Hypothalamic regulation of the sleep/wake cycle

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Abstract

Sleep is one of the most important physiological functions in mammals. It is regulated by not only homeostatic regulation but also circadian clock. Several neuropeptide-producing neurons located in the hypothalamus are implicated in the regulation of sleep. Among them, orexins (also known as hypocretins) producing neurons (orexin neurons) are a crucial component for wakefulness, because lack of orexin function results in narcolepsy, which is a sleep disorder characterized by cataplexy. Recent findings have identified substances that excite or inhibit neuronal activity of orexin neurons. Furthermore neuronal projections of the neurons which release these substances have been revealed. In addition to orexin, melanin concentrating hormone (MCH)-producing neurons in the lateral hypothalamic area (LHA) are also implicated in the regulation of sleep. MCH neurons are active during sleep but become silent during wakefulness. Recently developed innovative methods including optogenetics and pharmacogenetics have provided substantial insights into the regulation of sleep and wakefulness. *In vivo* optical recordings and retrograde and anterograde tracing methods will allow us to understand additional details regarding important interactions between these two types of neurons in the LHA and other neurons in the brain. Finally we discuss the circadian clock and sleep/wake cycle. Understanding of the neuronal networks and its circadian modulation of sleep/wake cycles remains to be investigated.

Contents

1. Introduction

At the beginning of the 20th century, the pandemic influenza virus induced encephalitis and many people suffered from sleep disorders. Some people exhibited serious insomnia, while others slept like the dead. A Viennese physician named von Economo investigated post-mortem brains of these patients and found that sleep and wake are regulated by specific brain areas within the anterior part of the hypothalamus (Von Economo, 1930). When the anterior parts of the hypothalamus were damaged, people suffered from an insomnia-like phenomenon. Comparatively, post-mortem brain analyses of patients who exhibited abnormally increased sleep periods revealed damage in the posterior parts of the hypothalamus. Although the mechanisms relating encephalitis to these sleep disorders have not been completely identified(Dale et al., 2004) , Economo's findings represented a monumental achievement of sleep research.

Seventy years later, two independent research groups identified the neuropeptide Orexin/Hypocretin (de Lecea et al., 1998; Sakurai et al., 1998). Soon after their findings, it was discovered that orexin neurons were related to the phenomenon of "Narcolepsy", which is a sleep disorder characterized by

excessive daytime sleepiness and sudden muscle weakness triggered by emotion. Canine narcolepsy was identified as a mutation in the orexin-2 (hypocretin-2) receptor (Lin et al., 1999). Furthermore, mice lacking the orexin peptide (Chemelli et al., 1999), the neurons containing orexin (hypocretin) (Hara et al., 2001), or orexin 2 receptor (Willie et al., 2003) exhibited symptoms similar to narcolepsy. Although prepro-orexin knockout (KO) mice exhibited narcolepsy symptoms, the circadian period of behavioral rhythms was unaffected (Mochizuki et al., 2004), suggesting that orexin neurons are not the center of regulation of circadian behavioral rhythms. In the case of humans, orexin concentration in cerebrospinal fluid was found to be low in narcolepsy patients (Nishino et al., 2000; Peyron et al., 2000; Thannickal et al., 2000). These results indicated that orexin peptide itself or orexin neurons regulate sleep and arousal states, and that orexin neurons are involved in maintaining arousal state in mammals.

In this review, we introduce roles for hypothalamic neurons in the regulation of sleep and wakefulness, with a particular focus on orexin- and melanin-concentrating hormone (MCH) producing-neurons located in the lateral hypothalamic area (LHA). Recent technical advances such as optogenetics,

pharmacogenetics, adeno-associated virus (AAV) gene delivery, and gene editing methods have uncovered several aspects of sleep/wakefulness regulation over the past decade. We also discuss the role of these technical methods in the understanding of sleep and wake regulation by LHA neurons.

2. Orexin neurons and receptors

There are two mature and bioactive orexin peptides, termed orexin A and B. The same precursor protein, named prepro-orexin protein, generates these neuropeptides. Orexin A is a 33 amino acid peptide of 3.5 kD, and orexin B is a 28 amino acid peptide of 2.9 kD that is 46% identical to orexin A peptide. The primary structure of orexin A is completely conserved among several mammalian species from mouse to human (Sakurai et al., 1998), suggesting its important physiological role. Orexin neurons are located in the LHA which contains about 3,000 neurons in the rat brain and 70,000 neurons in the human brain (Peyron et al., 1998; Thannickal et al., 2000). Their neurons widely project throughout the brain, including regions such as the paraventricular thalamic nucleus (PVH) and arcuate nucleus (Arc), and especially to the serotonergic dorsal raphe nucleus (DR), noradrenergic locus coeruleus (LC) and histaminergic tuberomammillary

