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The role of co-neurotransmitters in sleep and wake regulation

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Abstract

Sleep and wakefulness control in the mammalian brain requires the coordination of various discrete interconnected neurons. According to the most conventional sleep model, wake-promoting neurons (WPNs) and sleep-promoting neurons (SPNs) compete for network dominance, creating a systematic “switch” that results in either the sleep or awake state. WPNs and SPNs are ubiquitous in the brainstem and diencephalon, areas that together contain less than 1% of the neurons in the human brain. Interestingly, many of these WPNs and SPNs co-express and co-release various types of the neurotransmitters that often have opposing modulatory effects on the network. Co-transmission is often beneficial to structures with limited numbers of neurons because it provides increasing computational capability and flexibility. Moreover, co-transmission allows subcortical structures to bi-directionally control postsynaptic neurons, thus helping to orchestrate several complex physiological functions such as sleep. Here, we present an in-depth review of co-transmission in hypothalamic WPNs and SPNs and discuss its functional significance in the sleep-wake network.

1. Introduction

Multiple brain regions interact to modulate sleep and wakefulness, and distinct neuronal populations synthesize different neurotransmitters that regulate sleep-wake dynamics in a

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hierarchical manner. Wakefulness is governed by wake-promoting neurons (WPNs), including noradrenergic neurons of the locus coeruleus (LC), serotonergic neurons of the raphe nuclei (RN), histaminergic neurons of the tuberomammillary nucleus (TMN), and orexin/hypocretin-producing neurons of the perifornical nuclei (PFN)/lateral hypothalamic area (LHA)[1-8]. Sleep-promoting neurons (SPNs) include melanin-concentrating hormone-producing (MCH) neurons in the diencephalon and GABAergic neurons in the ventrolateral preoptic nuclei (VLPO)/intermediate nuclei, median preoptic nuclei (MnPO), and brainstem parafacial zone[9-13]. A prominent hypothesis of a sleep-wake control model suggests that WPNs and SPNs compete for network dominance through mutual inhibition, creating a systematic "switch" that results in the sleep or awake state[14-16]. However, this model does not fully account for the complexity of the neurobiology behind sleep/wakefulness, and research into the identity and functional significance of co-transmitting neurons in the sleep-wake network is still a work in progress[17].

Central nervous system (CNS) neurons can co-transmit different neurotransmitter types through distinct mechanisms, thereby allowing for versatile synaptic signaling[18]. Moreover, the neurophysiological effect of co-transmission is determined by differences in neurotransmitter concentrations at the presynaptic terminals and composition of postsynaptic receptors[19, 20]. Different signaling molecules may be packed in the same vesicle[21, 22], which can affect their postsynaptic receptivity and functions. For example, one of the best-studied co-transmission systems is the co-release of glutamate by primary GABAergic/glycinergic neuron terminals in the medial nucleus of the trapezoid body (MNTB), part of the sound localization pathway during the development process. Co-released glutamate activates post-synaptic NMDA receptors and reorganizes the MNTB-lateral superior olive inhibitory circuitry[23]. Disrupting glutamate co-transmission during this critical period impairs the tonotopic refinement of the auditory brainstem[24]. Additionally, co-transmission facilitates various functions in the adult brain. For example, starburst amacrine cells (SACs) in the retina co-release GABA and acetylcholine (ACh) through separate vesicles in a Ca^{2+}-dependent process[21]. This allows a small number of SACs to encode both motion and direction sensitivities using GABA and ACh signaling, respectively. Glutamate is co-released from dopaminergic neurons in the ventral tegmental area (VTA)[25]. Dopamine acts on a slow time scale by binding to G-protein-coupled receptors, whereas glutamate acts on a fast time scale when bound to ionotropic glutamate receptors and conveys temporally precise signals. Glutamate co-release is useful for accurate prediction-error signals, allowing reward to be encoded in the firing rates of dopaminergic neurons and mediating dopamine-dependent behaviors[26, 27].

Co-transmission, a concept introduced decades ago, is increasingly being incorporated into models attempting to explain the sleep-wake circuit, thus rapidly replacing models that privileged the "one neuron = one neurotransmitter" hypothesis. Most WPNs and SPNs release more than one neurotransmitter type, and neurotransmitter co-release is vital in increasing the computational capabilities of this relatively short-numbered neuronal population which modulates the sleep-wake circuit. In this review, we discuss recent findings on co-transmission in hypothalamic WPNs and SPNs and examine its functional significance in the sleep-wake circuit.

