Published in final edited form as:

Curr Top Behav Neurosci. 2015 ; 25: 367–378. doi:10.1007/7854_2014_364.

Optogenetic Control of Hypocretin (Orexin) Neurons and Arousal Circuits

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Abstract

In 1998, our group discovered a cDNA that encoded the precursor of two putative neuropeptides that we called hypocretins for their hypothalamic expression and their similarity to the secretin family of neuropeptides. In the last 16 years, numerous studies have placed the hypocretin system as an integrator of homeostatic functions with a crucial, non-redundant function as arousal stabilizer. We recently applied optogenetic methods to interrogate the role of individual neuronal circuits in sleep-to-wake transitions. The neuronal connections between the hypocretin system and the locus coeruleus (LC) seem to be crucial in establishing the appropriate dynamic of spontaneous awakenings.

Keywords

Hypothalamus; Sleep/wake cycle; Neuropeptides; Narcolepsy Awakenings; In vivo recordings; Knockout animals; Hypnotics

1 Introduction

The term “arousal” usually refers to the degree of vigilance and alertness during wakefulness, manifesting as increased motor activation, responsiveness to sensory inputs, emotional reactivity, and enhanced cognitive processing.

The brain mechanisms underlying the organization of the sleep–wake cycle and general level of arousal remain unclear. The reticular activating system originally described by Moruzzi and Magoun originates in the brain stem and provides excitatory input to the cortex (Moruzzi and Magoun 1949). Activation of the reticular activating system, which includes fibers containing neuromodulators norepinephrine (NE), serotonin (5-HT), dopamine (DA), histamine (His), and acetylcholine (ACh), as well as fast transmitters (i.e., glutamate and GABA), has long been known to elicit an arousal-like state in decorticated cats (Steriade and McCarley 1990). Intracellular recordings in vivo by Steriade and colleagues showed that individual principal cortical neurons transition from irregular “up and down” states during NREM sleep, to regular firing patterns during wakefulness. McCormick and colleagues have shown that these transitions can be elicited in vitro by addition of a mixture of the neuromodulators mentioned above (Steriade et al. 1993). Also recently, Tafti and colleagues have shown that a similar cocktail of neurotransmitters and modulators can induce

“desynchronization” of neuronal activity in neuronal cultures (Hinard et al. 2012). However, whether the neuronal components of the reticular activating system causally drive state transitions or merely correlate with cortical activity remained unclear.

Clearly, the neuronal circuits responsible for the transition from sleep to wakefulness need to be modulated and coordinated by multiple variables, including light, food, circadian rhythms, presence of a threat or predator, inflammation, or disease. The neurotransmitter hypocretin (Hcrt), also known as orexin (OX), appears to be a master integrator of these variables and critically modulates sleep-to-wake transitions (de Lecea et al. 1998; Sakurai et al. 1998). The hypocretin peptides, 28 and 33 amino acids in length, are derived from the same precursor and bind to 2G-protein-coupled receptors with different affinities. HcrtR1 (also known as OX1R) is expressed in deep layers of the cerebral cortex, hypothalamus, ventral tegmental area, and several brain stem nuclei, including a very prominent expression in the locus coeruleus (LC). In contrast, HcrtR2 (also known as OX2R) is expressed throughout the neocortex, septum, posterior hypothalamus, and raphe nuclei. Based on the fact that HcrtR-deficient dogs and mice are narcoleptic, but not mice with an HcrtR1 knockout allele, it appears that the two Hcrt receptors subserve different functions.