nucleus (TMN) (Allen and Cechetto, 1992; Nambu et al., 1999; Peyron et al., 1998). There are two types of orexin receptors, orexin receptor-1 and -2 (OX1R and OX2R) (Marcus et al., 2001; Trivedi et al., 1998), which are G-protein coupled receptors. OX1R is coupled with Gq and OX2R is coupled with both Gq and Gi/o (Zhu et al., 2003). Brain expression of these receptors has been well characterized. For instance, OX2R is dominantly expressed in the Arc, ventrolateral hypothalamus (VMH), LHA, and TMN. OX1R is dominantly expressed in the LC. In addition, both OX1R and OX2R are expressed in the raphe nucleus and ventral tegmental area (VTA). From previous reports, orexinergic neurons have been considered important components for the regulation of sleep-wakefulness via activation of these neurons.

3. Physiological roles of orexin neurons

3-1 Input pathways to orexin neurons

Several types of neurons project to orexin neurons and many neurotransmitters that affect the activity of orexin neurons have been identified. For example, NPY and agouti-related peptide (AgRP), which are expressed in the Arc of the hypothalamus, are involved in food intake behavior (Broberger et

al., 1998; Clark et al., 1984; Elias et al., 1998; Stanley and Leibowitz, 1985). Projections to orexin neurons from NPY/AgRP neurons in the Arc were previously identified (Broberger et al., 1998; Elias et al., 1998). Serotonergic neurons in the median/paramedian raphe nucleus also project to orexin neurons (Sakurai et al., 2005), and GABAergic neurons in the ventrolateral preoptic nucleus (VLPO), sends axons to orexin neurons in the LHA (Sakurai et al., 2005; Sherin et al., 1996; Yoshida et al., 2006). The VLPO plays an important role in the initiation and maintenance of non-rapid eye movement (NREM) sleep, and these neurons are activated by adenosine and prostaglandin D2 (Chamberlin et al., 2003; Kumar et al., 2013; Scammell et al., 1998; Szymusiak et al., 1998), which are thought to be sleep-inducing substances. Indeed, cell-specific lesions within the VLPO significantly reduce both NREM and REM sleep (Lu et al., 2000), which might explain the insomnia in Economo's patients. Neuronal projections from the bed nucleus of the stria terminalis (BST), supraventricular zone, and dorsomedial hypothalamus (DMH) were also identified to target orexin neurons (Sakurai et al., 2005; Yoshida et al., 2006), and these upstream neurons receive neuronal projections from the suprachiasmatic nucleus (SCN), the central circadian pacemaker (Leak and Moore, 2001). Neuronal activity of orexin

neurons is regulated indirectly by the SCN, but a direct neural pathway between the SCN and orexin neurons has not been identified so far. Recently, Cre-dependent rabies virus glycoprotein (RVG) expression, which is a monosynaptic retrograde tracing method, revealed anatomical afferent pathways to orexin and MCH neurons (Gonzalez et al., 2016). It is important to know how specific neurons form neuronal circuits and projections in the brain to regulate sleep/wakefulness. In this regard, the “tracing the relationship between input and output” method (TRIO) could be a powerful tool for understanding neuronal circuits related to sleep/wake regulation (Schwarz et al., 2015).

3-2 Pharmacological evidence of orexin inputs

Electrophysiological methods such as the slice patch clamp technique represent powerful tools to understand the roles of input pathways, and they have been used for the identification of substances that affect the activity of orexin neurons. To do this, transgenic mice expressing enhanced green fluorescent protein (EGFP) under the control of the orexin promoter were generated to identify orexin neurons, termed *orexin-EGFP* mice (Yamanaka et al., 2003a; Yamanaka et al., 2003b). This is a useful tool since only a small

number of orexin neurons are distributed in the LHA, and there are no morphological features to distinguish orexin neurons from other neurons. Several research groups have investigated the effects of various neurotransmitters on orexin neurons. Orexin neurons are depolarized by AMPA and NMDA (glutamate receptor agonists), while muscimol ($GABA_A$ receptor agonist) and baclofen ($GABA_B$ receptor agonist) hyperpolarize orexin neurons (Xie et al., 2006; Yamanaka et al., 2003b). Serotonin (5-hydroxytryptamine; 5-HT) and noradrenaline hyperpolarize all orexin neurons through the $5HT_{1A}$ receptor and the alpha2A (a2A) receptor, respectively. The 5-HT_{1A} receptor and a2A receptor are both coupled to inhibitory Gi proteins and subsequently activate G-protein-coupled inwardly rectifying potassium channels (Muraki et al., 2004; Yamanaka et al., 2006). Recently, Chowdhury et al. showed that 5-HT nerve terminal activation in the LHA using optogenetics both directly and indirectly inhibits orexin neurons. Direct inhibition is mediated through 5-HT and via the $5HT_{1A}$ receptor. Indirect inhibition is mediated via facilitation of GABAergic inhibitory inputs onto orexin neurons (Chowdhury and Yamanaka, 2016). Dopamine also hyperpolarizes orexin neurons, which might be partially through indirect a2A receptor action (Muraki et al., 2004). A muscarinic agonist,