2.1 Histamine and GABA of the Tuberomammillary Nuclei

The hypothalamic TMN contains almost all histaminergic neurons (about 64,000) found in the adult mammalian brain[28, 29]. Moreover, TMN neurons also synthesize GABA[30] and have been shown to express galanin, enkephalins, thyrotropin release hormone, and substance-P in some species[30, 31]. Accumulating evidence suggests that the interaction of histamine and GABA transmission is relevant to the sleep-wake circuit.

Histamine in neurons is synthesized by decarboxylation of L-histidine by histidine decarboxylase (HDC), transported into vesicles by vesicular monoamine transporter-2 (VMAT2), and inactivated by histamine-N-methyltransferase and monoamine oxidase-B following release[32, 33]. Histaminergic neurons can innervate multiple brain regions such as the basal forebrain, LHA, and neocortex. Pharmacological studies in animals have shown that histamine is a critical wake-promoting neurotransmitter; however, the underlying mechanisms are unclear. Bilateral histamine injection into the TMN increases arousal and sleep latency in cats[33]. In contrast, irreversible HDC inhibitor (alpha-fluoromethylhistidine) administration reduces wakefulness and increases non-rapid eye movement (NREM) and rapid eye movement (REM) sleep in rats and cats[34].

Previous studies have demonstrated that glutamate decarboxylase-67 (GAD67) and vesicular GABA transporter (VGAT), the enzymes needed for GABA synthesis and transmission, respectively, are expressed in the TMN[31, 35]. VMAT-positive neurons in TMN-explants also expressed GABA in *in vitro* cultures, indicating histamine and GABA co-localization[36]. The same study reported that GABA and VMAT2 were located in different granular deposits, suggesting that they are packed in distinct presynaptic vesicles. Although the hypothesis of separate packing needs confirmation by electron microscopy[36], this finding indicates that histamine and GABA are capable of acting independently on the diverse populations of postsynaptic neurons. This is not surprising because the TMN projects to many parts of the CNS and co-regulates various brain functions, such as learning and memory, appetite, metabolism, and thermoregulation, besides promoting wakefulness[37-40].

At the cellular level, co-transmitted GABA can potentially influence the overall postsynaptic excitability by acting on a different time course than that of histamine. The histamine-1 receptors (H1Rs) are coupled to G-protein ($G_{q/11}$) and phospholipase C (PLC), which activate secondary messengers, diacylglycerol (DAG) and triphosphoinositol (IP3). IP3 facilitates Ca^{2+} release from the endoplasmic reticulum (ER), leading to Ca^{2+}-dependent depolarization through cation channels. Histamine-2 receptor (H2R) activation results in CREB transcription factor phosphorylation through G_s, adenylyl cyclase, and PKA cascade, leading to an enhanced excitatory response[41]. Compared with the slow-acting G-protein coupled receptors (GPCRs), synaptic ionotropic GABA-A receptors regulate neuronal inhibition on a millisecond time scale[42]. Therefore, if GABA binds synaptic receptors, it may produce inhibitory postsynaptic potentials (IPSPs) before the onset of H1R and H2R-mediated depolarization. Initially, IPSPs activate hyperpolarization-activated cation currents (I_h) and steadily depolarize the resting potential[43]. It is possible that the delayed H1R- and H2R-mediated inward currents enhance post-inhibitory rebound spiking and create a biphasic inhibition-excitation signal commonly found in other monoamine-GABA co-

transmission cases[44-46]. During low TMN activity, this mechanism may attenuate spontaneous and subthreshold excitatory postsynaptic potentials (EPSPs) in receiving WPNs. Only when sufficient presynaptic TMN neurons are discharged may the rebound spiking generate a postsynaptic excitatory switch. We hypothesize that an increase in the signal-to-noise ratio is an essential mechanism governing accurate and rapid sleep-to-wake transitions while preventing uncommitted transitions.

Moreover, co-transmitted GABA acts as an inhibitory regulator for an overactive histaminergic system. Yu *et al.* demonstrated that mice with a Cre recombination VGAT knock-down (*HDC-VGAT KD*) as well as those with VGAT knock-out (*TMN-ΔVGAT)* displayed hyperactivity and an increased and sustained wakefulness at night (dark phase)[35]. Even after sleep deprivation, *HDC-VGAT KD* and *TMN-ΔVGAT* mice maintained the hyperactive states and their hours of sleep were less than controls. As noted by the same study, specific optogenetic activation of *TMN-ΔVGAT/HDC-Channelrhodopsin* in neocortex and caudate-putamen slices resulted in the absence of tonic inhibitory conductance usually generated by VGAT-dependent GABA release from the TMN onto extrasynaptic ionotropic GABA-A receptors. These findings corroborate the hypothesis that co-transmitted GABA plays a critical role in preventing histamine-induced overexcitement of post-synaptic neurons and promotes optimal wakefulness[35]. Conversely, in Williams, Chee *et al.*, optogenetic stimulation of TMN neurons evoked histamine release in the ventrolateral TMN (vlTMN) and ventrolateral preoptic nucleus (VLPO) but evidence of GABA co-release was lacking [47]. Thus, it is possible that TMN co-transmission is target-specific and a study incorporating a larger number of brain areas is sought to clarify this matter.