2 Hypocretins as Master Regulators of Arousal State Transitions

Hcrt-producing cells are constituted by a group ~3,200 neurons in the mouse lateral hypothalamus (~6,700 and 50,000–80,000 in the rat and human brain, respectively) (Modirrousta et al. 2005). These neurons receive functional inputs from multiple systems distributed in the cortex, limbic system, subcortical areas including the hypothalamus itself, thalamus, and ascending projections from the brain stem cholinergic nuclei, the reticular formation, the midbrain raphe nuclei, and the periaqueductal gray. In turn, these neurons project throughout the central nervous system, including to arousal and reward centers of the brain, to neurons expressing hcrtr receptors. The afferent and efferent projections of hcrtr neurons suggest that they play a role in multiple hypothalamic functions including regulating the sleep/wake cycle and goal-oriented behaviors. Interestingly, we have found that a specific efferent projection from hcrtr neurons to noradrenergic LC neurons mediate sleep-to-wake transitions and possibly more general aspects of arousal.

Juxtacellular recordings of Hcrt neuron showed that they are generally quiescent during quiet wakefulness, SWS, and REM sleep but show high discharge rates during active wake and in anticipation of REM sleep-to-wake transitions (Lee et al. 2005; Mileykovskiy et al. 2005; Takahashi et al. 2008). In addition, they show high discharge rates during arousal elicited by environmental stimuli (e.g., an auditory stimulus and goal-oriented behavior) (Takahashi et al. 2008). These studies suggest that hcrtr neurons participate in sleep-to-wake transitions, but cannot conclude whether the activity of Hcrt cells accompanies wakefulness or drives the transition.

Loss of function studies has clearly demonstrated the necessity of hcrtr signaling for the integrity of behavioral states in mice, dogs, and humans (Sakurai 2007). This finding appeared to be of particular importance for our understanding of narcolepsy. Narcolepsy is a sleep disorder characterized by intrusions of sleep into wakefulness, as well as signs of

dysregulated REM sleep and cataplexy, a sudden loss of muscle tone during wakefulness elicited by positive emotions. Narcoleptic patients with cataplexy have a complete absence of *hcr* gene transcripts in the hypothalamus as well as non- or barely detectable levels of *hcr* in the cerebrospinal fluid. Strong evidence supports that this is due to cell loss, as other markers that colocalize with *Hcr* are also absent or significantly reduced in narcoleptic patients (e.g., dynorphin). Moreover, Mignot and colleagues also highlighted the importance of *Hcrtr2* in canine narcolepsy, as a breed of narcoleptic dogs displayed mutations in the *Hcrtr2* gene (Lin et al. 1999). Chemelli et al. (1999) demonstrated that a mutation of the *Hcr* gene in mice resulted in behavioral arrests during the dark (active) period that resembled cataplexy-like attacks. Later studies have shown that *Hcrtr2* mutants also display these arrests, whereas *Hcrtr1* knockout mice do not show overt sleep abnormalities. Importantly, pharmacological or genetic rescue of *hcr* gene expression alleviates narcolepsy symptoms in *Hcr*-deficient mice (Mieda et al. 2004; Liu et al. 2011; Willie et al. 2011).

Intracerebroventricular (i.c.v.) infusion of *hcr* peptides or *hcr* agonists causes an increase in the time spent awake and a decrease in SWS and REM sleep [review in (Sakurai 2007)]. Stereotactic injection of the *Hcrtr1* peptide in the LC, laterodorsal tegmentum, basal forebrain, or the lateral hypothalamus increased wakefulness and locomotor activity often associated with a marked reduction in SWS and REM sleep (Hagan et al. 1999). In vivo injection of *Hcrtr1* in the LC resulted in dramatic changes in sleep architecture (Bourgin et al. 2000). More recently, genetic disinhibition of *hcr* neurons using a selective GABA-B receptor gene deletion only in *hcr* neurons induced severe fragmentation of sleep/wake states during both the light and dark periods without showing an abnormality in total sleep/wake durations or signs of cataplexy (Matsuki et al. 2009). Collectively, these data suggest that the *hcr* peptides are important to define boundaries between sleep and wake states, as shown by the fragmentation of sleep and wake state in animal models of narcolepsy.

