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Twenty-Three Years of Hypocretins: The “Rosetta Stone” of Sleep/Arousal Circuits

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Abstract

The discovery of the hypocretins/orexins (HCRTs) has revolutionized sleep science in the last two decades. A combination of anatomical tracing methods, optogenetics, and pharmacology is delineating a blueprint of functional inputs and outputs of the HCRT system. Here, we discuss several models of HCRT action that involve the integration between physiological variables, circadian output, and sleep homeostasis. Generation of activity maps during the sleep-wake cycle at the cellular level will allow investigators to decipher computational frameworks modeling operations of HCRT networks.

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Introduction

The hypocretins (HCRTs) were discovered by two groups at essentially the same time using disparate methods [1, 2], reported in 1998. Our group, led by J. Gregor Sutcliffe at the Scripps Research Institute, was interested in collecting hypothalamic-enriched mRNAs in order to identify markers expressed in subnuclei and to dissect their function [3]. Using cDNA subtractive hybridization, we discovered an mRNA that encoded a predicted pre-protein containing two putative peptides exclusively expressed in a group of neurons in the lateral/paraventricular hypothalamus (Fig. 1) [1]. Because of their hypothalamic location and sequence similarity with the gut hormone secretin, these peptides were named hypocretins, hypocretin-1 (HCRT1) and hypocretin-2 (HCRT2) [4].

Fig. 1. The first glimpse of HCRT. An in situ hybridization autoradiography showing hypocretin expression in the hypothalamic region of the rat brain. Originally published by Gautvik et al. [3]. Copyright (1996) National Academy of Sciences.

In parallel, Masashi Yanagisawa, Takeshi Sakurai, and colleagues at the University of Texas Southwestern Medical Center screened for novel ligands of orphan G-protein-coupled receptors (GPCRs) using a cell-based reporter system [5]. Because GPCRs are powerful targets for drugs, it was thought that identifying their natural ligands would be fundamentally important in the development of treatments for a variety of diseases [6]. They discovered two peptides and, because injecting them into the brain at high doses promoted feeding, named them the orexins (A and B). It was later learned that the orexins were the same as the HCRTs [2]. Despite attempts to reconcile the nomenclature, and the popular name of orexin in the pharmaceutical industry, we note that the HCRTs were discovered first and, because their main function appears to be in wake-stability, and not feeding, we refer to them as the hypocretins.

A broad body of literature has implicated the HCRT system as a key player in arousal stability. Most notably, mutations in the HCRT receptor-2 (HCRT2) gene were found to be causative of narcolepsy with cataplexy in two breeds of dogs, while HCRT knockout mice showed behavioral arrests in the active period that were reminiscent of cataplexy attacks [7–9]. Initial microdialysis and *c-fos* immunoreactivity studies demonstrated that HCRT neurons showed a circadian activity pattern, where firing rates peaked during the active (dark) phase in rodents [10]. Juxtacellular recordings in head-fixed or freely moving animals revealed that HCRT neural activity is phasic and firing rates peak approximately 10–20 s prior to a sleep-to-wake transition [11, 12]. The advent of optogenetics has allowed for millisecond-timescale control of distinct neural populations, with the first in vivo use of this technology being applied to the HCRT neurons to causally test their role in vigilance state stability and transitions [13]. This study demonstrated that light-evoked stimulation of HCRT neurons powerfully promotes wakefulness in mice, an effect dependent on HCRT release (and not other co-released neurotransmitters). The effect on arousal was frequency-dependent, as stimulations below 5 Hz failed to induce sleep-to-wake transitions. Subsequent studies using designer receptors exclusively activated by designer drugs

(DREADDs) confirmed these findings [14]. HCRT neurons expressing the excitatory Gq-coupled DREADD increased *c-fos* expression upon clozapine-N-oxide (CNO) administration, which was associated with increased wakefulness [14]. Conversely, when HCRT neurons were made to express the Gi-coupled inhibitory DREADD, CNO injection promoted sleep. Further optogenetic silencing experiments have demonstrated that long-term silencing of hypocretin neurons promotes slow-wave sleep in mice, highlighting their essential role in wake maintenance [15]. Many arousal systems are involved in arousal induction (e.g., norepinephrine, dopamine, acetylcholine) but these systems are highly redundant and none, except HCRT, show a narcolepsy-like phenotype when inhibited. These differences make HCRT a unique neuronal ensemble orchestrating arousal stability.