At other neuronal terminals, histamine and GABA may elicit synergistic, inhibitory actions on target neurons. In addition to activating metabotropic histamine receptors, recent *in vitro* electrophysiological studies have shown that histamine acts through positive allosteric sites on heteromultimeric GABA-A receptors to enhance GABA-dependent tonic inhibition[48-51]. In fact, while histamine is mainly known for eliciting an excitatory-modulatory response, TMN stimulation has been shown to also evoke fast IPSPs, with kinetics that resembles GABA-A receptors in supraoptic oxytocin neurons[52, 53]. Furthermore, iontophoretically administered histamine directly reduces neuronal firing in the anterior and intralaminar thalamic nuclei, perigeniculate nuclei (PGN), and other regions[54, 55]. Lee *et al.* hypothesize that increased histamine release during wakefulness can dampen a thalamic oscillation in sleep-wake transition by PGN neuron inhibition[55]. The inhibitory effects via enhanced Cl- conductance may be mediated by GABA-histamine binding on GABA-A receptor. Further research is needed to clarify how an interaction between the two neurotransmitters regulates circuit-specific and selective inhibition[49, 56].

Recent identification of cortical GABAergic interneurons by Kilduff *et al.* demonstrated an increased Fos-immunoreactivity during recovery and spontaneous sleep[57, 58]. This led to the speculation that inhibitory effects of GABA-histamine on GABAergic interneurons that contain GABA-A receptors may underlie an indirect excitation of arousal centers. These interneurons are sleep-active and co-express nitric oxide (NO), neuropeptide Y(NPY), and somatostatin (SST)[57, 58]. The authors also speculated that a combination of co-transmitters (GABA, NO, NPY, and SST) contributes to synchronization of cortical EEG activity during

sleep[58]. NO has been previously implicated in REM regulation[59], although its role in interneurons remains unclear. Thus, the hypothesis that inhibitory TMN inputs during wakefulness modulate the activity of NO/GABA cortical interneurons needs testing.

In conclusion, the mechanisms underlying histamine modulation of wakefulness in the context of GABA co-transmission still remain elusive. Nevertheless, mounting evidence suggests that GABA is an active component of histaminergic neurotransmission (Figure 1A), and GABA and histamine co-transmission may promote bi-directional postsynaptic modulation of coincidental excitation, inhibitory regulation, and synergistic inhibition. These mechanisms may promote appropriate sleep-to-wake transitions and wakefulness while suppressing sleep-related neuronal activity.

2.2 Dynorphin, Glutamate, and Orexin in the Lateral Hypothalamic Area

The orexinergic (hypocretinergic) neurons in the LHA promote and stabilize wakefulness by innervating WPNs of the basal forebrain, LC, TMN, and RN. Orexinergic neurons co-express dynorphin and/or glutamate. Co-release of orexin and dynorphin or orexin and glutamate may modulate activity of LHA neurons through distinct feedback mechanisms.

Orexinergic neurons (~70,000) play a critical role in sleep-wake homeostasis. Selective optogenetic activation of orexin neurons increases the probability of NREM or REM transition to wakefulness in mice[60], and selective optogenetic inhibition of these cells induces NREM sleep in mice[61, 62]. Orexin or Orexin-receptor (OxRs) knock-out animal models mimic human narcolepsy[63]. Neuropeptides Orexin-A and B are synthesized by prepro-orexin cleavage and pre-synaptically packaged into large dense core vesicles. Orexin-A binds to both Ox1Rs and Ox2Rs, whereas Orexin-B binds mainly to Ox2Rs[64]. G-protein activation coupled with the receptors increases neuronal excitability by inhibiting G protein-coupled inward-rectifying K^+ channels (GIRKs), inducing Ca^{2+} influx through voltage-dependent calcium channels (VDCCs) and influencing Na^+/Ca^{2+} exchange[64-66]. Ox1Rs and OxR2s occur in distinct anatomical locations. For example, LC neurons and TMN neurons exclusively express Ox1Rs and Ox2Rs respectively, whereas RN neurons express both.