The biological function of *hcr* peptides is clearly necessary to maintain appropriate arousal and sleep. Studies in *Hcr* receptor knockout mice seem to indicate that most of the effects of *Hcr* on sleep are mediated through *Hcrtr2* signaling. This is based on the fact that *Hcrtr1* ko mice do not appear to have overt sleep abnormalities, whereas *Hcrtr2* ko mice and dogs with mutations in *Hcrtr2* show narcolepsy with cataplexy. The role of *Hcrtr1* remains unclear because double *Hcrtr1* and *Hcrtr2* mutant mice show more episodes of behavioral arrests resembling cataplexy than single *Hcrtr2* knockouts (Willie et al. 2003). It has been proposed that the control of wakefulness and NREM sleep to wake depends critically on *Hcrtr2R* (Mochizuki et al. 2011), while the dysregulation of REM sleep (unique to narcolepsy–cataplexy) results from the loss of signaling through both *Hcrtr1R* and *Hcrtr2R* (Mieda et al. 2011). However, their implications in the regulation of narcolepsy, in particular cataplexy and sleep attack, remain unclear.

Importantly, activity in other arousal systems is strongly perturbed during cataplexy. LC neurons cease discharge and serotonergic neurons significantly decrease their activity, while cells located in the amygdala (Gulyani et al. 2002) and the TMN showed an increased level of firing (John et al. 2004). This association suggests that both *Hcrtr1* (LC, raphe) and *Hcrtr2* (TMN, raphe) are involved in the maintenance of appropriate muscle tone. Recent studies also highlighted the role of altered cholinergic systems in triggering cataplexy in

narcoleptic mice (Kalogiannis et al. 2010, 2011). Therefore, an important, unresolved goal is to identify the functional wiring of hcr neurons, as well as the dynamics of synaptic release from hcr terminals to precisely delineate the downstream projections (Li et al. 2014) that control arousal, sleep states, muscle tone, and goal-oriented behaviors.

Recordings in awake behaving animals show that LC neurons fire tonically at 1–3 Hz during awake states, fire less during SWS sleep, and are virtually silent during REM sleep (Berridge 2008). The LC also fires phasically in short bursts of 8–10 Hz during the presentation of salient stimuli that may increase wake duration. Like hcr neurons, alterations in discharge rate precede changes in sleep-to-wake transitions (Aston-Jones and Bloom 1981a, b), suggesting that these cells are important for transitions to wakefulness or attention.

Interestingly, physical lesions of the LC do not elicit consistent changes in cortical EEG or behavioral indices of arousal (Cirelli et al. 1996). Genetic ablation of dopamine beta-hydroxylase, an enzyme required for NE synthesis, also does not disrupt sleep–wake states (Hunsley et al. 2006). This suggests the presence of redundant neural circuitry, external to the LC structure, supporting cortical activity and compensatory developmental mechanisms. However, central injections of pharmacological antagonists of α_1 - and β -noradrenergic receptors (Berridge and Espana 2000) have substantial sedative effects. Stimulation of neurons in the LC using local microinjections of a cholinergic agonist (bethanechol) produces rapid activation of the forebrain EEG in halothane-anesthetized rats (Berridge and Foote 1991). Recently, the LC-NE system was shown to be critical for maintaining the increased membrane potential of cortical neurons in awake compared to sleep states (Constantinople and Bruno 2011). Taken together, these studies imply that the LC-NE system desynchronizes cortical activity and increases cortical membrane potential to increase arousal.

3 Optogenetic Dissection of hcr and LC-NE Control of Arousal

We applied optogenetics to reversibly and selectively manipulate the activity of hcr and LC neurons in freely moving animals (Adamantidis et al. 2007; Carter et al. 2009a, 2010, 2012; Carter and de Lecea 2011; Rolls et al. 2011). Optogenetics uses actuator opsin molecules (e.g., channelrhodopsin-2 (ChR2) or halorhodopsin-NpHR) to selectively activate or silence genetically targeted cells, with flashes of light at specific wavelength (Fig. 1).