carbachol, induced both activation and inhibition of neuronal activity in orexin neurons (Sakurai et al., 2005; Yamanaka et al., 2003b). Glycine inhibits the electrical activity of orexin neurons directly and indirectly (Hondo et al., 2011; Karnani et al., 2011). Interestingly, orexin itself increases neuronal activity via OX2Rs, indicating a positive feedback regulation by orexin (Yamanaka et al., 2010).

Since intracellular calcium concentration is increased when neurons are active, calcium-imaging methods can also be used for recording neuronal activity. Tsujino et al. (Tsujino et al., 2005) established transgenic mice in which orexin neurons specifically express a genetically encoded calcium indicator (GECI) protein, yellow cameleon 2.1 (YC2.1) (Miyawaki et al., 1999). This method is useful because the activity of several orexin neurons can be monitored simultaneously using fluorescence imaging. It is well known that neuropeptides have important roles in the regulation of not only sleep/wakefulness but also feeding and drinking behaviors (Kojima et al., 1999; Kunii et al., 1999; Shimada et al., 1998; Xu et al., 2004), and these neuropeptides modulate orexin neuron activity. Among them, cholecystokinin (CCK-8S) and arginine-vasopressin (AVP) activate orexin neurons via the CCK_A receptor and V1A receptor, respectively

(Tsujino et al., 2005; Tsunematsu et al., 2008). Neurotensin (NT) and oxytocin also activate orexin neurons. Furthermore, orexin neurons are affected by corticotrophin-releasing factor (CRF) (Winsky-Sommerer et al., 2004), ATP (Wollmann et al., 2005), NPY (Fu et al., 2004), and CO_2 levels (Williams et al., 2007). Not only neuronal projections but also humoral factors regulate activity in orexin neurons. For example, glucose, leptin, and ghrelin are involved in the regulation of feeding behavior. Interestingly these regulate neuronal activity of orexin neurons (Yamanaka et al., 2003a). Specifically, decreases in extracellular glucose concentration (mimicking hypoglycemia) increase action potentials in orexin neurons, whereas increases in glucose concentration (mimicking hyperglycemia) decrease neuronal activity (Burdakov et al., 2005; Yamanaka et al., 2003a). These results suggest that orexin neurons are glucose-sensing neurons. Application of leptin, a negative regulator of feeding behavior released from adipocytes, strongly and persistently inhibits orexin neuron activity (Yamanaka et al., 2003a). In comparison, application of ghrelin, a positive regulator of feeding behavior released from the stomach, induces depolarization and increased activity in orexin neurons (Yamanaka et al., 2003a). These findings indicate that metabolic signals related to feeding also influence the

activity of orexin neurons, and that this mechanism acts as a metabolic sensor. This fact might reflect the functional relationship between feeding behavior and sleep/wakefulness. We summarized all substances that affect the activity of orexin neurons in Table 1 and input and output pathway of orexin neurons in Figure 1.

3-3 Ablation of orexin neurons

Hara et al. generated transgenic mice in which orexin neurons are ablated by specific expression of a truncated CAG repeat sequence of the Machado-Joseph disease responsive gene (*ataxin-3*). In these mice, orexin neurons are gradually ablated after birth. These transgenic (*orexin/ataxin-3*) mice show a phenotype similar to human narcolepsy (Hara et al., 2001). In addition to this, both orexin 1 and 2 receptor knockout mice exhibit all symptoms observed in narcolepsy (Kalogiannis et al., 2011). Although these mice are good animal models to elucidate the roles of orexin neurons in sleep/wake regulation, they are not proper models for human sleep disorder, because orexin neurons in *orexin/ataxin-3* mice are ablated at the beginning of their life. Usually narcolepsy patients exhibit a post-pubertal onset of symptoms. To establish a model more