Interestingly, nearly all mice and rat orexinergic neurons contain dynorphin[67]. In the LHA, only orexinergic neurons co-expressed dynorphin[67]. Dynorphins are neuropeptides derived from prodynorphin and act as endogenous ligands for k-opioid receptors. Dynorphins show high affinity for other opioids (μ and δ) and non-opioid receptors (NMDARs)[68, 69]. Opioid receptors inhibit adenylyl cyclase by coupling to inhibitory G-proteins. They also stimulate GIRK channels and inhibit presynaptically expressed N-type Ca^{2+} channels, which increase K^+ conductance and decrease Ca^{2+} conductance[70]. Thus, dynorphins are mainly inhibitory peptides. However, at sub-nanomolar dynorphin concentrations, opioid receptors may bind to stimulatory G-proteins and prolong action potential duration[69, 71], making dynorphin a multi-functional neurotransmitter in the CNS. Electron micrograph (EM) images show orexin and dynorphin co-localization in large dense core vesicles, suggesting co-packaging and co-release on the same postsynaptic target[72]. Many orexin-innervated SPNs and WPNs contain dynorphin fibers and opioid receptors[67]. Thus, orexin and dynorphin likely coordinate closely to regulate diverse physiological functions such as arousal.

The significance of dynorphin and orexin co-release remains largely unknown, but both types of neurotransmitters have been shown previously to influence the sleep-wake circuit. In the LC, the selective k-opioid receptor agonist depresses the excitatory synaptic inputs into noradrenergic neurons *in vitro*[73]. A recent *in vivo* electrophysiological study supports these findings: dynorphin attenuated LC neuronal discharge through presynaptic inhibition *in vivo*[74]. In the RN, the k-opioid receptor agonist reduces EPSPs and decreases extracellular 5-HT[75, 76]. In the basal forebrain, dynorphin inhibits cholinergic neurons through pre- and postsynaptic mechanisms[77]. Thus, despite being co-released, dynorphin seems to have an opposing role to that of stimulatory orexin[78]. In the VTA, orexin and dynorphin tune dopaminergic output by simultaneously inhibiting and activating different neuronal subsets[79]. A study on reward circuitry in mice showed that when applied separately, orexin excited while dynorphin inhibited dopaminergic VTA neurons[72]. However, upon co-application, no net change in firing rate was observed, suggesting that the effects of these co-released neuropeptides compensated each other during saturating concentrations. At the behavioral level, orexin disruption using orexin-1 receptor antagonist (SB334867) blunted the reward effects of LHA electrical stimulation, eliminated cocaine-induced impulsivity, and reduced cocaine self-administration[72]. However, disruption of both orexin and dynorphin, using SB334867 and K-opioid receptor antagonist (norbinaltorphimine), reversed these effects, suggesting that the dynamic interactions between the two neuropeptides may regulate reward-driven behavior. While this study focused on reward circuitry, it has strong implications on the wake-circuit as well because (1) VTA is a wake-promoting center[80, 81] and (2) other WPNs innervated by orexin-dynorphin fibers may be similarly regulated (Figure 1B).

What, then, is the purpose of co-releasing signals with opposing effects? Antagonistic neurotransmitter co-release may allow pre-synaptic neurons to balance excitation and inhibition of postsynaptic neurons. Thus, LHA neurons may control firing rates and prevent postsynaptic overexcitation during wakefulness. Similar to the effects in the VTA, dynorphin or orexin application in the basal forebrain produces opposite effects on cholinergic neurons[77]. When co-applied at a membrane potential of −40mV, dynorphin exerts a stronger effect than orexin, resulting in net neuronal inhibition. At a more hyperpolarized potential (−70mV), the opposite effect is observed. Thus, depending on the membrane potential, orexin and dynorphin co-release results in either net inhibition or excitation. In an overstimulated neuron, this mechanism may prevent excitotoxicity and signal saturation. Moreover, co-released dynorphin may act on presynaptic LHA neurons themselves to attenuate overexcitation. Orexinergic neurons form synapses and directly activate neighboring orexinergic neurons through Ox2Rs[82], allowing signal amplification and maintenance of wakefulness. However, a positive feedback circuitry may be prone to instabilities (runaway excitation). Co-released dynorphin directly inhibits local orexinergic neurons through multiple mechanisms, including GIRK channel activation and calcium current reduction[83]. While orexin and glutamate (see below) promote positive feedback, dynorphin may lead to negative feedback and optimize LHA neuronal activity.

In other regions, dynorphin may act in concert with orexin to increase postsynaptic neuronal excitability. During sleep, TMN neurons are hypothesized to receive inhibitory inputs from GABA/galaninergic sleep-promoting neurons of the VLPO, which express Fos protein

during sleep but not during wakefulness [84, 85]. Dynorphin suppresses these inhibitory inputs and attenuates TMN neuron inhibition[86]. A similar mechanism is employed by NPY neurons in the hypothalamic arcuate nucleus. Orexin directly excites NPY neurons while dynorphin attenuates GABAergic inputs, resulting in enhanced excitation[83]. In this case, co-released dynorphin and orexin function synergistically.