Optogenetics provided the ideal tools to interrogate causal relationships between the activity of genetically identified neurons and brain states. Before optogenetics, it was difficult to selectively stimulate or inhibit specific hcr and LC-NE populations with a temporal resolution relevant to sleep or wakefulness episodes and to achieve spatial selectivity to probe those cells without affecting surrounding cells or fibers of passage. This was particularly challenging in species where sleep and wakefulness are not consolidated such as laboratory rodents. Mice and rats have sleep cycles that last between 6 and 12 min and show frequent awakenings during the light (rest) cycle. This temporal scheme makes it impossible to use pharmacological interventions, as these span timescales that extend many sleep/wake cycles.

To deliver the optogenetic probes to hcrt or LC neurons, we used lentiviral and cre-dependent adeno-associated viral (AAV) gene delivery tools, under the control of cell-type specific (Adamantidis et al. 2007). To deliver light to the hcrt or LC field, we designed optical–neural interfaces in which optical fibers were chronically implanted on the mouse skull, as described elsewhere. Using this strategy, we were able to control hcrt neural activity both in vitro and in vivo with millisecond-precise optical stimulation (Adamantidis et al. 2007). The high temporal and spatial precision of stimulation allowed us to mimic the physiological range of hypocretin neuron discharge rates (1–30 Hz) (Hassani et al. 2009). Indeed, we used light-pulse trains for our optogenetic stimulation that were based on parameters on the actual frequency analysis of hcrt neurons in vivo (8–12 Hz). We found that direct unilateral optical stimulation of hcrt neurons increased the probability of transitions to wakefulness from either SWS or REM sleep. Interestingly, high-frequency optical stimulation (5–30 Hz light-pulse trains) reduced the latency to wakefulness, whereas 1 Hz trains did not, suggesting a frequency-dependent synaptic release of neurotransmitter (glutamate) and neuromodulators, including hcrt or dynorphin from the terminals. We further showed that the effects of stimulating hcrt neurons could be blocked by injection of an HcrtR antagonist or by genetic deletion of the hcrt gene, suggesting that hcrt peptides mediate, at least in part, optogenetically induced sleep-to-wake transitions and that fast transmission through glutamatergic terminals of Hcrt cells appears to be dispensable for Hcrt function. These experiments show that hcrt release from hcrt-expressing neurons is necessary for the wake-promoting properties of these neurons. It should be noted, however, that optogenetic stimulations may not reflect the actual patterns of activity in vivo, even if the frequencies are selected based on in vivo recordings. This is because it is unlikely that the volume of Hcrt cells stimulated by light will ever be activated synchronously. This was further supported by data showing that optical silencing of hcrt neurons promotes SWS (Tsunematsu et al. 2011, 2013).

These results were recently confirmed by Sasaki and collaborators (Sasaki et al. 2011), who used a chemogenetic approach called designer receptors exclusively activated by designer drugs (DREADDs) to activate and suppress hcrt neural activity. DREADD technology is based on the introduction of a mutated muscarinic receptor that is only activated in the presence of a synthetic ligand (clozapine N-oxide (CNO)). DREADDs allow bimodal modulation of neural activity with temporal resolution of several hours (Armbruster et al. 2007) and therefore is used as a complementary method of optogenetics in longer timescales. They found that activation of hcrt neural activity increased wakefulness, while suppression of hcrt activity promoted SWS.

We then showed that in sleep-deprived animals, optogenetic stimulation of Hcrt neurons failed to increase the probability of awakenings, suggesting that hcrt control of sleep–wake transitions is under the dependence of sleep homeostasis (Carter et al. 2009b). However, the effect of optogenetic stimulations of hcrt persisted in mice that are unable to synthesize histamine, suggesting that the histaminergic system is not required for the effect of hcrt on sleep-to-wake transitions. This effect does not rule out a possible role of the Hcrt–TMN connection in other features of sleep dynamics, as evidenced by the rescue of sleepiness in narcoleptic mice when replacing Hcrt2. Finally, we showed that downstream arousal centers such as the LC neurons increased their activity (as measured by c-Fos expression) in

response to hcrtr optogenetic stimulation. Because previous work showed an excitatory effect of hcrtr on LC-NE neurons (Bourgin et al. 2000), we investigated the hcrtr–LC connection and focused our experimental investigations on the noradrenergic LC as a new target for optogenetic manipulation.

