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Review

The hypocretins/orexins: novel hypothalamic neuropeptides involved in different physiological systems

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Abstract. The hypothalamus regulates diverse physiological functions, including the control of energy metabolism, circadian rhythms, stress and anxiety, sexual and reproductive behaviors. An overview of the most prevalent hypothalamus-enriched mRNAs revealed that this area of the brain specializes in producing intercellular signaling molecules. Two new secreted peptides derived from a single neuropeptide precursor, named hypocretins and orexins by two different groups, are synthesized in a small set of neurons in the perifornical area of the hypothalamus. Intracerebroventricular injection of the hypocretins/orexins increases food consumption in rats. Here we review recent progress in identifying the role of the hypocretins/orexins in the control of energy balance and in other physiological systems.

Key words. Leptin; neuropeptide Y; perifornical area; lateral hypothalamus; food consumption; obesity.

The hypothalamus can be considered an ensemble of nuclei with diverse specialized functions that include the control of energy metabolism, circadian rhythms, stress and anxiety, sexual and reproductive behaviors. Most of the information on the function of the hypothalamus originated from lesioning studies [1–4]. These studies investigated the physiological effects of nuclei ablation, as well as the anatomical interactions between different nuclei within the hypothalamus and other regions of the brain. In particular, lesions in the lateral hypothalamus revealed this area as a feeding center [1], whereas lesions in the ventromedial hypothalamic area resulted in increased food intake, and established this area as a satiety center [3].

Elucidation of the chemical nature of peptidic hormones that control pituitary action boosted interest in the molecular composition of hypothalamic neurons. Early work on oxytocin, vasopressin, somatostatin and other peptides showed that a very specific subset of hypothalamic neurons effectively control homeostasis and modulate the release of peripheral hormones. More recently, the discovery of new hormones, including leptin [5], glucagon-like peptide (GLP) [6], cocaine- and amphetamine-regulated transcript (CART) [7], neuropeptide Y (NPY) [8] and agouti-related protein (AGRP) [9] has uncovered a complex network of signaling molecules that integrate information about the energy state in hypothalamic nuclei and control food intake and energy balance. Recent comprehensive reviews on the role of these molecules in feeding have been published elsewhere [8, 10, 11].

Leptin, the product of the obese (*ob*) gene, regulates adipose tissue mass through hypothalamic effects on satiety and energy expenditure [10]. In rodents, homozygous mutations in genes encoding leptin or the leptin receptor cause early onset morbid obesity, hyperphagia and reduced energy expenditure. These rodents also show hypercortisolemia, alterations in glucose

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homeostasis, dyslipidemia, and infertility due to hypogonadisms. In humans, a homozygous missense mutation in the leptin gene has been associated with extreme obesity [12]. Patients with mutations in the leptin receptor are also obese and display reproductive abnormalities [13].

Extensive work has accumulated evidence for the role of NPY in feeding. Administration of this 36-amino-acid transmitter into the cerebral ventricles, or locally in the lateral hypothalamus induces food intake [8, 14]. Mutant mice deficient in NPY, however, have normal food intake and body weight, and become hyperphagic following food deprivation [15]. NPY-deficient mice decrease their food intake and lose weight when treated with recombinant leptin, indicating that NPY may modulate feeding, but is not essential for certain feeding responses or leptin actions.

In the search for novel factors involved in feeding control, Maratos-Flier and colleagues [16] used differential display to identify mRNAs differentially expressed in the hypothalamus of *ob*/+ compared with *ob*/*ob* mice. One mRNA that was overexpressed in the hypothalamus of *ob*/*ob* mice encoded the neuropeptide melanin-concentrating hormone (MCH). Fasting further increased expression of MCH mRNA in both normal and obese animals and injection of MCH into the lateral ventricles of rats increased food consumption. Moreover, mice deficient in MCH are hypophagic and lean [17], indicating that this signaling molecule is involved in food consumption. Another mRNA that was upregulated in *ob*/*ob* mice encoded AGRP [9]. Recombinant AGRP is a potent, selective antagonist of Mc3r and Mc4r, melanocortin receptor subtypes implicated in weight regulation [18, 19]. Ubiquitous expression of human AGRP complementary DNA in transgenic mice caused obesity without altering pigmentation. These results suggested that MCH and AGRP participate in the hypothalamic regulation of body weight, probably downstream from leptin.