Repeated pharmacological application of either dynorphin or orexin in mice brain slices *in vitro* resulted in desensitization of cholinergic neurons, whereas the same cholinergic neurons showed a sustained electrophysiological response when exposed to dynorphin and orexin simultaneously[77]. Melanin-concentrating hormone (MCH) neurons desensitize faster when exposed to dynorphin than orexin, and when dynorphin and orexin are repeatedly co-applied, orexinergic excitation remains[83]. In contrast to previous studies suggesting a collective role of dynorphin and orexin in modulating MCH neurons, a more recent study in mouse brain slices showed that optogenetic stimulation of orexinergic neurons inhibits most MCH neurons through a dynorphin-independent mechanism[87]. In this study, a k-opioid receptor antagonist (nor-binaltorphimine), intended to block the effect of dynorphin, did not cause changes in MCH cell membrane potential. Conversely, GABA-A receptor blocker (Gabazine) abolished the orexin-driven inhibition of MCH neurons[87]. The authors further speculated that orexinergic inhibition of MCH neurons is mediated by local GABAergic interneurons containing orexin receptors[87]. Therefore, the effect of co-transmission originating from LHA neurons varies and depends on the targeted neuron. Understanding the interactions of these neuropeptides in postsynaptic signaling may provide further insights on their downstream modulatory effects[88, 89].

Along with dynorphin, over half of orexinergic neurons synthesize glutamate[90], suggesting that orexinergic neurons may be heterogeneous[91]. However, the exact topographic organization and significance of orexinergic neurons remain unknown. Orexinergic neurons containing glutamate are directly involved in the wake circuit. For instance, orexinergic terminals in the LC contain vesicular transporter for glutamate (VGLUT2). They also contain synaptophysin, a protein found in small synaptic vesicles of glutamatergic terminals[92]. In the TMN, EM studies show orexin immunoreactivity in large dense-core vesicles; the same orexinergic terminals contain glutamate in synaptic vesicles[93]. This is consistent with the notion that small amino acid neurotransmitters and neuropeptides are differentially stored and regulated. Orexin and dynorphin are synthesized and processed in the rough ER and Golgi apparatus. Then, dense core vesicles, containing the neuropeptides, are transported to the release site, resulting in a relatively slow replenishment rate[94]. In contrast, glutamate can be refilled into small synaptic vesicles directly at the axonal bouton. Additionally, while dense-core vesicles are released extra-synaptically and act more diffusely (volume transmission), glutamate tends to congregate in the active zone near the synapse, providing a limited but fast synchronous excitation-release coupling[94-96]. The spatial and temporal differences (fast versus slow) of co-neurotransmitters may modulate various input-output operations on the downstream wake circuit.

Co-released orexin and glutamate may produce distinct excitatory spike outputs in postsynaptic neurons (Figure 1B). A recent study explored the relationship in histaminergic neurons using optogenetic manipulation of upstream orexinergic neurons[97]. Orexin-

dependent excitation occurred only at high-frequency stimulation (20 Hz), whereas glutamate-dependent excitation occurred at all frequencies. In contrast to large dense core vesicles, small synaptic vesicles containing glutamate are docked near voltage-gated Ca^{2+}-channels, requiring less calcium influx to be released[98]. In the same study, glutamate produced a brief, rapid excitation through AMPAR, whereas orexin induced a delayed but linearly-increasing excitation through Ox2Rs. These two outputs were temporarily and pharmacologically separable, suggesting a parallel and non-redundant co-transmission signaling at the postsynaptic level[97]. The authors further speculated that orexin may act as an integral controller because the orexin-dependent output and input are proportional. Meanwhile, glutamate acts as a derivative controller, which spikes only at stimulus onset, possibly detecting input changes. Together, these two components increase the computational capacity of the LHA-TMN circuit and may stabilize wakefulness. An interesting experiment would be to selectively knock out glutamate in orexinergic neurons *in vivo* and observe whether glutamate co-transmission loss affects wakefulness. Co-released glutamate and orexin act independently on histaminergic neurons. However, it is unclear whether these independent effects are observed in other postsynaptic and local orexinergic neurons; this should be investigated to shed light on mechanisms governing target-dependent computation. For instance, Apergis-Schoute *et al.* used optogenetics in mouse brain slices, showing that glutamate released from orexinergic neurons was not required for activating local GABAergic interneurons[87]. Lastly, investigating how dynorphin cross-communicates with glutamate at both the molecular and physiological level is essential in unraveling the wake circuit. For example, while dynorphin mainly binds to opioid receptors, it has also been implicated in blocking NMDA receptors, which may decrease glutamate-induced excitation and plasticity[99].