similar to human narcolepsy, Tabuchi et al. generated a new line of mice (Tabuchi et al., 2014). They used the tet-off system to control the expression of diphtheria toxin A fragment (DTA), which is an inhibitor of elongation factor 2, to induce cell death. Tetracycline operator (TetO) -DTA mice, which express DTA in the presence of tetracycline transactivator (tTA), were bred with *orexin-tTA* mice, which express tTA in orexin neurons under the control of the prepro-orexin promoter to generate bigenic mice (*orexin-tTA; TetO DTA* mice). In these bigenic mice, DTA was exclusively expressed in orexin neurons. In the tet-off system, without doxycycline (DOX), DTA is expressed in orexin neurons and they are ablated. However in the presence of DOX, orexin neurons are intact since tTA cannot induce DTA expression. Thus, these mice enable ablation of orexin neurons at a desired timing. DOX is supplied through the diet, typically a DOX-containing chow containing 100 mg/kg DOX. *Orexin-tTA; TetO-DTA* mice are fed DOX-containing chow until 10 weeks after birth, then chow is replaced with normal chow, which does not contain DOX. During the ablation of orexin neurons, the sleep/wake patterns of *orexin-tTA; TetO-DTA* mice was analyzed. *Orexin-tTA; TetO-DTA* mice with the gradual ablation of orexin neurons showed a fragmentation of sleep/wakefulness. Cataplexy bout frequency was

significantly increased as compared to *orexin/ataxin-3* mice, indicating that some compensatory mechanisms during development attenuated narcolepsy symptoms in *orexin/ataxin-3* mice. Importantly, the 95% decrease in the number of orexin neurons induced by 2 weeks of DOX(-) might be sufficient to trigger cataplexy. Black et al. performed a therapeutic experiment using *orexin-tTA; TetO-DTA* mice and found that γ-Hydroxybutyrate (an approved therapeutic for narcolepsy) and R-baclofen ($GABA_B$ agonist) decreased the amount of cataplexy (Black et al., 2014). These mice represent a useful tool to understand the physiological mechanisms underlying narcolepsy.

3-4 Optical stimulation of orexin neurons

Pharmacological experiments, knockout mice, and conditional ablation of orexin neurons provided much of our basic understanding of sleep/wakefulness regulation via orexin neurons. However it is still unclear how the neuronal activity of orexin neurons regulates sleep/wakefulness *in vivo*. Recent optogenetic approaches have revealed additional functional and physiological roles of orexin neurons in the regulation of sleep/wakefulness *in vivo*.

The microbial protein channelrhodopsin-2 (ChR2) was isolated from *Chlamydomonas reinhardtii*. ChR2 is a blue light-gated monovalent non-selective cation channel. It opens a channel by sensing blue light (470 nm) and induces depolarization of the membrane potential (Nagel et al., 2003). Using this technique, Adamantidis et al. performed a pioneering experiment. They expressed ChR2 in orexin neurons using a lenti-virus vector and photo-stimulated orexin neurons *in vivo* (Adamantidis et al., 2007). Electroencephalographic (EEG) and electromyographic (EMG) electrodes were implanted in freely moving mice to determine sleep/wakefulness stages, and an optical fiber was implanted in the LHA to photostimulate orexin neurons. Blue light illumination into the LHA increased activity of orexin neurons and increased the probability of transition to wakefulness from either NREM or REM sleep.

Other microbial protein, halorhodopsin (HaloR), isolated from *Natronomonas pharaonic*, is also used for optogenetics to silence neurons (Zhang et al., 2007). Tsunematsu et al. established transgenic mice in which orexin neurons expressed HaloR under control of the human prepro-orexin promoter (orexin/Halo mice). When they illuminate orange light (590nm) into the LHA during the day *in vivo*, it induces an increase in slow wave ratio in EEG with

reduced amplitude of EMG, characteristic of NREM sleep (Tsunematsu et al., 2011). However, these effects were not observed when orexin neurons were inhibited by light during the night, which is the active period for mice. These results indicate that acute inhibition of orexin neurons leads to a time-of-day-dependent induction of NREM sleep.

Many types of optogenetic tools have been established (Figure 2). A light-activated proton pump, Archaerhodopsin (Arch/ArchT), is also used for inhibition of neuronal activity by green light (550nm) (Chow et al., 2010; Han et al., 2011; Tsunematsu et al., 2013). Optical stimulation can modulate not only membrane excitability but also GPCR signaling cascades (OptoXRs) (Airan et al., 2009), cAMP (PAC) (Schroder-Lang et al., 2007), Ca^{2+} (Melanopsin, BACCS) (Ishii et al., 2015; Qiu et al., 2005), gene expression (GAVPO) (Wang et al., 2012), and gene manipulation (Magnets) (Nihongaki et al., 2015). The Knockin-mediated Enhanced Gene Expression (KENGE)-tet system is also a good method to express these proteins in specific type of neurons (Tanaka et al., 2012). If mice expressing the gene encoding tTA protein under the control of a specific promoter are crossed with mice expressing optogenetic tools such as ChR2 downstream of the tetO sequence, ChR2 expression can be enhanced in

specific cells. The timing and amount of expression can also be regulated by DOX application.

