THE HYPOCRETINS: SETTING THE AROUSAL THRESHOLD

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Over a short period in the late 1990s, three groups converged on the discovery of a neuropeptide system, centred in the dorsolateral hypothalamus, that regulates arousal states, influences feeding and is implicated in the sleep disorder narcolepsy. Subsequent studies have illuminated many aspects of the circuitry of the hypocretin (also called orexin) system, which also influences hormone secretion and autonomic homeostasis, and have led to the hypothesis that most human narcolepsies result from an autoimmune attack against the hypocretin-producing neurons. The biochemical, physiological and anatomical components that regulate the switch between waking and sleeping are becoming clear. The rapidity with which the hypocretin story has emerged is a testament to both the conceptual and the technical evolution of genomic science in the past two decades.

OPEN-SYSTEM ANALYSIS
Analysis of all the mRNAs that are expressed by a tissue without regard to whether the mRNAs have previously been identified. The contrasting approach is to measure the expression of known mRNAs, as is done in cDNA array (chip) studies.

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DOI: 10.1038/nrn808

Recent studies have led to the discovery of a neuropeptide system that regulates arousal states and energy metabolism. The hypocretins (also called orexins) are two carboxy-terminally amidated neuropeptides of related sequence. They are produced from a common precursor, which is expressed only in a few thousand neurons of the rat dorsolateral hypothalamus. Two G-protein-coupled receptors (GPCRs) for the hypocretins have been identified, and these have different distributions within the central nervous system (CNS) and different affinities for the two hypocretins. The hypocretins have been detected in secretory vesicles at synapses of fibres that project to areas within the posterior hypothalamus that are implicated in feeding behaviours and hormone secretion. Hypocretin fibres also project to diverse targets in other brain regions and the spinal cord, including several areas that have been implicated in cardiovascular function and sleep–wake regulation. The peptides are excitatory when applied directly *in vivo*, and to cultured neurons and slices *in vitro*, although there is also evidence for some inhibitory signalling. Administration of the hypocretins stimulates food intake, affects blood pressure, hormone secretion and locomotor activity, and increases wakefulness while suppressing rapid eye movement (REM) sleep.

Inactivating mutations in the gene for hypocretin receptor 2 (*Hcrtr2*) in dogs result in narcolepsy. Mice in which the preprohypocretin (*Hcrt*) gene has been inactivated have a narcolepsy-like phenotype. Most human patients with narcolepsy have greatly reduced levels of hypocretin peptides in their cerebrospinal fluid (CSF), and no, or barely detectable, hypocretin neurons in their hypothalami, indicating the possibility of an autoimmune attack against these neurons. One aspect of hypocretin activity is the direct excitation of cholinergic forebrain neurons and brainstem monoaminergic REM-off neurons in the locus coeruleus, dorsal raphe nucleus and tuberomammillary nucleus (TMN), which together suppress slow-wave sleep. The hypocretins also modulate the activity of cholinergic REM-on neurons in the brainstem, which gate REM entry. So, the dominant activities of the hypocretin system are maintenance of the waking state and suppression of REM entry.

Discovery and properties of the hypocretins

The first glimpse of the hypocretins came from an OPEN-SYSTEM search for undiscovered homeostatic regulatory peptides. Gautvik and colleagues[1] conducted a systematic subtractive hybridization survey aimed at identifying messenger RNA species that are expressed only in discrete nuclei within the rat hypothalamus.

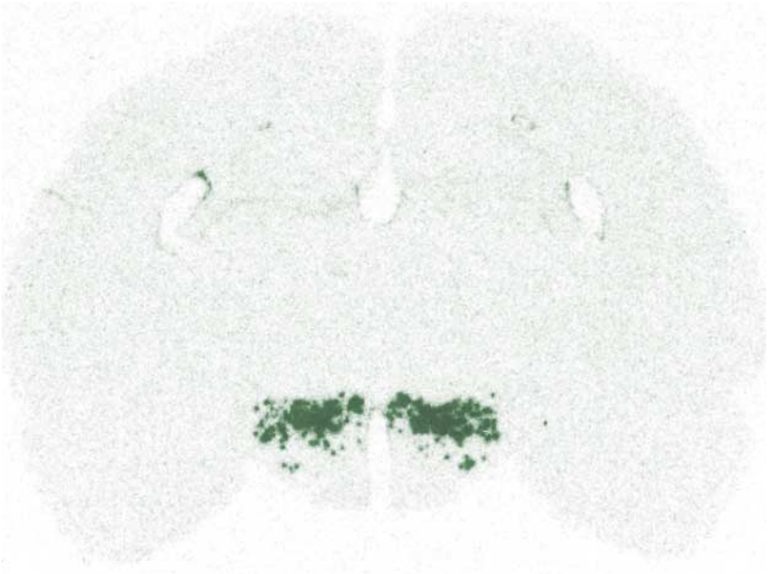

Figure 1 | **The first glimpse of the hypocretin system.** *In situ* hybridization in a coronal section of the rat brain with complementary DNA that was isolated in a subtractive hybridization study. A few thousand neurons were detected in the dorsolateral hypothalamus. Reproduced, with permission, from REF. 1 © 1996 National Academy of Sciences, USA.

They found that 40% of the mRNA species that were highly enriched in the hypothalamus encoded hormones and releasing factors.

Among the new hypothalamic mRNAs that were identified was a species with a pattern of expression, detected by *in situ* hybridization, that was restricted to a few thousand neurons that were bilaterally distributed within the dorsolateral hypothalamus[1,2] (FIG. 1). The complementary DNA sequences of the rat and homologous mouse mRNAs each encoded a 130-residue, putative secretory protein with an apparent signal sequence and two further phylogenically conserved sites for potential proteolytic maturation followed by modification of the carboxy-terminal glycines by peptidylglycine α-amidating monooxygenase[2]. These features indicated that the product of this hypothalamic mRNA was a preprohormone for two carboxy-terminally amidated, secreted peptides (FIG. 2a). One of these, hypocretin 2 (Hcrt2), was, on the basis of the putative preprohormone amino-acid sequence, predicted to contain 28 residues. The other, hypocretin 1 (Hcrt1), had a defined predicted amidated carboxyl terminus but, because of uncertainties as to how the amino terminus might be proteolytically processed, an undefined amino-terminal extent[2]. The carboxy-terminal 19 residues of these two putative peptides shared 13 amino-acid identities (FIG. 2b), indicating that the peptides had related structures and functions. This region of Hcrt2 contained a 7-amino-acid match with secretin.