2.3 Co-transmitters and Melanin-Concentrating Hormone in the Diencephalic Areas

MCH neurons are located mainly in the LHA, incerta-hypothalamic area (IHy), and zona incerta (ZI)[100-103]. The dorsomedial part of the TMN also contains non-histaminergic MCH neurons[104]. MCH neurons interact with neighboring orexinergic neurons to modulate physiological functions such as learning and memory, stress and anxiety, and energy homeostasis[105-107]. Additionally, MCH neurons promote sleep by innervating WPNs and REM-generating pons, although the mechanism of this process is elusive[108-110]. Some evidence suggests that the different signaling patterns of MCH neurons on sleep promotion could relate to the interplay of MCH, GABA and glutamate release from these neurons. Besides GABA and glutamate, MCH neurons co-express other neurotransmitters, including nesfatin, cocaine-amphetamine-regulated transcript (CART), neuropeptide-EI, and neuropeptide-GE[101, 111-113]. Further work is still necessary to pinpoint how each neurotransmitter type contributes to sleep regulation.

MCH binds two GPCRs: MCHR1s and MCHR2s. Studies using MCHR1-expressing, non-neuronal CHO cells show that MCH activates diverse intracellular signaling pathways by coupling to G_i, G_o, and G_q proteins[114] Studies in hypothalamic neuronal cultures suggest that MCH-MCHR1 predominantly plays an inhibitory role in the CNS. One such study showed that MCH inhibited VDCCs through the $G_i/_o$-mediated pathway[115]. MCH also causes miniature excitatory postsynaptic currents (mEPSC) depression by modulating

presynaptic glutamate release and postsynaptic glutamate receptors[116]. MCHR2 function remains largely unclear because although humans express both receptors, rodents express only MCHR1s[117]. However, some studies suggest that MCHR2s activate G_q protein[118].

To date, it remains unclear whether MCH neurons promote only REM or also NREM sleep. Some studies have shown that optogenetic stimulation of MCH neurons increased NREM and REM sleep in mice[119] and rats[120]. However, others show that it extended only REM duration[121, 122]. This may be because of a variation in MCH neuron clusters (zona incerta *vs.* perifornical/LHA/dorsomedial TMN) and channelrhodopsin expression rate. The first two studies[119, 120] used chronic stimulation (1 min stimulation / 4 min of no stimulation cycles for 24 h). The latter studies[121, 122] used acute stimulation.

Curiously, acute stimulation (stimulation at the onset of a stable NREM or REM sleep episode) extends REM sleep in both MCHR1-expressing and MCHR1-knock out mice, suggesting that other co-released neurotransmitters, and not MCH, modulate REM behavior[121]. As also noted, GABA co-release evoked bicuculline-sensitive inhibitory postsynaptic currents (IPSCs) in postsynaptic neurons *in vitro*. This is consistent with the findings that among the co-transmitters GABA, neuropeptide-EI, and MCH, a site-specific microinjection of GABA promotes the most rapid increase in REM sleep and shows no change in NREM sleep[123]. In contrast, increased NREM sleep seen in the experiments using chronic stimulation may derive from additional recruitment of MCH and other slow-acting inhibitory co-neuropeptides, such as neuropeptide-EI, neuropeptide-GE, nesfatin-1, and CART. As such, the intraventricular MCH peptide administration induces a dose-dependent increase in NREM and REM sleep[124]. Blocking MCH1 receptors with antagonists decreased the time spent in both types of sleep[125]. Similarly, a near-complete MCH neuron ablation by cell-specific diphtheria toxin-A expression decreased NREM sleep but showed no effect on REM sleep[122]. However, Vetrivelan *et al.* and Naganuma *et al.* demonstrated that selective activation of MCH neurons using chemogenetics causes an increase in REM sleep without altering NREM sleep in mice[126, 127].

Given the contradicting nature of these above-mentioned results, further investigation is necessary to clarify whether different MCH neuronal subpopulations modulate particular sleep components. MCH subpopulations are shown to have distinct intrahypothalamic localization and projection patterns[128]. For example, MCH neurons bordering the cerebral peduncle are devoid of CART whereas MCH subpopulations near the zona incerta, the perifornical area, and the area between the ventromedial hypothalamus (VMH) and dorsomedial hypothalamus (DMH) co-express CART[128].