Differential Contributions of HCRT1 and HCRT2

HCRT acts through two GPCRs to exert neuroexcitatory effects. HCRT receptor-1 (HCRT1; also known as orexin receptor-1) has a higher affinity for HCRT1 over HCRT2 by an order of magnitude, whereas HCRT2 (also known as orexin receptor-2) displays equal affinity for both HCRT1 and HCRT2 [16]. Consistent with HCRT projections, HCRTs are expressed widely throughout the brain [17]. However, HCRT1 and HCRT2 have distinct, if overlapping distributions, with some wake-promoting target regions primarily expressing HCRT1 (e.g., locus coeruleus [LC], cholinergic neurons of the laterodorsal tegmental nucleus and pedunculopontine tegmental nucleus), some expressing HCRT2 (e.g., tuberomammillary nucleus [TMN]), and some expressing both (e.g., dorsal raphe nucleus [DRN]) [17, 18]. The function of HCRT1 and HCRT2 appears to differ significantly based on the phenotype of mice deficient for either receptor [19]. HCRT2 mutations lead to severe sleep disruptions similar to a full narcolepsy phenotype, whereas mice with single HCRT1 mutations do not have an overt sleep phenotype. Animal studies with antagonists indicate that HCRT1 blockade leads to impaired brain reward function [20]. These differences have led the pharmaceutical industry to develop compounds that selectively affect sleep-wake architecture, reward and motivation, and will be discussed in other chapters.

HCRT Neurons as Hubs of Arousal Integration

The essential nature of HCRT for optimal sleep/wake and its hierarchical relationship with downstream effector regions makes HCRT a logical starting point and the “Rosetta Stone” to decipher the neuronal underpinnings of sleep and wakefulness and to develop models that would contribute to their understanding. In the context of sleep processes conceptualized as a relaxation oscillator, HCRT is particularly important for stabilizing sleep-to-wake transitions. This function is revealed most clearly through the narcolepsy-like fragmented sleep-wake phenotype of HCRT-deficient animals [7]. Indeed, increasing

HCRT neuronal activity precedes awakening [11, 12] and acute stimulation evokes wake [21]. In the flip-flop model of sleep-state switching, vigilance state is controlled by balanced activity between mutually inhibiting wake- and sleep-promoting loci [22]. In this framework, HCRT neurons support wake consolidation by increasing activity across multiple wake-promoting targets (e.g., LC^{NE}, VTA^{DA}, DRN^{5-HT}, TMN^{HA}, BF^{ACh}). Inhibitory input from sleep-promoting preoptic area (POA) GABAergic neurons (POA^{GABA}) onto HCRT cells may suppress wakefulness to facilitate wake-to-sleep transitions. While attractive, the sleep-switch model does not capture many of the features of sleep-to-wake transitions, as will be discussed below. For more specific examinations of circuit interactions during sleep-to-wake transitions, computational frameworks have proven useful for the generation and refinement of testable hypotheses [23].

Quantitative Modeling

As pointed out by Adamantidis et al. [24], cataplexy associated with HCRT deficiency may be interpreted as a dissociated state, containing elements of both rapid-eye-movement (REM) sleep and wakefulness, triggered when executive neural circuits driving REM sleep become inappropriately activated during wakefulness. Alternatively, the underlying mechanisms of muscle atonia during cataplexy may be different from those of REM sleep, suggesting instead, that cataplexy is an entirely distinct state potentially unrelated to either REM sleep or wakefulness [24]. Either case is difficult to fit into the sleep-switch model that suggests mutually exclusive vigilance states. To solve these issues, we proposed a theoretical framework based on three main concepts [25]: (1) hierarchical organization of neuronal circuits controlling sleep and wakefulness; (2) redundancy; and (3) feedback control. This model allows coexistence of brain states that may usually be short-lived unless an important piece of the control mechanism (i.e., the HCRT integrator) is missing.