The detection of the two hypocretin peptides within the brain allowed their structures to be determined by MASS SPECTROMETRY[3] (FIG. 2). The sequence of endogenous Hcrt2 was as predicted from the cDNA sequence. The amino terminus of Hcrt1 corresponds to a genetically encoded glutamine that is derivatized as pyroglutamate. Hcrt1 contains two intrachain disulphide bonds. Human HCRT1 is identical to the rodent peptide, whereas human HCRT2 differs from rodent Hcrt2 at two residues[3].

Phylogenic studies, facilitated by the genomics database revolution, have detected genes that encode conserved preprohypocretins in pufferfish and frog species, indicating that the gene arose early in the chordate lineage[4]. Sequence similarities with various members of the incretin family, especially secretin, indicate that the *Hcrt* gene was formed from the secretin gene by three genetic rearrangements: first, a duplication of the secretin gene; second, deletions of the amino-terminal portion of the 5′ duplicate and the carboxy-terminal portion of the 3′ duplicate to yield a secretin with its amino and carboxyl termini leap-frogged (CIRCULARLY PERMUTED); and third, a further duplication of the permuted gene, followed by modifications, to form a gene that encoded two related peptides[4] (FIG. 2c).

Consistent with the hypothesis that the hypocretins and secretin are phylogenically related, portions of their three-dimensional solution structures, as determined by nuclear magnetic resonance, are similar despite their leap-frogged primary sequence, consisting of two adjacent α-helices (6–7 and 9–14 amino acids in length) separated by a short 2–3-amino-acid turn[5,6]. The longer helix corresponds to the region of identity between the two peptides.

The hypocretin receptors

The second important step in illuminating the hypocretin system was taken by Sakurai *et al.*[3], who prepared transfected cell lines that stably expressed each of 50 orphan GPCRs, and then measured Ca^{2+} fluxes in these cell lines in response to fractions from tissue extracts. One of the cell lines responded to a substance in a brain extract. Mass spectrometry showed that this substance was a peptide with a sequence that was later identified as that of endogenous Hcrt1. The initial orphan GPCR, Hcrtr1, bound Hcrt1 with high affinity, but Hcrt2 with 100- to 1,000-fold lower affinity. A related GPCR, Hcrtr2, which was identified by searching GenBank database entries with the Hcrtr1 sequence, had a high affinity for both Hcrt2 and Hcrt1 (REF. 3).

The mRNAs that encode the two hypocretin receptors are both enriched in the brain and are moderately abundant in the hypothalamus, but have different distributions within the brain[7,8]. Hcrtr1 mRNA is prominent in the prefrontal and intralimbic cortex, hippocampus, paraventricular thalamic nucleus, ventromedial hypothalamic nucleus, dorsal raphe nucleus and locus coeruleus. Hcrtr2 mRNA is detected in the cerebral cortex, septal nuclei, hippocampus, medial thalamic groups, raphe nuclei and various nuclei of the hypothalamus, including the TMN, dorsomedial nucleus, paraventricular nucleus and ventral premammillary nucleus.

Localization of the hypocretins

The hypothalamus is a phylogenically ancient region of the mammalian brain, organized as a collection of distinct, autonomously active nuclei. Several of these nuclei

MASS SPECTROMETRY
A technique in which a compound is bombarded with an electron beam of sufficient energy to fragment the molecule. The cations that are produced are accelerated in a vacuum through a magnetic field, and sorted on the basis of mass-to-charge ratio. The ratio is roughly equivalent to the molecular weight of the fragment.

CIRCULARLY PERMUTED
The rearrangement of a string of nucleotides or amino acids in which all elements of the string maintain their orientation and immediate neighbours, except for those elements that were at the ends of the string before the rearrangement and those that form the new ends, as if the string had formed a circle and then opened at a different point. At the genetic level, this process is thought to occur by gene duplications to form a tandem structure, followed by deletions from opposite ends of the tandem.

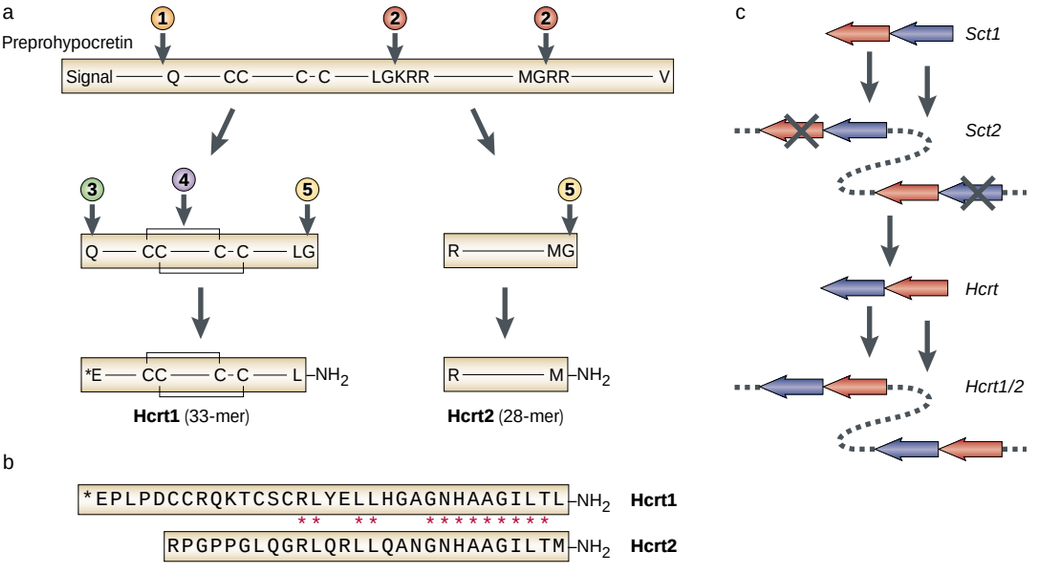

Figure 2 | **Preprohypocretin and the hypocretins. a** | Maturation of preprohypocretin. Only amino-acid residues that are key to the processing of the prepropeptide are shown. After removal of the secretion signal (1), the prohypocretin is cleaved at two pairs of tandem basic amino acids (KR, RR; 2). The genetically encoded glutamine (Q) is derivatized to form pyroglutamate (*E; 3), two intrachain disulphide bonds (C–C) are formed (4), and the carboxy-terminal glycines (G) are modified by peptidylglycine α-amidating monooxygenase (5), leaving carboxy-terminal amides on the resulting 33-mer and 28-mer peptides, Hcrt1 and Hcrt2, respectively. **b** | The primary amino-acid sequences of rat Hcrt1 and Hcrt2. The *E at the amino terminus represents the pyroglutamate residue; the asterisks between the sequences indicate the positions of identity between the two peptides. The disulphide bonds are as in **a**. Notice the carboxy-terminal amide groups. **c** | Model of the evolution of hypocretins Hcrt1 and Hcrt2 from secretin (Sct). Adapted from REF. 4.

have been implicated as regulatory centres for autonomic and endocrine homeostatic systems. The perifornical region has been associated with nutritional homeostasis, blood pressure and thermal regulation, neural control of endocrine secretion and arousal[9–14]. So, these activities ranked among those that might be affected by the hypocretins.