In a follow-up study, we genetically targeted LC-NE neurons by stereotaxic injection of a Cre recombinase-dependent adeno-associated virus (rAAV) into knock-in mice selectively expressing Cre in tyrosine hydroxylase (TH) neuron (Carter et al. 2010). We found that both NpHR and ChR2 were functional and could inhibit and activate, respectively, LC-NE neurons both in vitro and in vivo. We found that optogenetic low-frequency (1–10 Hz) stimulation of LC-NE neurons caused immediate (<5 s) sleep-to-wake transitions from both SWS sleep and REM sleep. Detailed time/frequency analysis revealed that 20 pulses delivered over 5 s were sufficient to induce an awakening (Carter et al. 2010). Stimulation of LC neurons during wakefulness increased locomotor activity and the total time spent awake, confirming the strong arousal effect. LC-NE stimulation can be so robust as to wake an animal from isoflurane Hypnotics (Vazey and Aston-Jones 2014). In contrast, NpHR-mediated silencing of LC-NE neurons decreased the duration of wake episodes but did not block sleep-to-wake transitions when animals were asleep. Taken together, this study showed that activation of LC-NE neurons is necessary for maintaining normal durations of wakefulness (NpHR experiment) and sufficient to induce immediate sleep-to-wake transitions, sustained wakefulness, and increased locomotor arousal. Thus, we proposed that the LC-NE neurons act as a fast tuning system to promote sleep-to-wake transitions and general arousal. Interestingly, we found that sustained optical activation of LC-NE neurons induces locomotor arrest (Carter et al. 2010), an effect likely caused by the depletion of norepinephrine from LC terminals or overexcitation of downstream motor nuclei. Such behavioral arrests share common symptoms with cataplexy, catatonia, or behavioral freezing both in animal models of narcolepsy and in human patients (Scammell et al. 2009). Thus, we hypothesized that behavioral arrests in narcoleptic mice could be caused by the lack of inhibitory control of LC neurons, possibly exerted under normal conditions by GABAergic neurons sensitive to Hcrtr (and likely expressing HcrtrR2) in the peri-LC region.

Most recently, we tested the hypothesis that LC activity gates hcrtr neuron's effects on sleep-to-wake transitions (Carter et al. 2012). We took a dual optogenetic approach to stimulate hcrtr neurons while concomitantly inhibiting or stimulating noradrenergic LC neurons during SWS sleep. Silencing LC neurons during hcrtr stimulation blocked hcrtr-mediated sleep-to-wake transitions. To test whether an increase in LC excitability would facilitate Hcrtr-induced awakenings, we used a variant channel rhodopsin known as step functional opsin (SFO). This channel can be activated by short pulses of blue light (1–10 ms) and can stay open up to 1 min, letting the flow of cations into the cell without necessarily reaching the depolarization threshold. Thus, when an SFO was expressed in LC neurons and LC neurons were primed with a 10-ms pulse, Hcrtr stimulation was much more effective at eliciting sleep-to-wake transitions (Carter et al. 2012). Taken together, our results show that the LC serves as a necessary and sufficient downstream effector for hcrtr-mediated SWS-to-wake transitions during the inactive period.

4 Hcrt and LC-NE System Dynamics

Across our experimental studies, we observed that optogenetic manipulation of hcrt and LC-NE neurons affects sleep-to-wake transitions with dramatically different temporal dynamics (Adamantidis et al. 2010; Carter et al. 2009b, 2010, 2012). Acute optical activation of hcrt neurons causes sleep-to-wake transitions over a time period of 10–30 s, while stimulation of LC neurons causes sleep-to-wake transitions in less than 5 s. The delayed effect of Hcrt on transitions may be explained by in vitro recordings of laterodorsal tegmental neurons in response to Hcrt, which show maximal depolarizations 20–30 s after bath application of Hcrt. Another explanation was provided by a mathematical conductance-based model of Hcrt and LC neurons (Carter et al. 2012; de Lecea and Huerta 2014). According to this model, Hcrt would slowly depolarize LC neurons' subthreshold during a time window that is consistent with variable integration, that is, a "safety" window that would prevent spontaneous awakenings to irrelevant signals. If all conditions are met and the depolarization continues, then LC neurons produce spikes that may reach the awakening threshold.