From these peptide discoveries and the lesioning studies it was also clear that additional signaling molecules remained to be identified.

Cloning of hypothalamic-enriched sequences

We used subtractive hybridization to obtain a collection of the most prevalent hypothalamus-enriched mRNAs [20]. Hypothalamic cDNA sequences were hybridized with complementary RNA from cerebellum and hippocampus, non-hybridizing cDNAs were amplified and cloned, and the nucleotide sequences of 100 clones from the subtracted library were determined. In contrast with the striatum, in which a similar analysis was performed [21], 40% of hypothalamus-enriched sequences encoded proteins with a confirmed or putative secretory signal peptide, suggesting that the hypothalamus specializes in producing hormones and intercellular signaling molecules. Interestingly, a large proportion of the hormones encoded in these sequences (i.e., CART, galanin, H35) are involved in the control of energy balance.

The hypocretins (orexins): two hypothalamus-specific peptides

Analysis of the expression pattern of subtracted hypothalamus-enriched sequences [20] revealed that one of these was expressed exclusively by a bilaterally symmetric structure within the posterior hypothalamus (fig. 1). Its 569-nucleotide sequence [22] 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 (fig. 2). This region of one of the peptides contained a 7/7 match with secretin (residues marked with asterisks in fig. 2), suggesting that the prepropeptide gave rise to two peptide products that were structurally related both to each other and to secretin. Interestingly, the region where secretin and the two peptides were more similar has been shown to be important for binding specificity [23]. Thus, these peptides were named hypocretin (hcrt) 1 and 2 to reflect their hypothalamic origin and the similarity to secretin. The identity between hcrt peptides and secretin is not preserved with the other members of the glucagon, vasoactive intestinal peptide, secretin (incretin) family. However, alignments of the sequences of the two new

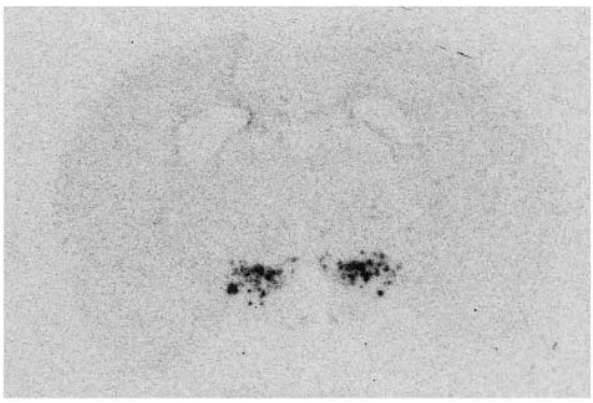

Figure 1. Detection of hypocretin (orexin) mRNA in large neurons in the dorsal-lateral hypothalamus by in situ hybridization to a coronal section from rat brain.

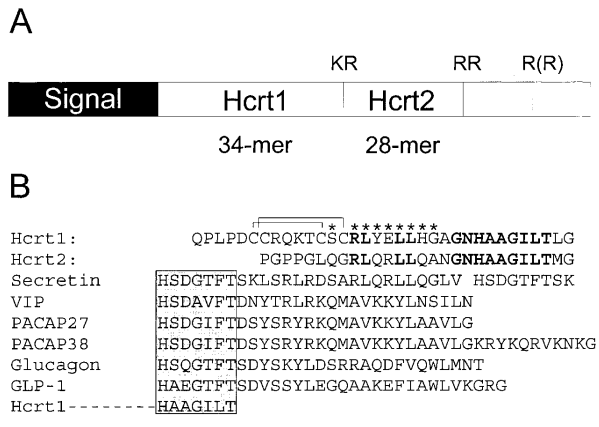

Figure 2. Alignment of the two hypocretin peptides with members of the secretin peptide family. (*A*) Diagram of preprohypocretin, indicating the dibasic residues that may be used as substrates for prohormone convertases. (*B*) Amino acid alignment of incretin family members. Identical residues between hypocretin 1 (Hcrt1) and hypocretin 2 (Hrct2) are indicated in bold. Identities between at least one of the hypocretins and at least one of the secretin family members (secretin, PACAP27, glucagon, GLP-1) are indicated by an asterisk. The N-terminal regions of the secretin family members (shaded box) have been aligned with the C-terminal hypocretin residues to extend the region of identity. Disulfide bridges between cysteine residues in Hcrt1 were determined by Sakurai et al. [24].

peptides with other family members reveals additional sequence similarities between N-terminal residues in the family members and the C-terminal residues of the hcrts. This suggests that the hypocretins represent members of the family whose primary amino acids are circularly permuted. A clone of rat preprohypocretin was subsequently isolated in an independent study by Sakurai et al. [24] and called preproorexin (see below).