The perifornical hypothalamus contains a collection of neurons that express melanin-concentrating hormone (MCH), a peptide that has been implicated in feeding-related behaviour[15]. Double-label colocalization studies[16–19] have shown that the MCH and hypocretin neurons are distinct but spatially intermingled. However, there is a nearly one-to-one correspondence between neurons in the lateral hypothalamus that express the opioid receptor agonist dynorphin and the hypocretin neurons[20]. Hypocretin neurons receive direct projections from neurons in the suprachiasmatic nucleus (SCN)[21], which generates the circadian rhythm.

Antisera against both chemically synthesized and recombinant hypocretin fragments[2,16,22,23] have revealed a few thousand immunoreactive neurons between the fornix and the mammillothalamic tracts, and prominent projections from these cells (FIG. 3a) to other neurons in the perifornical and posterior hypothalamus. There are further prominent projections with apparent terminal fields within septal nuclei in the basal forebrain, the preoptic area, the paraventricular nucleus of the thalamus, the central grey, the locus coeruleus, the pedunculopontine tegmental nucleus (PPT) and the spinal cord. The cumulative set of projections is consistent with the combined patterns of expression of the two hypocretin GPCRs, perhaps indicating that there are no other receptors for these peptides. The projection fields in humans are similar to those in rodents[24].

TETRODOTOXIN
A potent marine neurotoxin that blocks voltage-gated sodium channels. Tetrodotoxin was originally isolated from the tetraodon pufferfish.

Hypocretins are neuroexcitatory

Electron-microscopic examination revealed that hypocretin immunoreactivity is associated with dense-core vesicles[2,16,25]. These can be seen traversing the Golgi network, along myelinated axons and at presynaptic terminals apposed to dendritic shafts (FIG. 3b). The accumulation of the hypocretins within dense-core vesicles at axon terminals indicated that they might have intercellular signalling activity. In support of this idea, bath application of synthetic Hcrt2 to mature hypothalamic neurons evoked increases in the frequency of postsynaptic currents[3]. The number of responsive neurons in different brain regions is consistent with projection and receptor densities: around 33% of hypothalamic neurons respond to Hcrt2, compared with 5% of cerebral cortex neurons and 15% of spinal cord neurons[23,26]. Around 80% of neurons within the hypothalamic paraventricular nucleus are excited by Hcrt2 (REF. 27). Similar results have been obtained by applying Hcrt1 to locus coeruleus slices[28].

Hcrt2 has a potent effect at both presynaptic and postsynaptic receptors: in the presence of TETRODOTOXIN, it increases the frequency, but not the amplitude, of miniature postsynaptic currents (presynaptic effect) and evokes an increase in cytoplasmic Ca^{2+} by opening plasma-membrane Ca^{2+} channels in arcuate postsynaptic

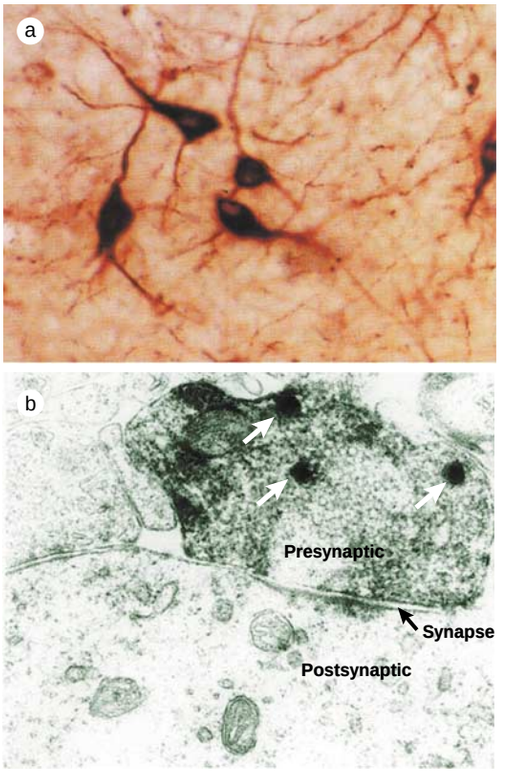

Figure 3 | **Localization of hypocretin. a** | Projections from hypocretin neurons. Photomicrograph of the perifornical area of the hypothalamus treated with an antibody directed against preprohypocretin, showing immunoreactive cell bodies and hypocretin-containing fibres, the paths of which have been traced throughout the central nervous system. **b** | Electron micrograph showing electron-dense, hypocretin-immunoreactive material in dense-core vesicles (white arrows) in a dendritic terminal. The presynaptic site is loaded with synaptic vesicles that contain neurotransmitter; the postsynaptic site contains mitochondria and electron-dense granules that are filled with hypocretin-immunoreactive material. Reproduced, with permission, from REF. 2 © 1998 National Academy of Sciences, USA.

neurons (postsynaptic effect)[26]. Most synaptic activity in hypothalamic circuits is attributable to axonal release of GABA (γ-aminobutyric acid) or glutamate. Hypocretin, acting at axon terminals, can increase the release of each of these transmitters, as shown by whole-cell patch-clamp recording[26].

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 Ca^{2+} channel[26,29]. Responses to hypocretin are completely blocked by the protein kinase C (PKC)-specific inhibitor bisindolylmaleide and by phospholipase C inhibitors, indicating that the hypocretins operate through a family of GTP-binding proteins (G_q) that activate PKC and mobilize intracellular Ca^{2+}. G_q- activated signalling cascades result in the phosphorylation of Ca^{2+} channels, which can increase Ca^{2+} conductance and neuronal excitability[30,31]. The non-amidated forms of the peptides are not electrophysiologically active[30]. The adrenal glands also express Hcrtr2 (REFS 32,33). Treatment of human adrenal membranes from fetal or adult tissue with HCRT1 increases the labelling of G_s and G_i in both preparations, and also of G_q in the adult tissue. So, although most hypocretin signalling is excitatory, it can be inhibitory in some cases[34]. Acting as excitatory peptides, the hypocretins can enhance the activities of both excitatory and inhibitory neurons.