3-5 Pharmacogenetic regulation of orexin neurons

Since optogenetics confers a high temporal resolution for manipulation, it is widely used in many neuroscience researches. However, to manipulate neurons located in deep brain areas, insertion of an optical fiber is necessary. This invasion destroys neuronal networks in the brain. To overcome this difficulty, Sasaki et al. used Designer Receptors Exclusively Activated by Designer Drugs (DREADDs) to manipulate the activity of orexin neurons (Sasaki et al., 2011). Modified muscarinic receptors (hM3Dq for excitation and hM4Di for inhibition) can be manipulated by application of a specific synthetic ligand, clozapine-N-oxide (CNO), which can cross the blood–brain barrier (Alexander et al., 2009; Armbruster et al., 2007). Using this method, they manipulated the activity of orexin neurons *in vivo*, and observed changes in sleep and wakefulness patterns (Sasaki et al., 2011). This method is a less-invasive technique to manipulate specific types of neurons. However the temporal resolution of manipulation is relatively low compared with optogenetics, as the

effects last for several hours once CNO is injected in mice. Causality between the activity of neurons and induced behavior is lower than that revealed via optogenetics due to low temporal accuracy to control activity of cell function. To overcome this difficulty, up-conversion luminescent materials, such as lanthanide chemical compounds, would be one solution (Hososhima et al., 2015; Wang et al., 2011). Near infrared (NIR) light (650–1450 nm) can penetrate brain tissue, and up-conversion nanoparticles, composed of rare-earth elements such as Yb and Er, are excited by NIR and emit visible green light (550 nm), which can excite variants of ChR2 such as C1V1. These could be used as next-generation optogenetic tools for *in vivo* experiments, representing fiber-less optogenetics.

4. Optical recording of orexin neurons *in vivo*

There are many good fluorescent probes that can be measure cytosolic Ca^{2+} concentrations with high temporal resolution and sensitivity (Chen et al., 2013; Horikawa et al., 2010; Miyawaki et al., 1999; Nakai et al., 2001). They are useful tools to measure indirectly neuronal activity from specific neurons in the brain. Recently, Inutsuka et al. successfully measured Ca^{2+} responses from

orexin neurons in conscious and freely behaving mice using fiber photometry with G-CaMP6, which is a GECI (Inutsuka et al., 2016). Fluorescence intensity of orexin neurons was increased when mechanistic or heat stimulation was administered to conscious mice. However, these responses were not detected under anesthetic conditions. Gonza´lez and colleagues also performed optical recordings from orexin or MCH neurons in freely moving mice (Gonzalez et al., 2016). Orexin GCaMP6s activity was increased when mice received tail air puff exposure. Moreover, MCH and orexin GCaMP6s activity was increased when a novel object was placed into the cage. This fiber photometry method would be a powerful method to understand important characteristics of orexin or other types of neurons in the regulation of sleep-wakefulness in conscious mice.

Fluorescence calcium indicators are useful tools to measure several events happening in a cell, but require an excitation light, which brings several disadvantages, such as autofluorescence, phototoxicity, or photobleaching (Magidson and Khodjakov, 2013). Furthermore, as long as we use excitation light for measurement of fluorescence probes, we need to be careful of the spectrum of optogenetic tools. Recently Nagai's group established luminescence-based calcium probes termed Nano-lanterns by using

bioluminescence resonance energy transfer (BRET) (Saito et al., 2012; Takai et al., 2015). It does not require excitation light, and emission light is tremendously bright as compared to conventional luminescence proteins such as firefly luciferase. In addition, Yang et al. developed a very bright and genetically encoded calcium sensor that is ratiometric via BRET (Yang et al., 2016). These bioluminescence tools would be applicable for *in vivo* experiments for long period (Ono et al., 2015a; Ono et al., 2015b; Yamaguchi et al., 2001) combined with optogenetics.