The mouse preprohcrt nucleotide sequence [22, 24, 25] differed in 39 positions relative to the rat sequence. Of these differences, 19 were within the putative protein-coding region, only 7 of which affected the encoded protein sequence. One difference obliterated a potential proteolytic cleavage site, eliminating two of the four possible rat maturation products. However, hcrt1 and hcrt2 were absolutely preserved between rat and mouse. Both hcrts terminated with glycine residues, which typically are substrates for peptidylglycine alpha-amidating monooxygenase, leaving a C-terminal amide in the mature peptide. We mapped the mouse *Hcrt* gene to chromosome 11, a region that shows conserved synteny with human chromosome 17q21–q24. Indeed, Sakurai et al. [24] assigned the human gene by radiation hybrid mapping to 17q21. These workers isolated the human homologue: hcrt1 is identical among rats, mice, and humans; human hcrt2 differs from rodent hcrt2 at two residues.

In Northern blot studies with RNA from brain and different peripheral tissues, we detected the 700-nucleotide hcrt mRNA only in brain samples and within the brain only in hypothalamus. In samples of RNA from brains of developing rats, hcrt mRNA was detected at low concentrations as early as embryonic day 18, but increased in concentration dramatically after the third postnatal week [22, 25].

Hypocretin in cell bodies, fibers, and synaptic vesicles

We raised polyclonal antisera against chemically synthesized peptides corresponding to regions within the rat preprohcrt sequence and to bacterially expressed, histidine-tagged preprohcrt [22, 25–27]. In Western blots using these antisera and, as target extracts, bacteria expressing the fusion protein, we observed a single prominent immunoreactive band with a migration of approximately 19 kDa with the hyperimmune serum, but not with the preimmune serum. Control extracts contained no immunoreactive targets, confirming the specificity of the antisera for hcrt. Immunohistochemical studies detected prominent granular immunoreactivity within widely spaced, large polymorphic neurons exclusively in the perifornical nucleus, the dorsal, lateral and posterior hypothalamic areas, and the subincertal nucleus at the hypothalamic-thalamic border, coincident with the in-situ-hybridization-positive cells (fig. 3A,B) [22, 26]. This coincident staining and its elimination when the sera were preincubated with their immunogens, together with the Western blot studies, provided strong evidence for the specificity of the antiserum for hcrt. Similar results have been obtained by other investigators using these and independently prepared antisera [24, 26–28].

Approximately 1100 reactive cell bodies were observed in the rat brain between the fornix and the mammillothalamic tracts, 1 mm lateral to the midline, at the level of the median eminence [22, 24, 26, 28]. The hcrt neurons span the perifornical nucleus and the magnocellular nucleus of the lateral hypothalamus from the medial hypothalamus across the supra-fornical region at mid- to posterior hypothalamic levels into the myelinated axons of the retrochiasmic optic radiation (fig. 3A,B). In addition to the hypothalamic neurons, the antisera detected a prominent network of axons located within the posterior hypothalamus and beyond. Fiber projections were observed in apparent terminal fields within septal nuclei in the basal forebrain, the preoptic area, the paraventricular nucleus of the thalamus, the central gray, and the locus ceruleus. Very dense projections have also been described in lamina I of the spinal cord [29]. Less prominent fiber projections were observed in apparent terminal fields within the colliculi,

the laterodorsal tegmental nucleus, and the nucleus of the solitary tract. Hypocretin-containing fibers have also been detected in the pineal complex (J. Mikkelsen, personal communication). A complete mapping of these extensive projections from a relatively small number of neurons is given by Peyron et al. [26].