The hypocretins and feeding

Our understanding of the regulation of food intake has advanced greatly over the past decade. Leptin, encoded by the *obese* gene, is a peptide hormone that is produced by peripheral adipocytes. Its concentration in the circulation is proportional to fat stores and it serves as an anorexigenic (appetite-suppressing) signal, in part by binding to leptin receptors in the hypothalamus. There are leptin receptors in arcuate nucleus neurons that express neuropeptide Y (NPY) and agouti-related peptide (AgRP), and others that express pro-opiomelanocortin (POMC) and cocaine- and amphetamine-regulated transcript (CART). There are also leptin receptors in MCH neurons of the lateral hypothalamus. Genetic and physiological studies have shown that NPY, AgRP, α-melanocyte-stimulating hormone (α-MSH, a POMC-derived peptide), CART and MCH all contribute to the regulation of food intake.

Sakurai and colleagues[3] found that intracerebroventricular (i.c.v.) administration of either Hcrt1 or Hcrt2 increased short-term food consumption in rats. Furthermore, rats that had been deprived of food for 48 hours had increased concentrations of hypocretin mRNA and peptides in the hypothalamus[3,35]. These observations led to the proposal of the alternative name, orexin, for the hypocretin peptides. Feeding can be elicited by local administration of Hcrt1 to the paraventricular nucleus, the dorsomedial nucleus, the lateral hypothalamus or the perifornical area[36]. The i.c.v. administration of Hcrt2 also increases food intake in sheep[37].

Despite these observations, the central importance of the hypocretins to feeding activities remains uncertain. There is little doubt that the hypocretin system influences and is influenced by primary nutritional-homeostasis circuits, but other findings indicate that the hypocretins are not crucial players in feeding activities.

On the plus side, hypocretin-immunoreactive fibres form synapses on neurons in the arcuate nucleus that contain NPY, an important orexigenic (appetite-stimulating) peptide, and with POMC-expressing neurons, which produce α-MSH, a satiety factor[17,18,25]. Hypocretin neurons express leptin receptors[19,25], and preprohypocretin mRNA expression is reduced in *obese* (*ob*/*ob*) mice[38], which lack leptin. Hypocretin neurons receive inputs from NPY- and AgRP-positive neurons in the arcuate nucleus, which themselves express leptin receptors, and NPY stimulates *C-FOS* expression by hypocretin neurons, whereas NPY receptor antagonists block the feeding effect of hypocretins[39,40]. Hypocretin-expressing cells respond

C-FOS
An immediate early gene that is rapidly turned on when many types of neuron increase their activity. It can therefore be used to identify responsive neurons.

to circulating leptin by reducing Hcrt1 concentrations and c-*Fos* expression[41–43].

The i.c.v. injection of antibodies against Hcrt1 reduces feeding activity[44] and attenuates the feeding response to injected NPY[42]. The i.c.v. administration of ghrelin, the orexigenic ligand of the growth hormone secretagogue receptor, activates c-*Fos* expression by hypocretin neurons[45], although this result is also consistent with a response to the transient reduction in core body temperature that is induced by this substance (see below). In addition, hypocretin neurons express Stat3 (signal transducer and activator of transcription 3), a transcription factor that is induced by leptin[19]. Hypocretin cells are sensitive to glucose and food deprivation[46–50]: the activity of hypocretin neurons and their expression of hypocretin mRNA and c-*Fos* increases during hypoglycaemia; hypocretin mRNA decreases during glucopenia; and c-*Fos* expression increases during fasting. These results are consistent with a complex circuitry of appetite-controlling signalling molecules in the arcuate and lateral hypothalamus, in which hypocretin might have a role.

However, not all data support this idea, particularly when the other activities of the hypocretins are considered (see below). Ida *et al.*[51] found that Hcrt1-induced increases in food intake were small relative to those induced by NPY infusion. Other groups could not find any effect of Hcrt2 on feeding (REFS 52,53). One report found increases in food intake after i.c.v. infusion, but not after local intrahypothalamic injection of Hcrtr2 (REF. 54). Others have measured no alteration in Hcrt1 peptide concentration or hypocretin mRNA in the rat hypothalamus in response to either fasting or a high-fat diet, and no effect on hypocretin mRNA of experimentally induced diabetes[55–57]. However, hypocretin mRNA increases after leptin administration to fasted mice[56]; this study also found no effect of the *obese* mutation on hypocretin expression.

It is difficult to attribute physiological effects to i.c.v. administration of high doses of hypocretin, which might activate circuits other than those that would be activated by local axonal release of the transmitter[26]. Also, the hypocretins might be orexigenic only in some physiological states (perhaps in relation to circadian rhythms or stress). In this regard, it might be significant that the hypocretins activate dopamine-mediated stereotypical behaviours. Hcrt1 peptide concentrations in the hypothalamus are under circadian control and are highest during the awake, dark period in nocturnal rodents[58]. During fasting, Hcrt1 accumulation in the CSF does not exceed normal concentrations for the awake period, indicating that some of the food-uptake effect might result from arousal rather than direct feeding pressure[59]. Continuous administration of Hcrt1 for seven days in rats does not significantly alter daily food intake, body weight, blood glucose, total cholesterol or levels of free fatty acids[60], indicating that many of the effects of hypocretin might be limited to immediate, short-term stimulation of feeding behaviour due to increased wakefulness. We will revisit this issue later.

PENETRANCE
The proportion of genotypically mutant organisms that show the mutant phenotype. If all genotypically mutant individuals show the mutant phenotype, then the genotype is said to be completely penetrant.

Autonomic and endocrine homeostasis

Hypocretin neurons receive inputs from brainstem areas that are associated with cardiovascular function, and project to the ventrolateral medulla, the locus coeruleus, the lateral paragigantocellular nucleus, the nucleus of the solitary tract and other areas that have been implicated in the regulation of blood pressure and heart rate[61]. Projections to the arcuate nucleus also indicate a possible role in the regulation of hormone release. In the ovine hypothalamus, there are hypocretin terminals on the neurons that produce gonadotropin-releasing hormone, indicating that hypocretin might modulate reproductive endocrinology[62]. In addition, projections to the raphe magnus and subcoeruleus indicate a role for hypocretins in the regulation of body temperature. The dense hypocretinergic projections to the ventrolateral preoptic area, TMN, pontine reticular formation (PRF), PPT, laterodorsal tegmental nucleus (LDT) and locus coeruleus indicate involvement in states of arousal[16,28,63]. Strongly hypocretin-immunoreactive projections have been described in regions of the spinal cord that are related to the modulation of pain[23], and hypocretin-like immunoreactivity has also been detected in the intestinal epithelium[64].