One explanation is that hcrt neurons may act as an upstream integrator of arousal during hypothalamic-related functions, while the LC-NE system acts as a primary effector for arousal, stress, and attention (de Lecea and Huerta 2014). However, the neuronal effector systems are likely redundant and activated by distinct sets of inputs. Therefore, we cannot rule out that blocking other arousal systems, such as the central histaminergic and cholinergic systems, would also severely affect hcrt-induced behavioral state transitions in other experimental conditions.

Besides the short-term effects of photostimulation, it is of interest that experiments applying sustained ~1–4-h photostimulation of hcrt neurons showed increased sleep-to-wake transitions without changing the total duration of wakefulness (Carter et al. 2009b; Rolls et al. 2011), whereas long-term photostimulation of LC-NE neurons significantly increased wakefulness duration (Carter et al. 2010). It thus seems that Hcrt neurons are very sensitive to sleep pressure and do not elicit awakenings efficiently in conditions of sleep deprivation. Interestingly, Muhlethaler and colleagues have observed that the sensitivity of Hcrt neurons to noradrenergic innervation depends on sleep pressure (Grivel et al. 2005). Together, these results suggest that the hcrt system may regulate sleep–wake boundaries, while LC-NE neurons may rather control wake duration by increasing cortical membrane potential and desynchronizing the cortical EEG. Also, interestingly, we showed that control of the boundaries between sleep states is crucial for the consolidation of long-term memory (Rolls et al. 2011).

Obviously, the Hcrt–LC connection is not the only circuit involved in sleep-to-wake transitions. The role of histamine as a neuromodulator and powerful actor in sleep/wake cycles has long been documented. Anatomical studies revealed strong connectivity between lateral hypothalamic neurons and histaminergic cells in the tuberomammillary posterior hypothalamus. Trivedi et al. showed that His cells express Hcrt2 (Trivedi et al. 1998). Moreover, Haas and colleagues demonstrated in slice experiments that His cells can be depolarized by Hcrt infusion (Eriksson et al. 2001). More recently, Burdakov and colleagues (Schone et al. 2014) have shown that optogenetic activation of Hcrt2 is crucial for a reliable

output of histaminergic neurons. These data, together with those of Mochizuki et al.'s discussed above, suggest an essential role of Hcrt-His circuitry in maintaining appropriate wakefulness. Thus, a picture is emerging in which the interaction of Hcrt with a network of neuromodulators defines the dynamic of sleep-to-wake transitions (Fig. 2). According to this scheme, Hcrt plays a fundamental role as a non-redundant neuromodulator of neuromodulators, providing the appropriate timescale of integration across multiple variables and transmitting this information into different timescales encoded by other transmitters such as norepinephrine, acetylcholine, or histamine.

5 Concluding Remarks

As optogenetics unravels the role of individual transmitters in sleep/wake dynamics, it may be possible to build a larger-scale framework predictive of sleep architecture based on the activity of ensembles of neurons. Such a framework could have important consequences on the treatment of sleep disorders and other neuropsychiatric conditions in which arousal state transitions are impaired.

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Fig. 1. Schematic of a mouse implanted with optical fibers connected with *blue* and *yellow* lasers for combinatorial optogenetic interrogations (Carter et al. 2012)

Fig. 2.
Schematic of the interactions between Hcrt and arousal circuits. Hcrt neurons integrate information from metabolic, circadian, and limbic structures and convey the integrated information to a network of effectors, each of which has a different role in establishing the dynamic of a behavioral state transition