Antibodies to hcrt2 were also used to detect hcrt in monkey and human brain [30]. As in the rat, numerous hcrt-positive cells were detected in the posterior hypothalamus, scattered over the lateral hypothalamic area, across the dorsal hypothalamic area and perifornical area extending from the levels of the ventromedial nucleus to the rostral mammilary complex. Moore et al. [31] detected moderate dense axonal plexuses across most of the medial hypothalamus and extending dorsally in midline thalamus, laterally in the diagonal band and substantia innominata of the human brain. In the rostral medial hypothalamus, moderately dense inner-

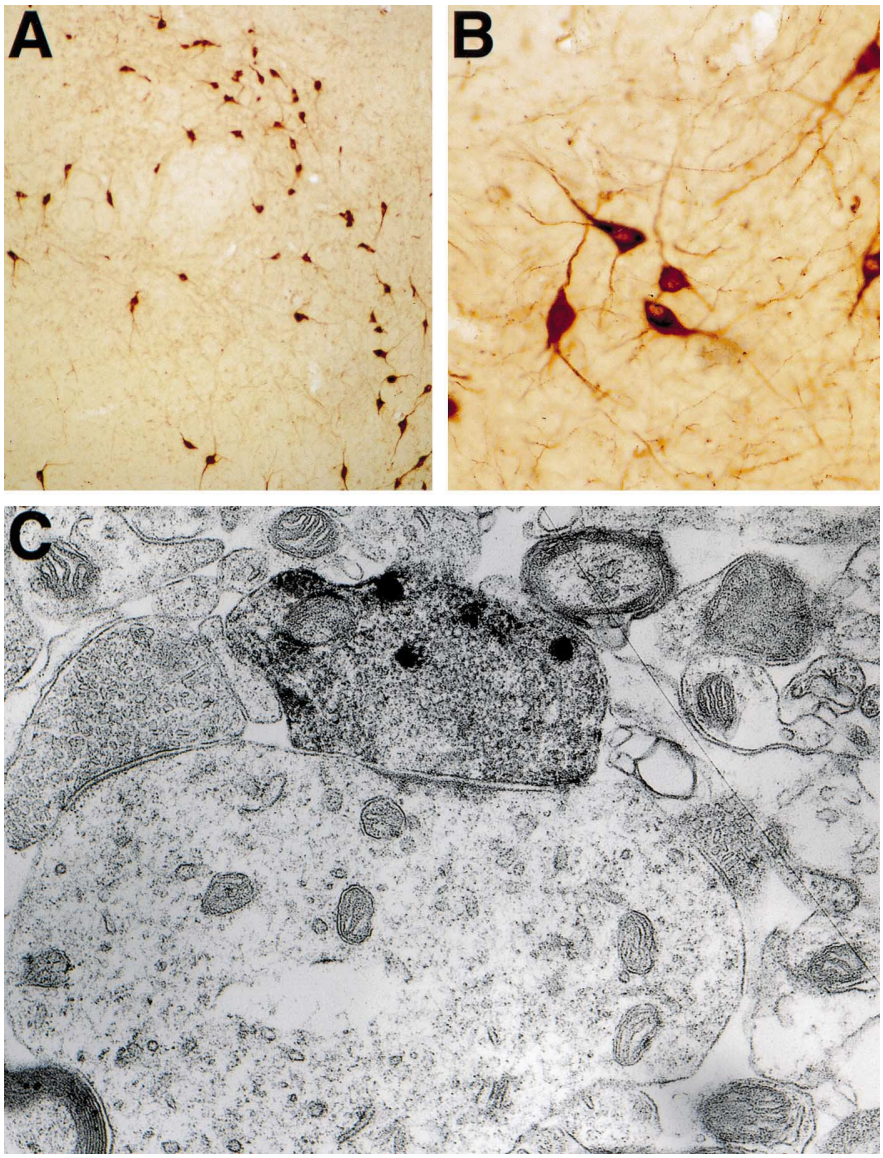

Figure 3. Immunocytochemical detection of hypocretin peptides. (*A*) Photomicrograph of the perifornical area in the lateral hypothalamus stained with antibody 2123 to preprohypocretin. All hypocretin-immunoreactive cell bodies are located around 1 mm^3 of the fornix. (*B*) Higher magnification of a set of hypocretin-immunoreactive somata and processes. (*C*) Electron micrograph showing electrodense hypocretin-immunoreactive material in large dense core vesicles of a hypothalamic axon terminal.

vation of the anterior hypothalamic area extended into the shell of the suprachiasmatic nucleus. This pattern of innervation extends over the retrochiasmatic area, ventromedial and dorsomedial nuclei, and arcuate and posterior hypothalamic nucleus.