In accordance with the wide distribution of hypocretin terminals, i.c.v. administration of the hypocretins affects several functions other than feeding. Both Hcrt1 and Hcrt2 elevate mean arterial blood pressure, heart rate and oxygen consumption[65–68]. Hcrt1 increases body temperature independently of peripheral thermogenesis[69], increases water consumption and stimulates gastric acid secretion in the gut[70,71]. Hcrt1 also increases locomotor activity and wakefulness, while decreasing slow-wave and REM sleep[28,72–75].

The peptides also stimulate the secretion of luteinizing hormone in ovarectomized and proestrus female rats and hypothalamic explants of male pituitaries[76,77]. Hcrt1 decreases the concentrations of circulating growth hormone and prolactin, while increasing corticosterone, adrenocorticotropic hormone (ACTH) and insulin levels[28,78–81]. Hcrt2, but not Hcrt1, increases the concentration of circulating thyroid-stimulating hormone[82], and has direct effects on the pituitary, adrenal and pineal glands[32,83,84]. Hcrt2 is excitatory on superficial dorsal horn neurons of the spinal cord[85] and has an analgesic effect in models of pain[86]. There are several examples in which either Hcrt1 or Hcrt2, but not both, elicits a response, indicating that the two peptides are not redundant. In some cases, the effects can be explained by differential involvement of Hcrtr1 or Hcrtr2; in others, differential resistance of the peptides to degradation might provide the explanation.

The hypocretins and narcolepsy

The third revelation concerning the hypocretins came from genetic linkage studies in a colony of Doberman Pinschers in which narcolepsy was inherited as an autosomal-recessive, fully PENETRANT phenotype. Fine mapping and cloning of the defective canine narcolepsy gene, facilitated by advances in chromosome-mapping

technology, showed that it encodes the hypocretin receptor, Hcrtr2 (REF. 87). The mutation in the Doberman lineage is an insertion of a short interspersed repeat (SINE element) into the third intron of *Hcrtr2* that causes aberrant splicing of the Hcrtr2 mRNA (exon 4 is skipped) and results in a truncated receptor protein. In cells that have been transfected with the mutant gene, the truncated Hcrtr2 protein does not localize properly to the membrane and, therefore, does not bind its ligands[88]. Analysis of a colony of narcoleptic Labradors revealed that their *Hcrtr2* gene contained a distinct mutation that resulted in the skipping of exon 6, also leading to a truncated receptor protein. A third family of narcoleptic Dachshunds carries a point mutation in *Hcrtr2* that results in a receptor protein that reaches the membrane but cannot bind the hypocretins.

Sleep is characterized by complex patterns of neuronal activity in thalamocortical systems[89–91]. The fast, low-amplitude electroencephalographic (EEG) activity of the aroused state is replaced by synchronized high-amplitude waves that characterize slow-wave sleep. This pattern develops further into the high-frequency waves that define paradoxical, or REM, sleep. Switching between these states is controlled in part by the activities of neurons in the hypothalamic ventrolateral preoptic nucleus and a series of areas referred to as the ascending reticular activating system, which is distributed across the PPT–LDT, locus coeruleus, dorsal raphe nucleus and TMN, and regulates cortical activity[92].

Human narcolepsy is a sleep disorder that strikes around 1 in 2,000 adults, appears between the ages of 15 and 30 years, and involves four characteristic symptoms: excessive daytime sleepiness with irresistible sleep attacks during the day; cataplexy (brief episodes of muscle weakness or paralysis precipitated by strong emotions such as laughter or surprise); sleep paralysis, a symptom that is considered to be an abnormal episode of REM sleep atonia, in which the patient is suddenly unable to move for a few minutes, most often on falling asleep or waking up; and hypnagogic hallucinations, or dream-like images that occur at sleep onset. These last symptoms have been proposed as pathological equivalents of REM sleep.

Continuous recording of the behaviour of mice in which the hypocretin gene was inactivated by homologous recombination in embryonic stem cells revealed periods of ataxia, especially during the dark period[93]. EEG recordings showed that these episodes were not related to epilepsy, and that the mice suffered from cataplectic attacks — a hallmark of narcolepsy. In addition, the mutant mice spent almost twice as much time in REM sleep during the dark period as did their wild-type littermates, and their EEGs showed episodes of direct transition from wakefulness to REM sleep, another event that is unique to narcolepsy. Similar symptoms, with the exception of cataplexy, were found in rats in which the hypocretin neurons of the lateral hypothalamus were inactivated by SAPORIN TARGETING[94]. Mice with an inactivated *Hcrtr2* gene have a milder narcoleptic phenotype than the *Hcrt* knockouts; *Hcrtr1* knockouts show only a sleep fragmentation phenotype, whereas double *Hcrtr1* plus *Hcrtr2* mutants recapitulate the full *Hcrt* knockout phenotype[95], indicating that signalling through both receptors contributes to normal arousal.

It was unlikely, *a priori*, that human narcolepsy would have as straightforward an explanation as the canine disorder. Studies with monozygotic twins have shown that human narcolepsy is weakly penetrant: in only 25% of cases does the monozygotic twin of an affected individual also develop the disorder. Sporadic narcolepsy is highly correlated with particular class II human leukocyte antigen (HLA)-DR and HLA-DQ histocompatibility haplotypes in about 90% of patients, but most people with these haplotypes are not narcoleptic[96]. Because many autoimmune disorders are HLA-linked and because of the late and variable age of disease onset, it has long been considered that narcolepsy is likely to be an autoimmune disorder, but the targets of the immune attack were not known. Lessons from animal studies have once again pointed to an understanding of human physiology.

Nishino *et al.*[97] studied hypocretin concentrations in the CSF of normal controls and patients with narcolepsy. In control CSF, hypocretin concentrations were highly clustered, indicating that tight regulation of the substance is important. However, of nine patients with narcolepsy, only one had a hypocretin concentration within the normal range. One patient had a greatly elevated concentration, whereas seven patients had no detectable circulating hypocretin. In an expanded study, hypocretin was undetectable in 37 of 42 narcoleptics and in a few cases of Guillain–Barré syndrome[98]. CSF hypocretin was within the normal range in most neurological diseases, but was low, although detectable, in some cases of CNS infections, brain trauma and brain tumours.