5. Melanin-concentrating hormone (MCH) neurons in the LHA

The sleep-active neurons in the preoptic area (VLPO and MnPN) have an important role to initiate and maintain sleep. In addition to these neurons, other sleep-active neurons outside the preoptic area have also been found. One such group is melanin-concentrating hormone (MCH) neurons (Hassani et al., 2009). MCH is produced in neurons sparsely distributed throughout the LHA. MCH is a 19 amino acid neuropeptide synthesized as a prepro-hormone encoding two additional peptides: neuropeptide EI and neuropeptide GE (NGE) (Nahon, 1994). MCH neurons are active during sleep, especially in REM sleep

(Hassani et al., 2009). Pharmacological infusion of MCH peptide induces both non-REM and REM sleep (Monti et al., 2013; Verret et al., 2003). Furthermore, MCH neurons project throughout the brain and densely innervate the cholinergic and monoaminergic arousal centers (Bittencourt et al., 1992). MCH receptor1 (MCHR1) is a GPCR linked to Gq, Gi, and Go subunits (Hawes et al., 2000). MCH decreases cAMP levels in a cell (Chambers et al., 1999; Lembo et al., 1999) and cellular electrophysiological studies have revealed both presynaptic and postsynaptic inhibitory effects of MCH (Gao and van den Pol, 2001; Wu et al., 2009). MCHR1 is expressed in the hippocampus, subiculum, basolateral amygdala, shell of the nucleus accumbens, ventromedial nucleus, arcuate nucleus, TMN, dorsolateral pons (including the dorsal raphe), and LC (Saito et al., 2001). These results indicate that MCH neurons play an important role in sleep/wakefulness regulation.

MCH knockout mice are awake and more active, and also have less NREM sleep (Willie et al., 2008; Zhou et al., 2005). MCH1R$^{-/-}$ mice show a hyperactive phenotype (Shimada et al., 1998), and exhibit a significant decrease in total REM sleep duration and REM sleep episode duration during fasting (Willie et al., 2008). Recently, Jego et al. reported that acute activation of MCH

neurons by ChETA (a ChR2 variant with faster deactivation kinetics) (Gunaydin et al., 2010) or ChR2-mutant step-function opsin (SSFO) (Berndt et al., 2009) at the onset of REM sleep extended the duration of REM, but not non-REM, sleep episodes (Jego et al., 2013). In addition, Konadhode et al. demonstrated that activation of MCH neurons by ChETA stabilized REM sleep, while silencing of them (eNpHR3.0: to improve the membrane localization of NpHR) reduced the frequency and amplitude of hippocampal theta rhythms without affecting REM sleep duration (Konadhode et al., 2013). Furthermore, Tsunematsu et al. reported that activation of MCH neurons by ChR2 in NREM sleep induced REM sleep, but activation during wakefulness was ineffective (Tsunematsu et al., 2014). They also ablated MCH neurons using the DTA method (which is the same method applied to orexin neurons to generate narcoleptic mice), and found that ablation of MCH neurons increased time in wakefulness and decreased time in NREM sleep, while REM sleep amount was intact. These results indicate that MCH neurons are important for sleep regulation.

MCH neurons are excited by orexin, AMPA agonists, NMDA, and cannabinoid type-1 receptor (CB1R) agonists (Huang et al., 2007; Huang and van den Pol, 2007; van den Pol et al., 2004). Recently, however,

Apergis-Schoute et al. demonstrated that optical stimulation of orexin neurons using ChR2 inhibited action potentials in MCH neurons via $GABA_A$ receptors (Apergis-Schoute et al., 2015). Comparatively, MCH neurons inhibit orexin neurons and neighboring GABA neurons (Gao and van den Pol, 2001). MCH neurons are also inhibited by MCH, GABA, noradrenaline (NA) (effect mediated by alpha-2 receptors), serotonin, acetylcholine (muscarinic), neuropeptide Y (NPY) (Gao et al., 2003), and histamine (Parks et al., 2014). Dopamine (DA) inhibits MCH neurons through the alpha-2 receptor (Alberto et al., 2011) and also via D1- and D2-like receptors (Conductier et al., 2011). These data suggest that there is mutual inhibitory interaction between orexin neurons and MCH neurons in the LHA. Interaction of orexin and MCH neurons is important for the regulation of sleep/wakefulness, although specific regulatory mechanisms *in vivo* need to be investigated in the future in more detail.

6. Circadian regulation of sleep/wake cycles

Although we have discussed the functions of orexin and MCH neurons in homeostatic sleep/wake regulation, they are also controlled by the circadian clock. In mammals, circadian regulation of sleep/wakefulness across a day