In electron microscopy studies [22, 25, 26], immunoreactivity was observed within cell soma in the lateral hypothalamus on the Golgi apparatus and large dense-core granules (fig. 3C). Similar vesicles were observed in dendrites, within myelinated axons, and at presynaptic terminals opposite non-immunoreactive dendritic shafts.

Hypocretins are neuroexcitatory

The putative structures of the hypocretins, their expression within the dorsolateral hypothalamus and accumulation within vesicles at axon terminals suggested that they might have intercellular signaling activity. To test this hypothesis, we and others applied a synthetic peptide corresponding to the amidated form of hcrt2 to rat hypothalamic neurons that had been cultured for 10 days, and recorded postsynaptic currents under voltage clamp [22, 27]. At 1 μM, the peptide evoked a substantial, but reversible, increase in the frequency of postsynaptic currents in 75% of the neurons tested, indicative of an excitatory effect. The remaining 25% of the cells showed no response to hcrt2. There was little response by hypothalamic neurons that had been in culture for only 3–5 days, suggesting that a certain degree of synaptic maturity was required for the effect. hcrt2 elicited no response in cultures of synaptically coupled hippocampal dentate granule neurons (cells that do not express hcrt in vivo and where immunoreactive axons are rare), which demonstrated target selectivity and suggested that there may be specific receptors for hcrt2. In slices, approximately one-third of all medial and lateral hypothalamic neurons tested, but not hippocampal neurons, show a striking nanomolar sensitivity to hypocretin. The peptide has a potent effect at both presynaptic and postsynaptic receptors. Most synaptic activity in hypothalamic circuits is attributable to axonal release of gamma-aminobutyric acid (GABA) or glutamate. Hypocretin, acting directly at axon terminals, can increase the release of each of these amino acid transmitters, as seen with whole-cell patch-clamp recording.

van den Pol et al. [27] have determined the second-messenger systems involved in hypocretin signaling. Both hcrt1 and hcrt2 evoke rises in Ca^{2+}, as measured by fura-2 imaging, in about one-third of hypothalamic neurons, probably by opening a plasma membrane calcium channel. Hypocretin responses are completely blocked by the protein kinase C (PKC)-specific inhibitor bisindolylmaleide, suggesting that hypocretin may work via G_q-activated PKC, resulting in phosphorylation of Ca^{2+} channels, reported to increase Ca^{2+} conductance [32].

The endogenous peptides and their receptors

Sakurai et al. [24] prepared transfected cell lines stably expressing each of 50 orphan G-protein-coupled receptors. Calcium fluxes were measured in response to fractions from tissue extracts. A peptide from rat brain extracts, called orexin A, acted at one of the receptors, and its sequence matched that of the C-terminally amidated form of hcrt1 with the N-terminal glutamine derivatized as pyroglutamate. There were two intrachain disulfide bonds (fig. 2B). A peptide from bovine brain had the same structure. A less active peptide in the rat brain extract, orexin B, matched hcrt2.

The initial orphan receptor, hcrtr1, bound hcrt1 (orexin A) with high affinity, but hcrt2 (orexin B) with 100- to 1000-fold lower affinity. However, a related receptor, hcrtr2, identified by searching database entries with the hcrtr1 sequence, had high affinity for both hcrt2 and hcrt1 [24]. The mRNAs encoding the two receptors are both enriched in the brain and moderately abundant in the hypothalamus but display remarkably different distributions. Within the hypothalamus, hcrtr1 mRNA is most abundant in the ventromedial hypothalamic nucleus whereas hcrtr2 is predominantly expressed in the paraventricular nucleus. High levels of hcrtr1 mRNA have also been detected in tenia tecta, the hippocampal formation, dorsal raphe, and locus ceruleus. hcrtr2 mRNA is mainly expressed in cerebral cortex, nucleus accumbens, subthalamic and paraventricular thalamic nuclei, and anterior pretectal nucleus [33]. The combined pattern of expression of these receptors is consistent with the map of hcrt-containing fibers. In addition, 5% of cultured cortical neurons were excited by hcrt2 [27].