At the circuit level, classical co-neurotransmitters may work in synergy with MCH peptides to enhance postsynaptic inhibition (Figure 1C). In fact, GABA or glutamate co-transmission may be necessary to inhibit postsynaptic neurons[126, 129]. GABA from MCH neurons evokes IPSPs[121]. Moreover, some MCH neurons reveal a glutamate-mediated feedforward inhibition of neurons in lateral septal nucleus (LS). Optogenetic stimulation of MCH neurons directly releases glutamate onto LS neurons and GABAergic interneurons/afferents[129]. By activating GABAergic interneurons, MCH neurons can robustly inhibit LS neurons. In both studies led by Jego and Chee, MCH peptides themselves failed to inhibit

postsynaptic neurons[121, 129]. This is not too surprising because MCH peptides modulate presynaptic neurons to reduce transmission rather than to directly affect postsynaptic membrane potential or conductance[116]. These studies have highlighted the critical role of fast-acting co-transmitters in modulating the postsynaptic network.

The mechanism underlying the presynaptic release of co-neurotransmitters remains largely unknown. A recent study using single-cell qPCR analysis in mice demonstrated that MCH neurons and neighboring orexinergic neurons express mRNA for VGLUT2 *(Slc17a6+)*[91], supporting previous findings showing that these two neuronal populations are functionally glutamatergic[97, 129]. Jego *et al.*[121] suggested that MCH neurons also release GABA. Interestingly, the study from Mickelsen *et al.* with single cell analysis showed that although MCH neurons and about 50% of orexinergic neurons are capable of synthesizing GABA *(Gad1+),* they lack the machinery for vesicular GABA release *(Slc32a1-)*[91]. These latter results led to the speculation that GABA release, at least from MCH neurons, likely occurs through a non-synaptic or non-canonical release pathway[91]. Meanwhile, using double transgenic reporter mice, Blanco-Centurion *et al.* showed that there are four major populations of neurons in the hypothalamus: VGAT+, orexin+/VGLUT2+, orexin-/VGLUT2+, and MCH+ neurons[130]. These and other recent studies point to the complexity of neurotransmitter phenotypes in the LHA neuronal populations and the need to clarify the role of neurochemical heterogeneity in eliciting dynamic homeostatic changes. These complexities also provide some background for explaining the challenges in treating sleep dysfunction in the clinical practice.

3. Conclusion

In this review, we focus on the complexity and relevance of co-transmission in modulating the sleep-wake network. Although current methods are still subpar to investigate how neuronal populations control different behaviors in dynamic conditions (e.g., studies using animal models often focus on one behavioral paradigm at a time), the idea that WPNs and SPNs are unidirectional-signaling neurons has been repeatedly challenged. Furthermore, validation studies from animal models to humans remain an obstacle. Although molecular imaging technique improvement has allowed neurotransmitter circuit detection in vivo[131, 132], spatial/temporal resolution is too low to discriminate most subcortical structures and to observe their activities. However, recent improvements in human tissue techniques are widening the analytical scope of post-mortem studies. Moreover, sleep assessments are more widely accessible and have been incorporated to aging cohorts, enabling sleep-focused clinicopathological studies.

Co-transmission in histaminergic, orexinergic, and MCH-producing neurons increases the computational capabilities of these small neuronal populations (less than 300,000 out of 88 billion neurons[133]) by promoting postsynaptic synergistic excitation or inhibition, regulating negative or positive feedback, and modulating temporal responses. Co-transmission with fast-acting neurotransmitters may be a key to fine tune sleep-wake transitions and homeostasis, following the circadian rhythms and environmental cues. Thus, it is imperative to acknowledge the heterogeneous chemical identities of these neurons and investigate the consequences of the co-neurotransmitter imbalance. Co-transmission increases neuronal

flexibility and enables more specific neuron targeting. This review focused on the hypothalamus and sleep-wake regulation. However, co-transmission has also been described in other sleep-wake regulating regions, such as the LC (norepinephrine, dopamine, NPY, galanin) and the RN (serotonin, glutamate)[134-139], and in regulating other physiological functions, such as appetite, addiction, mood, and metabolism. Understanding how these co-transmitters interact to drive sleep and wakefulness in animal models and human subjects has immense implications for the next generation of treatments for sleep dysfunction and may explain the differential vulnerability of similar neuronal populations to neurodegenerative conditions[140].