depends on the central circadian clock termed the suprachiasmatic nucleus (SCN), which is located in the anterior part of the hypothalamus. SCN neurons exhibit circadian rhythms of frequency of neuronal activity, even in dissociated cell culture (Herzog et al., 1998; Welsh et al., 1995). Circadian neuronal activity rhythms are regulated by transcription-translation negative feedback loops involving several clock genes, such as *Per, Cry*, *Bmal1*, and *Clock* (Reppert and Weaver, 2002). When the SCN is electrically lesioned, circadian behavioral rhythms are eliminated (Stephan and Zucker, 1972). Interestingly, neuronal activity of SCN neurons is increased during subjective day and decreased during subjective night, regardless of the nocturnal and diurnal status of animals (Inouye and Kawamura, 1979; Sato and Kawamura, 1984). Thus, the switching of day-night information of neuronal activity in the SCN occurs somewhere and somehow in the brain, but the neuronal networks and mechanisms of this regulation remain unclear. Intriguingly, experimental mice usually exhibit nocturnal behavior in a laboratory, but once they were released into the natural environment, they often display diurnal behavioral activity (Daan et al., 2011). This phenomenon was confirmed in a laboratory. When mice were exposed to a reduced low food reward condition (they could obtain food pellets depending on

the number of running wheel revolutions) or to a low ambient temperature condition, behavioral activity time shifted from the dark phase into the light phase (Hut et al., 2011; van der Vinne et al., 2014), suggesting that the activity of outside of the SCN can be modified by metabolic changes. Furthermore, restricted feeding schedules or methamphetamine treatment induces behavioral rhythms that do not depend on the SCN (Honma et al., 1987; Stephan, 2002). These results indicate that elements outside of the SCN (peripheral clock) also regulate sleep/wake rhythms together with the SCN.

SCN neurons project to multiple areas in the brain to regulate a variety of physiological functions (Leak and Moore, 2001). Although projections to the orexin neurons were investigated using a retrograde tracer method (Gonzalez et al., 2016; Sakurai et al., 2005), strong projections from the SCN to orexin neurons have not been detected. Outputs from the SCN were observed to project to the subparaventricular zone (SPZ) and the dorsomedial nucleus of the hypothalamus (DMH). These neurons could also be involved in the circadian regulation of sleep/wakefulness (Chou et al., 2003; Lu et al., 2001).

The two-process model has been proposed for the regulation of sleep/wakefulness (Borbely, 1982; Daan et al., 1984). Process S and C are

driven by homeostatic mechanisms and the circadian clock, respectively. Process S represents sleep pressure, which increases as a function of how long we have been awake. Process C is regulated by the circadian clock, which depends on the phase of circadian rhythms in a body. Determination of the mechanisms of circadian regulation in sleep/wakefulness and its corresponding neuronal circuit or underlying long-range neuronal networks in the brain will be important for understanding the precise mechanisms of sleep and wakefulness regulation.

7. Conclusion

We discussed mechanisms of sleep/wake regulation in the hypothalamus, especially in regard to two peptide-producing neurons, orexin and MCH neurons. During the last decade, several findings were reported in sleep research, which was primarily driven by new and innovative techniques such as fluorescence imaging, optogenetics, and pharmacogenetics. Since sleep/wakefulness is observed only in the conscious animal whose neural networks are conserved, it is not easy to investigate the roles of neurons involved in the regulation of sleep/wakefulness. In addition,

sleep/wake-regulating neurons in the LHA, such as orexin and MCH neurons, comprise complex neuronal circuits in the brain. Incorporation of new techniques or approaches would open the next door for future sleep research.