The presence of orexin receptor mRNA in the hypothalamus supports its proposed role in feeding regulation, although the broad central distribution of orexin receptors may indicate additional functions for the hypocretins.

A role in feeding

Stereotactic ablation and physiological studies have previously implicated the dorsal-lateral hypothalamus in several homeostatic processes, including feeding behavior, blood pressure, thermoregulation and arousal [34–37]. Sakurai et al. [24] demonstrated that intracerebroventricular administration of either peptide increased food consumption in rats. Furthermore, rats

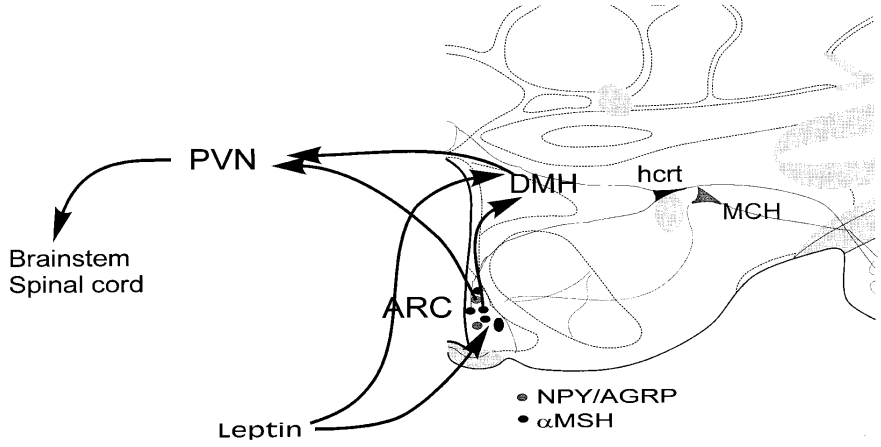

Figure 4. Model of interactions of regulatory signals in the hypothalamus. Leptin, which is mainly secreted by adipocytes in the periphery, exerts its central effects by binding to its receptors in the arcuate (ARC) and dorsomedial (DMH) hypothalamic nuclei. The arcuate nucleus, which contains cell bodies immunoreactive for NPY/AGRP and propiomelanocortin, projects axon terminals to the paraventricular nucleus, which regulates the hypothalamo-pituitary-adrenal and hypothalamo-pituitary-thyroid axes. The paraventricular nucleus (PVN) sends descending projections to the brainstem and spinal cord, regulating thermogenesis and inhibiting the effects of insulin. Arcuate neurons respond to leptin and are immunoreactive for the leptin receptor, suggesting that leptin acts through those neurons to generate a wide diversity of physiological effects [41]. Hypocretin- and MCH-immunoreactive cells in the lateral hypothalamic area receive input from the arcuate, and may integrate information from other brain structures, including the cerebral cortex, locus ceruleus and brainstem. Therefore, hcrt cells may integrate the complex network that regulates complex behaviors associated with food consumption.

fasted for 48 h increased the concentration of hypocretin mRNA by 2.4-fold. Based upon these two sets of observations the name, orexin, after the Greek word όρεξη (orex for appetite), was proposed for the hypocretins.

hcrt-immunoreactive fibers make synaptic contacts with neurons in the arcuate nucleus that contain NPY, an important orexigenic peptide, and with propiomelanocortin neurons, which produce α-melanocyte-stimulating hormone (α-MSH) raising the hypothesis that hypocretins modulate feeding indirectly. hcrt-positive cells also show leptin receptor (*ob*-r) immunoreactivity [30], and preprohypocretin mRNA expression is down-regulated in *ob*/*ob* mice [38]. Also supporting the idea that the hypocretins are involved in feeding is the co-localization of hypocretin immunoreactivity and the expression of *c-fos* induced by fasting in the lateral hypothalamus [30]. Although the localization of hcrt-immunoreactive neurons is reminiscent of the pattern obtained with antisera to MCH, double-labeling experiments have demonstrated that these two peptides do not co-localize in the same hypothalamic neurons [26, 39, 40]. Terminals from NPY- and AGRP-immunoreactive cells, at least some of which come from the arcuate nucleus that contains leptin receptors [41], engulf hypocretin-producing and MCH-producing cell bodies in the lateral hypothalamus [39, 40]. These results suggest a complex circuitry of appetite-controlling signaling molecules in the lateral hypothalamus, in which hypocretin may have a major integrating role (see fig. 4).