Peyron, Thannickal and their collaborators found few or no hypocretin-producing neurons in the brains of narcolepsy patients[99,100]. Whether the hypocretin neurons are selectively depleted, as is most likely, or only no longer express hypocretin is not known, although one report showed some indications of gliosis[100]. The co-distributed MCH neurons were unaffected. Furthermore, a single patient with a non-HLA-linked narcolepsy carries a mutation within the hypocretin gene itself. The mutation results in a DOMINANT-NEGATIVE amino-acid substitution in the secretion signal sequence that sequesters both the mutant and heterozygous wild-type hypocretin non-productively to the smooth endoplasmic reticulum[99].

These findings leave no doubt as to the central role of the hypocretin system in this sleep disorder. However, it is unlikely that mutations in the hypocretin gene or those of its receptors, *per se*, account for more than a small subset of the human narcolepsies, given the genetic considerations mentioned above. The HLA association, loss of neurons with signs of gliosis and age of disease onset indicate that autoimmune destruction of the hypocretin neurons could account for most cases of narcolepsy[101], although a non-immune-mediated degenerative process has not been ruled out. Whether hypocretin itself or some other protein that is selectively expressed by the hypocretin neurons is the target antigen has yet to be

SAPORIN TARGETING
Saporin is a ribosome-inactivating toxin. When coupled to Hcrt2 and administered locally, the conjugate targets and kills neurons bearing hypocretin receptors.

DOMINANT NEGATIVE
Describes a mutant molecule that can form a heteromeric complex with the normal molecule, knocking out the activity of the entire complex.

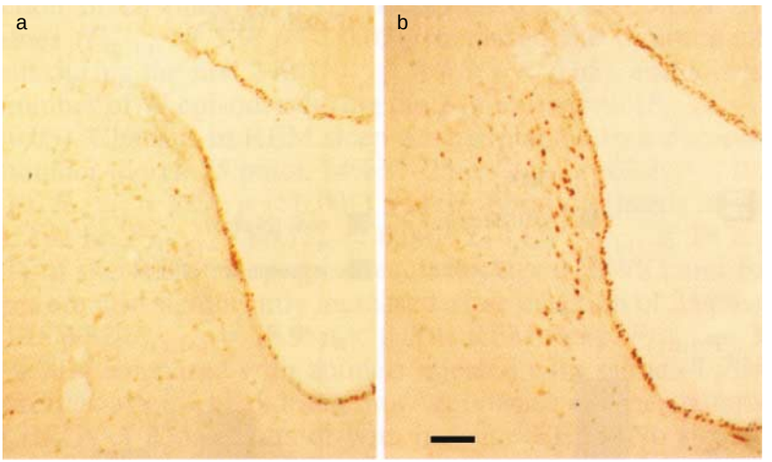

Figure 4 | **Local iontophoretic administration of Hcrt1 to the locus coeruleus stimulates expression of c-*Fos* in noradrenergic neurons. a** | c-*Fos* expression after saline administration. **b** | c-*Fos* expression after hypocretin 1 (Hcrt1) administration. Scale bar, 100 μm. Reproduced, with permission, from REF. 73 © 2000 Society for Neuroscience.

determined. The precipitating factor for the development of autoimmunity is also unknown. We know that there must be one (or more), because only a small percentage of individuals with the predisposing HLA haplotypes develop the disorder. The narcolepsies are probably a collection of disorders that are caused by defects in the production or secretion of the hypocretins or in their signalling, and these could have numerous genetic, traumatic, viral and/or autoimmune causes.

Hypocretins and arousal

Narcolepsy is associated with two sleep abnormalities: chronic sleepiness and rapid transition from the waking state to REM sleep. Data on the hypocretins give insights into both phenomena.

Among the neurons of the perifornical lateral hypothalamus, 53% increase their firing rates during both wakefulness and REM sleep, but decrease their activities during slow-wave sleep[102]. A further 38% of the neurons in this area are activated only during the awake phase. Hypocretin neurons express c-*Fos* during the waking period, and c-*Fos* expression is increased by sleep deprivation and methamphetamine[103]. The stimulant Modafinil, which is commonly used to treat the drowsiness that is associated with narcolepsy, elevates c-*Fos* expression in hypocretin neurons[93]. As mentioned earlier, hypocretin levels fluctuate with a circadian rhythm (remember the direct monosynaptic input from the SCN), being highest during waking, and peptide concentrations increase as a consequence of forced sleep deprivation[58] (although hypocretin mRNA levels are unaffected by sleep deprivation[104], possibly reflecting an effect on release rather than synthesis), indicating that the hypocretins and the activity of the hypocretin neurons serve as pressures that oppose sleep. The increase during forced wakefulness might indicate that the hypocretin system has executive wake-promoting activity, even when there is a need for sleep; alternatively, it could represent 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[105]. The noradrenergic neurons of the locus coeruleus, the serotonergic neurons of the dorsal raphe nucleus and the histaminergic neurons of the TMN are all so-called REM-off cells: they fire rapidly during wakefulness, slowly during slow-wave sleep and hardly at all during REM sleep[92]. Each of these structures projects to an array of targets in the forebrain, and their firing stimulates cortical arousal. The activity of these groups of monoaminergic neurons is one of the features that distinguishes wakefulness from REM sleep.

The noradrenergic locus coeruleus neurons fire constantly during wakefulness. In addition to their projections to the forebrain, these neurons send inhibitory projections to cholinergic REM-on generator neurons in the PPT–LDT, which fire during wakefulness and more rapidly during REM sleep (but do not fire during slow-wave sleep), and project to the PRF[106,107]. Hypocretin axons form synapses on these locus coeruleus neurons, which express Hcrtr1 postsynaptically[73,108]. Local administration of Hcrt1, but not Hcrt2, to the locus coeruleus suppresses REM sleep in a dose-dependent manner and increases wakefulness[73]. These effects are neutralized by an antibody that prevents the binding of Hcrt1 to Hcrtr1. Administration of Hcrt1 increases the firing rate of noradrenergic locus coeruleus neurons and induces the expression of c-*Fos* in these cells[73,109] (FIG. 4). Hcrt1 strongly excites serotonergic neurons of the dorsal raphe nucleus *in vitro*[110]. Both hypocretin peptides excite histaminergic neurons of the TMN in slice cultures, probably acting through Hcrtr2, and knockout mice that are deficient in histamine receptor 1 are impervious to hypocretin administration, indicating that at least some of the effects of the hypocretins are caused by the release of histamine and activation of postsynaptic H1 receptors[111–113].