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Figures

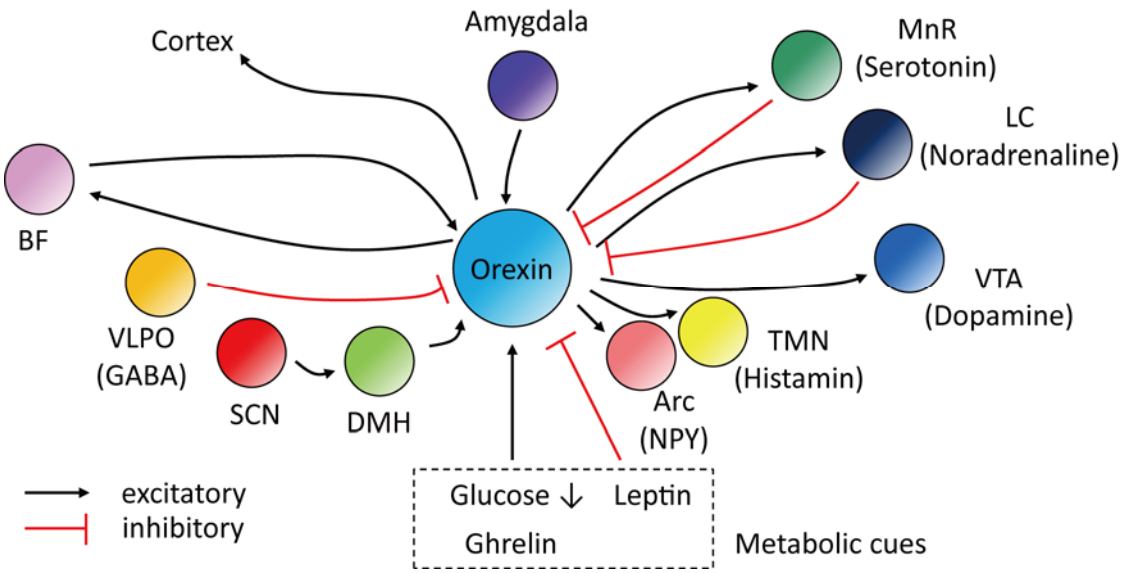

Figure1: Input and output pathways of orexin neurons in the lateral hypothalamus

Many neurons project to orexin neurons and orexin neurons project to a variety of brain areas. Black arrows and red lines indicate neuronal projections that excite and inhibit neuronal activity, respectively. Abbreviations: BF, basal forebrain; VLPO, ventrolateral preoptic nucleus; SCN, suprachiasmatic nucleus; DMH, dorsomedial hypothalamus; Arc, arcuate nucleus; TMN, tuberomammillary nucleus; VTA, ventral tegmental area; LC, locus coeruleus; MnR, median raphe nucleus.

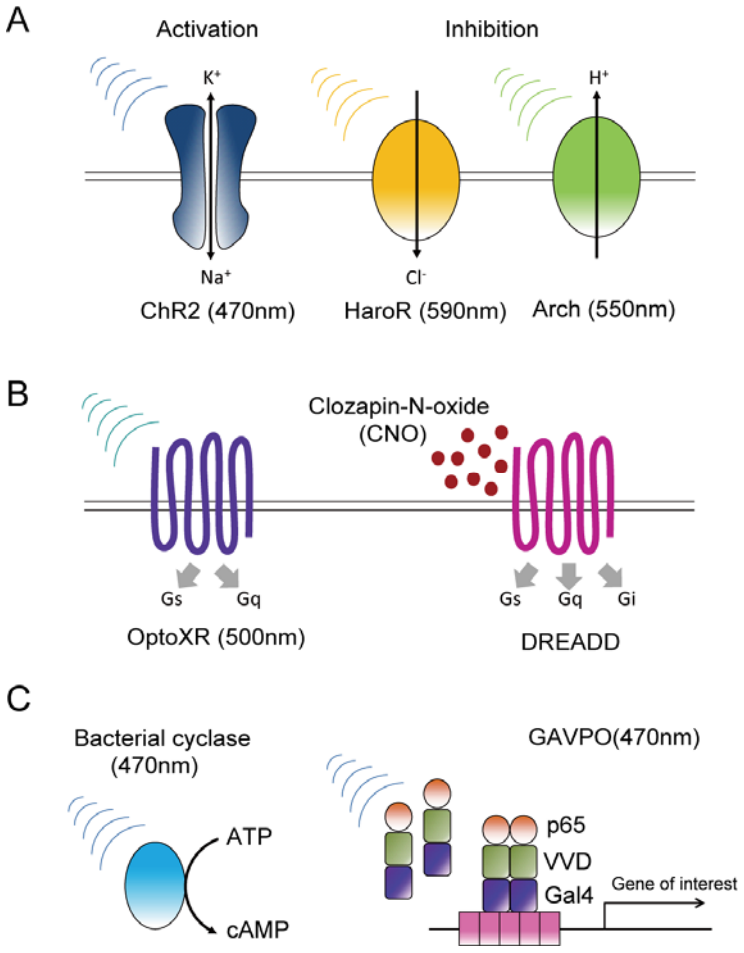

Figure 2: Variety of optogenetic tools for manipulation of cellular functions (A) Optogenetic tools for manipulation of membrane potential. (B) Optogenetic tools for manipulation of G protein-coupled receptors (GPCRs). Blue light exposure or CNO application can modulate GPCR signal cascades. (C) Optogenetic tools for manipulation of other cellular functions. Blue light can increase cAMP concentration or regulate transcription of genes of interest.

Substances which modulate activity of orexin neurons

Activation	**Inhibition**
Glutamate	GABA
Acetylcoline	Glycine
Orexin	Serotonin
Cholecystokinin	Noradrenaline
Arginine-vasopressin	Dopamine
Oxytocin	Acetylcholine
Corticotrophin-releasing factor	Glucose
Neurotensin	Leptin
Thyrotropin-releasing hormone	Neuropeptide Y
Glucagon-like peptide-1	Adenosine
Ghrelin	Nociceptin
ATP	Met-enkephalin
CO_2	
H^+	

Table 1