insight into a human disease for which little mechanistic information and still less rational basis for potential therapies had existed. Is narcolepsy the only condition or sleep-wakefulness the only physiological behavior impacted by the Hcrt/ORXs? The metabolic consequences of ORX deficiency or excess merit full attention. How do these peptides act to inhibit prolactin secretion and what is the physiological consequence? What importance can be attached to the apparently steroid-dependent effects of the peptides on reproductive hormone secretion²³? Is the depressive effect of Hcrt/ORX on GH secretion physiologically relevant and how is it manifested? Perhaps the apparent sympatho-stimulatory effect¹² of the Hcrt/ORXs will provide clues to significant effects in addition to those exerted on the arousal state and sleep-wakefulness. There might, in fact, be a convergence of autonomic and endocrine effects of the peptides that will further elucidate the integrative mechanisms controlling metabolic and circulatory homeostasis^{12,24,25}.

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