So, one mode of hypocretin function in arousal is the excitation of brainstem REM-off neurons during wakefulness, and this is accomplished, in part, by signalling through both Hcrtr1 and Hcrtr2. The involvement of both receptors is consistent with the more severe phenotype of the double-receptor knockout mice compared with either single-receptor knockout, and with the observation that sporadic cases of canine narcolepsy that are associated with lower or undetectable CSF hypocretin are more severe than are cases with the Hcrtr2 deficiency alone[114]. The excitation of these groups of monoaminergic cells contributes directly to forebrain arousal.

The hypocretin neurons also project to cholinergic brainstem REM-on neurons, including those in the LDT and the PRF, the projections of which contribute to cholinergic tone in the forebrain. This tone is elevated during both wakefulness and REM sleep, leading to desynchronization of the EEG. Cholinergic tone is low in slow-wave sleep, during which acetylcholine activity is further inhibited by the sleep-promoting peptide of

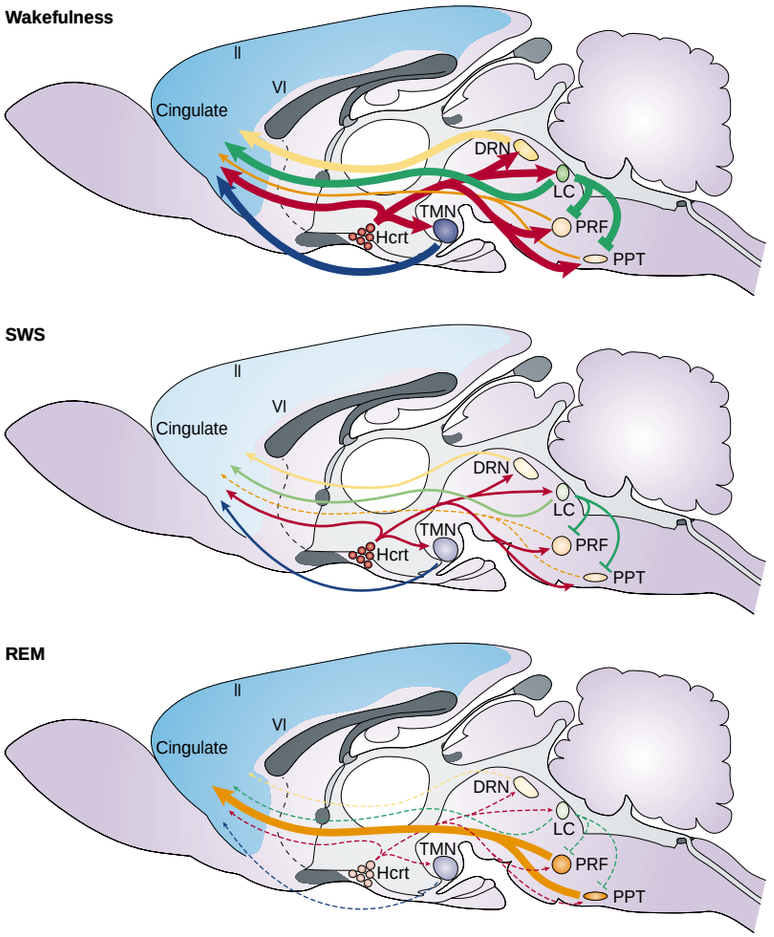

Figure 5 | **Schematic model of neurotransmitter circuits that are involved in the three states of vigilance.** During wakefulness, hypocretin (Hcrt) activity excites noradrenergic (green), histaminergic (deep blue) and serotonergic (yellow) neurons, which give rise to enhanced cortical activity and arousal. Slow-wave sleep (SWS) is characterized by synchronous intrinsic cortical activity, and most subcortical afferents show reduced activity. During rapid eye movement (REM) sleep, low hypocretin activity results in the disinhibition of REM-on cholinergic neurons (orange). DRN, dorsal raphe nucleus; LC, locus coeruleus; PPT, pedunculopontine tegmental nucleus; PRF, pontine reticular formation; TMN, tuberomammillary nucleus.

forebrain interneurons, cortistatin[115], contributing to the slow-wave synchrony of the EEG. Local injection of Hcrt1 into the LDT of freely moving cats increases wakefulness and decreases the number of REM episodes, but does not influence their length[116], indicating 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 sleep. This and the fact that deficiencies in the hypocretin system lead to increases in REM sleep make it more likely that an action on REM-on structures by hypocretin occurs only during waking periods, although an alternative model has been proposed[117]. The role of hypocretin in regulating the REM gate is a complex one in that, paradoxically, the REM-on structures receive both indirect hypocretin-initiated inhibitory signals from REM-off cells and direct projections from the hypocretin neurons themselves; their response to this push–pull pressure will vary according to the circumstances. One testable hypothesis that would rationalize this apparent paradox would be that the direct input is inhibitory, either acting postsynaptically in a G_i-coupled fashion or presynaptically to facilitate the indirect inhibitory input.

Furthermore, and importantly, the hypocretin neurons project to other brain areas that have been implicated in arousal. For example, the hypocretins, acting through Hcrtr2, excite cholinergic neurons of the basal forebrain, producing the cortical acetylcholine that is characteristic of the desynchronized EEG, which is associated with wakefulness and REM sleep[118]. Direct infusion of the hypocretins into the basal forebrain increases wakefulness[74,75]. So, the targets of the hypocretin neurons are highly distributed and their effects on wakefulness are twofold: maintenance of the waking state and suppression of REM entry during wakefulness.

One report has shown that systemic administration of a low dose (3 μg kg^{-1}) of Hcrt1 to narcoleptic Dobermans increases activity and wakefulness, and reduces REM sleep, and that chronic administration also consolidates sleep patterns[119]. This finding, which supports the idea that Hcrt1 crosses the blood–brain barrier[120], was at first puzzling, as these dogs are narcoleptic because they lack one of the two hypocretin receptors. However, the accumulating evidence that signalling through both receptors accounts for the arousal effects of the hypocretins makes the finding at least plausible, and indicates that small-molecule agonists of the hypocretin receptors might have therapeutic potential for human sleep disorders and might be preferable to the traditionally prescribed amphetamines. However, a second group did not detect an effect of Hcrt1 on the sleep architecture of narcoleptic dogs[121].

Arousal meets metabolism

The excitatory activities of the hypocretins on many of the components of the brain systems that maintain wakefulness and regulate entry into REM sleep, together with the findings that hypocretin insufficiencies lead to disorders of wakefulness and REM transitions, place the hypocretins as central elements of the sleep–wake apparatus (FIG. 5). These revelations provide us with a perspective on the inconsistent and sometimes contradictory findings concerning the central relevance of the hypocretins (orexins) to feeding-related activities.