Hierarchical Organization of Inputs to HCRT Neurons

We conceptualize HCRT as a critical integration node for the broad regulation of arousal. Information regarding circadian and homeostatic sleep pressure, environmental stressors and rewards, metabolic or immune status are all relevant to the decision to engage in arousal-dependent behaviors (e.g., goal-seeking, fight or flight, sleep) [26]. HCRT neurons receive this information from multiple sources, each input having an associated weight. Input weights may physically manifest through relative expression of the multitude of neurotransmitter and neuropeptide receptors expressed on HCRT neurons [27] or through changes in synaptic plasticity [28], as described previously. Input weights can be considered zero sum, whereby increased weighting of one input class (e.g., sleep pressure) will decrease weighting of other(s) (e.g., “arousing” environmental stimuli) [29]. An integrated representation of these data is output as phasic changes in HCRT neuron firing.

Intuitively, neuronal circuits encoding danger are expected to dominate over other neuromodulators associated with circadian rhythms; otherwise predators active in the op-

Fig. 2. Schematic of anatomical inputs, weighted molecular modifiers, and neurotransmitter and behavioral outputs of hypocretin (HCRT) neuronal circuits. Parameters such as sleep homeostasis may come in different molecular forms (protein phosphorylation as revealed by Sik3 mutants [42], redox state [40], or cellular metabolism [43]), and have different weights (e.g. $[w_1, w_2, \dots, w_n]$) in the computation. All of these factors are integrated into the HCRT system to provide a combination of excitatory outputs that define arousal states linked to different behavioral outputs. ACh, acetylcholine; DA, dopamine; GABA, gamma-aminobutyric acid; Glu, glutamate; HA, histamine; HCRT, hypocretin; NE, norepinephrine; NREM, non-rapid eye movement.

posite side of the circadian cycle would always be successful at catching their prey [30]. Indeed, we have shown that acute optogenetic stimulation of corticotropin-releasing hormone neurons in the paraventricular hypothalamus, mimicking the presence of an acute danger, provide robust and sustained activation of HCRT neurons [31]. Moreover, ablation of HCRT neurons dramatically decreased arousal elicited by corticotropin-releasing factor (CRF) stimulation, suggesting that the connection between the paraventricular nucleus and lateral hypothalamus (LH) is essential for hyperarousal response to stress. This effect occurred during both the dark and light cycles. Similarly, Giardino et al. [32] demonstrated that HCRT neurons receive afferents from CRF and cholecystokinin-positive neurons in the bed nucleus of the stria terminalis encoding opposite valence. Since CRF neurons in the bed nucleus of the stria terminalis (BNST) send more efferents to the LH, the net effect of stimulating HCRT neurons receiving afferents encoding positive and negative stimuli is aversive, based on real-time place preference assays following optogenetic stimulation of HCRT cells.

Together, these data suggest that anatomical inputs to HCRT have different weights in a fashion similar to the algorithm known as “Perceptron” [33]. Implementation of this

linear classifier would result in outputs to monoaminergic neurons, whose combinatorial effect produce a gradient of arousal-related behaviors. A schematic of a model of this concept is shown in Figure 2.

Redundancy

Our published model considered redundancy based on the concept of codebooks, or combinations of bits that represent probabilistic activation of arousal versus sleep-associated circuits [25]. In our theoretical framework, the least significant bit (LSB) denotes the procured behavioral state. The bits that precede the LSB are arranged in a decreasing order of importance with respect to the respective neurotransmitter inducing a sleep-to-wake transition. For instance, Carter et al. [34] showed that LC activity is necessary for HCRT-induced sleep-to-wake transitions; therefore, HCRT is hierarchically superior to LC. We define the probability that the wake state (i.e., $LSB = 1$) occurs with absolute certainty by $P[LSB = 1] = 1$, and define the probability of the i th codeword inducing a state on a subject by $P[C_i]$. *How many bits are necessary to define a brain state?* The degree of redundancy in the system may be quantified through the codebook paradigm since sleep-wake states can be expressed as a single probability with a minimal representation of 2 bits. For instance, LC stimulation at high frequency (10 Hz) can be represented with a vector (1,1). However, low frequency stimulation in the LC does not reliably induce an awakening; therefore, LC release will need the contribution of additional bits of information, increasing the dimensions needed to characterize the brain-state transition. Thus, redundancy is defined by the number of bits in the codeword that reliably define a brain-state or brain-state transition. A coarse codebook that characterizes brain states can be obtained by interrogating optogenetically pairs of brain structures (Fig. 3). Future maps of neuronal activity with cellular resolution will dramatically increase granularity of these models.