Feeding studies have demonstrated that both GABA and glutamate can regulate hypothalamic control of food intake [42], suggesting that one mode of hypocretin action is presynaptic modulation of GABA and glutamate circuits involved in energy regulation. Since hypocretin can modulate either glutamatergic or GABAergic neurons in the hypothalamus [27], the local microcircuitry is likely critical in hypocretin function. As already pointed out by others [27], this raises concerns about interpreting physiological effects of intracerebroventricular administration of high doses of hypocretin, which may activate circuits other than those that would be activated by local axonal release of the transmitter.

Since approximately one-third of all hypothalamic neurons respond to hypocretin and hypocretin axon terminals are found throughout the hypothalamus, hypocretin is likely to influence the general level of activity in many hypothalamic systems, including those related to MCH, α-MSH, AGRP, or CART [7, 9, 16, 18]. These are found in the same hypothalamic regions as high densities of hypocretin axons, suggesting that hypocretin cells may not only directly affect postsynaptic neurons, but may also modulate the synaptic input

to these areas at the axonal terminal. Thus, hypocretin may be an important link in the chain of regulatory cells that orchestrate behavioral, metabolic, and endocrine systems to maintain energy homeostasis.

Hypocretins in other physiological systems

The location of hcrt-producing cells in the perifornical and lateral hypothalamus and their projections [26] and the widespread distribution of orexin receptors [33] indicate that the peptides may have roles in other homeostatic processes. These cells receive inputs from brainstem areas associated with cardiovascular function, and output to the ventrolateral medulla, the locus ceruleus, the lateral paragigantocellular nucleus, the nucleus of the solitary tract and other areas that are consistent with a role in the regulation of blood pressure and heart rate [43]. Consistent with this idea, administration of a hypocretin dose that stimulates sucrose intake exerts a profound effect on blood pressure [44]. The projections to the raphe magnus and subceruleus suggest a role in the regulation of body temperature. Indeed, indirect calorimetric experiments in mice have shown that a single intracerebroventricular injection of 3 nmol of hcrt1 increases the metabolic rate [45], suggesting that the hypocretins modulate energy metabolism rather than food intake. It is noteworthy that in these experiments, hcrt2 had no effect on food consumption or metabolic rate. Another recent report showed that infusion of hcrt2 enhanced different types of behavior, including face washing, burrowing and searching activities, but had no significant effect on food intake [46].

The projections to the arcuate nucleus suggest a role in the regulation of hormone release. In particular, the distribution of hcrt fibers overlaps with the luteinizing hormone (LH)-releasing hormone (LHRH) neuronal system in the septopreoptic area and the arcuate nucleus-median eminence region. To determine whether the hypocretins regulate pituitary LH secretion by influencing LHRH release, the effects of hcrt1 and hcrt2 on LH secretion have been measured in ovariectomized and ovarian-steroid-treated ovariectomized rats [47]. Intracerebroventricular injection of hcrt1 or hcrt2 stimulates LH secretion in a dose- and time-related fashion in estradiol-benzoate- and progesterone-pretreated ovariectomized rats. On the other hand, both hcrts inhibit LH release in unprimed ovariectomized rats. This ovarian-steroid-dependent bimodal LH response suggests a role for the hypocretins in the control of reproduction [47]. The dense projections to the ventrolateral preoptic area and tuberomammillary nucleus suggest involvement in the state of arousal [48]. Very strong hypocretin-immunoreactive projections have been described in regions of the spinal cord related to modulation of pain [29]. These observations were supported by retrograde labeling and electrophysiological data showing that neurons in the spinal cord respond to hypocretins [29].

Besides the central effects of the hypocretins, other physiologically relevant actions of these peptides in the periphery cannot be ruled out. Intracisternal injection of hcrt1, but not hcrt2, stimulates gastric acid secretion [49]. Hypocretins have also been involved in the immune response. A recent study has shown that hcrt2 can modulate macrophage functions through the activation of Ca^{2+}-dependent K^+ channels [50].

Thus, at this stage we know that these new peptides function as intercellular messengers, and that they are involved in energy homeostasis. Further studies will be required to evaluate the involvement of these peptides in other functions.

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