Can we rationalize the relationship between the sleep–wake aspects of the hypocretins and feeding aspects of their activities? It is clear that the sleep–wake aspects of the hypocretins are dominant, and that a secondary involvement in metabolic concerns will therefore be subservient to the demands of the arousal cycle. Any measurements that relate to feeding must be interpreted in the context of time in the daily cycle, and in view of the alertness and physical activity of the subject.

There are a few further observations that might help us to understand the relationship between the arousal- and metabolism-related aspects of the hypocretins. Patients with narcolepsy have chronically low hypocretin concentrations, but they are not severely underweight; in fact, recent evidence indicates that narcolepsy

patients are significantly overweight, despite reduced calorie intake[122,123]. The *Hcrt* knockout mice are hypophagic, but they do not have lower weights than the unaffected controls[95]. Finally, mice that have been genetically engineered to lack hypocretin neurons have a phenotype similar to that of humans with narcolepsy, not only with respect to sleep/REM measures, but also in showing late-onset obesity despite eating less than their non-affected littermates[124]. Again, chronic hypocretin underactivity does not reduce body weight. Curiously, the obesity of the cell-ablation mice is more serious than that of *Hcrt* knockout mice, indicating that factors produced by the hypocretin neurons other than the hypocretins themselves might contribute to the metabolic abnormalities of these mice.

A model that accommodates the several and sometimes conflicting findings is one that considers the hypocretins as neither orexigenic nor anorexigenic *per se*. The hypocretin neurons are embedded in the appetite control centre and have reciprocal connections with important central components of metabolic regulation; they also sense peripheral reporters of metabolic status, including leptin and glucose, both directly and through their connections with other hypothalamic neurons. Likewise, the hypocretin neurons have reciprocal connections with and influence a long and probably incomplete list of endocrine and autonomic homeostatic circuits, many of which are related indirectly to aspects of metabolism and arousal. The hypocretin neurons receive information concerning the status of a significant portion of the systems that are homeostatically supervised by the CNS, including the circadian cycle.

So, how do the hypocretin neurons use this information? The crucial questions seem to be: first, what is the dominant theme that determines when these neurons are active; and second, which parts of the brain does their activity influence?

Electrophysiological measurement has shown that about 50% of perifornical neurons are wake-on, sleep-off, REM-on neurons, and that about 40% are wake-on, sleep-off, REM-off neurons[102]. These cells have yet to be classified in terms of whether they express hypocretin or MCH, but it is likely that the REM-on neurons express MCH, whereas the REM-off cells generally express hypocretin. This assessment is based, in part, on the REM-gating argument discussed above and, in part, on the expression of c-*Fos*. Under normal conditions, c-*Fos* expression cycles with a circadian rhythm in hypocretin neurons, increasing during the waking period and decreasing during the period that is dominated by sleep and REM. By contrast, c-*Fos* expression by MCH neurons is unaffected by the circadian cycle[102]. Perturbations that increase c-*Fos* expression in hypocretin cells include treatment with NPY, leptin, ghrelin, amphetamine or Modafinil, hypoglycaemia, and food and sleep deprivation. But these perturbations are abnormal and, sometimes, purely experimental, so they do not represent the main theme that the hypocretin neurons follow, which is to ebb and flow with the sleep–wake cycle.

Which areas of brain are influenced by hypocretin activity? The few thousand hypocretin cells have a vast array of projections. Many of these are reciprocal interactions with other homeostatic centres, particularly within the hypothalamus. However, the densest projections outside the hypothalamus are to structures that influence wakefulness, REM gating or both, such as the locus coeruleus, dorsal raphe nucleus, TMN, PPT, PRF and the basal forebrain.

When considered from these perspectives, a plausible model is that the communications that the hypocretin neurons conduct with various homeostatic systems, including those involved in regulating metabolic activities such as food intake, establish a SET-POINT threshold for the arousal outputs. In this sense, the hypocretins are not a signal for feeding *per se*, but provide a means by which metabolic and other homeostatic needs can affect arousal states. And the hypocretin neuronal projections allow the arousal set point to be widely disseminated, both within the CNS through the hypocretin peptides and throughout the periphery through the hormonal systems that are affected by the hypocretins.

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 a far-reaching influence on other set points, developing into ALLOSTASIS. Such effects are most substantial after gross perturbations, such as those involved in experimental or genetic manipulations and those experienced in disease. This view might allow us to understand phenomena that otherwise seem paradoxical, such as how removal of the hypocretins or the hypocretin neurons from a model rodent by genetic ablations or from human patients with narcolepsy results in an increase in body mass — when the hypocretins are absent, the metabolic thresholds are reset.

Discussion

It is a long way from gene expression, GPCRs and genetic mutations to systems biology. The discovery of the hypocretins (orexins), their receptors and their link with narcolepsy are attributable to the revolutions of genomics-based biology. But the placement of these peptides into organismic biology is the result of a more traditional analysis, albeit using tools provided by modern molecular biology, of several key questions. What criteria establish a peptide as a neurotransmitter? What are its electrophysiological properties? Where are the cells in which it is produced and where do they project? The studies of dozens of laboratories have put flesh on the skeleton of the original discoveries and have provided data about the many, sometimes contradictory, activities of this peptide neurotransmitter system. We have suggested a model of the hypocretin system that places it as the establisher of the arousal set point, residing side-by-side or, more appropriately, circuit-by-circuit, with other hypothalamus-based systems that converse with the hypocretin neurons and other systems to establish the set points for which they shoulder primary responsibility. Tests of this model will be conducted with both traditional and revolutionary tools.

SET POINT
In a homeostatically regulated phenomenon, the set point is the value that the system strives to maintain; for example, a body weight set point.

ALLOSTASIS
The maintenance of stability at any level outside the normal range is achieved by varying the internal milieu to match perceived and anticipated environmental demands. When demands on an individual are chronic, the set point for functioning is altered and might be maintained at that point indefinitely. Although this altered set point might seem appropriate to the conditions, it could be in the pathological range, in that any further perturbation can produce dysregulation.

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Acknowledgements
Supported in part by grants from the National Institutes of Health and Digital Gene Technologies. We thank A. Spier, C. Alvarez, V. Fabre, E. Thomas, P. Hedlund, C. Ploix and P. Danielson for helpful criticism of the manuscript, and our many collaborators who have shared this trail.

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