Feedback

Feedback refers to the architecture that provides robust control of a mechanism. Burdakov and colleagues [35, 36] have proposed multiple scenarios of feedback control in which the optimization of the desired and actual vigilance states would be driven by summation, integration, and amplification modules. Integration of information by HCRT neurons likely occurs over a multiple-seconds timescale as the nature of the afferent signals ranges from seconds (e.g., imminent danger) to hours (e.g., circadian rhythmicity or sleep pressure) [13, 37]. This integration could occur at the biochemical level in target neurons via phosphorylation [38]. Furthermore, different patterns of HCRT neuronal activity may elicit differential release of HCRT, glutamate, dynorphin, and likely other substances. These co-released factors can modulate the impact of HCRT signaling [39] or encode distinct components of HCRT neuron information [36]. HCRT neuronal activity can thus facilitate optimal behavioral response by promoting wake, autonomic arousal, and reward-seeking through widespread actions on diverse target regions.

Fig. 3. Generating a probabilistic model of brain states using data from optogenetically elicited sleep-wake transitions. **a** Multiple fibers can be implanted into different brain regions of mice targeting specific cell types associated with sleep-to-wake transitions. Each fiber would probe a structure (**a**; HCRT) or one of its targets (**b** [LC]; **c** [DR]). Optogenetic stimulation at these sites generates data that represent the probability of a sleep-wake transition. **b** Schematic of multiple interactions between wake-promoting nuclei, emanating from HCRT circuits. **c** The probability that a specific connection (e.g., HCRT to LC; $p_{i,j}^{(LC)}$) is involved in a sleep-to-wake transition is calculated using the vector defining the latency of a state transition elicited by optogenetic stimulations ($p_{i,1}, p_{i,2}, p_{i,3}$) multiplied by an adjacency vector that represents connectivity between these structures $d_{i,j}^{(LC)}$. i and j represent spatial dimensions; the current figure is on a plane, and the j dimension is used; the general formula allows the use of more dimensions to reflect the brain volume. ACh, acetylcholine; BF, basal forebrain; CRF, corticotropin-releasing factor; DA, dopamine; DR, dorsal raphe; GABA, gamma-aminobutyric acid; GP, global pallidus; HCRT, hypocretin; HA, histamine; LC, locus coeruleus; LDT, laterodorsal tegmentum; MCH, melanin-concentrating hormone; NAcc, nucleus accumbens; NT, neurotensin; SN, substantia nigra; VTA, ventral tegmental area.

Homeostatic Integration

Recent work in *Drosophila* suggests that redox potential is an important variable integrated by neurons associated with sleep-wake control [40]. Redox potential accumulates with the history of neuronal firing and is reduced during quiescence, following the same dynamics as a relaxation oscillator. This intriguing parallelism between redox potential and the homeostatic sleep-wake control, so called process S [41], leads to the reasonable hypothesis that specialized groups of neurons would be particularly sensitive to accumulation of free radical species, and oxidation of potassium channels within these neurons would alter the excitability of the network and lead to sleep oscillations. While attractive, this hypothesis needs substantial experimental confirmation. Nevertheless, a homeostatic mechanism of this nature could certainly be integrated in the model described above.

Conclusion

The Rosetta Stone, discovered in 1799, was instrumental in establishing an algorithm that deciphered hieroglyphs and opened a golden era of discovery of Ancient Egypt. Two hundred years later, the discovery of the hypocretin system has, similarly, allowed us to reveal the neuronal underpinnings of the sleep-wake cycle, identifying neuronal substrates that contribute to sleep-wake dynamics and develop new therapeutic tools that improve sleep. Convergence of neuronal activity maps with cellular resolution, functional interrogation, and computational tools that define and predict brain states will likely lead to golden era in sleep science.

Key Take-Home Points

- Ample evidence supports the notion that release of HCRT peptides stabilizes arousal.
- Integration of multiple variables at multiple timescales in HCRT neurons appears essential for HCRT function.
- Computational models of neuronal inputs to HCRT neurons based on hierarchy, redundancy, and feedback control may predict sleep-wake transitions with high accuracy.

Conflict of Interest Statement

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