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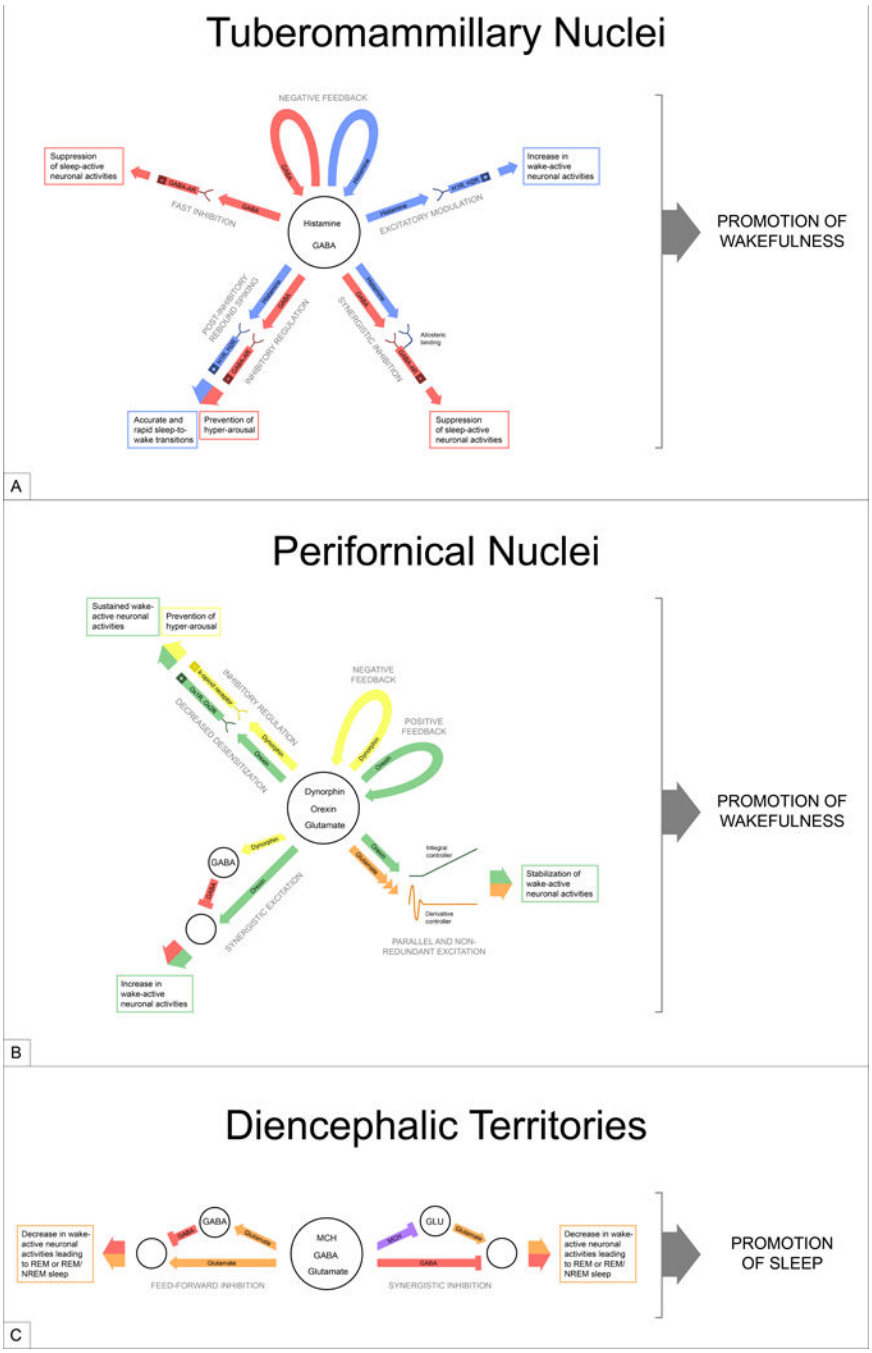

Figure 1. Possible mechanisms governing sleep and wakefulness via co-transmission.

(a) While TMN neurons are mainly known as histaminergic, they also synthesize and release GABA, which may segregate into different presynaptic terminals than histamine and act independently on distinct postsynaptic neurons. GABA may also work in opposition or synergy with histamine when released. **(b)** In addition to orexin, PFN neurons release inhibitory neuropeptides, dynorphin, and the fast-neurotransmitter, glutamate. The interaction between these neurotransmitters may allow PFN neurons to promote optimal wakefulness. **(c)** MCH neurons produce many neurotransmitters, including GABA and

glutamate. Although postsynaptic responses to fast-neurotransmitters are readily observable by photo-stimulation, this may not be the case for MCH and other co-neuropeptides. In all the three hypothalamic neuron populations **(a-c)**, the common motif is that the neurons synthesize “opposing” neurotransmitters can modulate postsynaptic neurons bi-directionally. In small neuron populations, this may allow target-specific neuronal influence and flexibility to regulate sleep and wakefulness. This diagram depicts only a few possible mechanisms. Abbreviations: H1R, H2R: histamine receptor-1, histamine-receptor-2; GABA-AR: GABA-A receptor; Ox1R, Ox2R: orexin-1 receptor, orexin-2 receptor; MCH: melanin-concentrating hormone; GLU: glutamate