BEHAVIORS AND NEURAL CIRCUITS IN SLEEP AND SEDATION

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BEHAVIORS AND NEURAL CIRCUITS IN SLEEP AND SEDATION

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Table of Contents

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EDITORIAL
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Editorial: Behaviors and Neural Circuits in Sleep and Sedation

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Editorial on the Research Topic

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INTRODUCTION

The function of sleep is an enduring mystery. Research over the last few years has attempted to unravel its complexity in the mammalian brain and determine the molecular and neuronal underpinnings of sleep state transitions.

This topic puts together new research that improves our understanding of the neural circuitry of sleep and its associated behaviors. Our long-term view is that understanding circuitry will drive our understanding of why we spend one third of our lives unconscious and vulnerable; unable to perform crucial biological functions from eating to hunting, foraging and reproduction. We seek to understand why such a state could be so vital as to be observed in all complex life.

CONTRIBUTIONS OF ARTICLES IN THIS SERIES

The gating of sleep requires the integration of permissive signals from the environment, such as satiety status and ambient temperature, but how these are assimilated is unclear (Harding et al., 2019). Guillaumin and Burdakov have considered the role of neuromodulation in slow local microcircuits, across the hypothalamus, with a particular focus on peptide neuromodulators. As these peptides have a persistent extracellular presence, they are uniquely placed to facilitate the integration of these context-dependent permissive signals. This is an appealing hypothesis as a distinct feature of the hypothalamus is the diversity and abundance of neurons using neuropeptides in transmission (de Lecea et al., 1998; Svensson et al., 2019). Understanding the functions of these peptides may help us explain, for example, why galanin expressing neurons in the preoptic area appear to have a role in both stereotyped parental behavior, such as pup grooming, as well as the induction of non-rapid eye movement sleep (NREM) (Kohl et al., 2018; Kroeger et al., 2018; Ma et al., 2019; Reichert et al., 2019).

Disruption of neuropeptide transmission is most well understood in the case of the peptide orexin and the loss of orexin producing neurons *in vivo* results in narcolepsy. This peptide can also induce action potentials in the absence of neurotransmitter co-release, suggesting functions beyond neuromodulation (Schöne et al., 2014; Mahoney et al., 2019). Coffey et al., specifically considered the role of sex and age on the severity of narcolepsy in a doxycycline-inducible model of mouse

narcolepsy, revealing interesting interactions with sex. We think this underlies the importance of considering these variables further in sleep research. The authors also propose that children with narcolepsy may suffer greater loss of orexin neurons than adults thus explaining the presence of more severe symptoms.

One of the most familiar aspects of natural sleep is that of waking-up and experiencing a lingering drowsiness, or "sleep-inertia." Luppi et al., considered how emergence from anesthesia, at a lower dose than required for induction (e.g., "neural-inertia"), has similarities to sleep-inertia. The authors have proposed a new model of orexin and noradrenergic circuitry to mediate this process and help explain why elderly and narcoleptic patients are more susceptible to neural inertia following anesthesia (Scammell, 2003; Kelz et al., 2008; Silva and Duffy, 2008). Similarly, Wang L et al. have shown that activation of basal forebrain cholinergic neurons blunts normal sensitivity to propofol and shortens recovery from anesthesia. These neurons also induce wake-like signals in the medial prefrontal cortex.

Within the hypothalamus, specific populations are now well associated to sleep control and the gating of environmental cues. Harding et al. considered the contribution of a specific subset of preoptic neurons, that express nitric oxide synthase, to normal sleep and thermoregulatory cycles. Many NOS1 neurons are NREM active and, when synaptic transmission is blocked, bi-directional changes in sleep-wake propensity occur across the light-cycle, alongside a shift to slightly warmer core temperatures. In agreement with previous work, these neurons appear to have a role in gating sleep in relation to thermoregulatory responses to ambient temperature (Harding et al., 2018). The role of nitric oxide (NO), however, remains unclear. NO is a gaseous and transient neuromodulator, with diffusion distances of up to a few tens of microns. As such, NO may influence glutamatergic transmission through cGMP mediated changes in excitability or indirectly via local vascular smooth muscle, supporting vasodilation (Förstermann and Sessa, 2011).

Reitz and Kelz, have asked us to carefully consider the "shared circuitry hypothesis"; the idea that sleep circuits are hijacked by the actions of general anesthetics. They detail the challenges of attributing anesthetic action to a single neuronal population, given the ability of some preoptic neurons to induce wakefulness, as well as our newfound understanding of exceptional cellular heterogeneity in the preoptic region (Moffitt et al., 2018; Vanini et al., 2020).

Cheng et al. have carried out extensive pharmacological assessment of the flip-flop hypothesis (Saper et al., 2010). Consistent with this model, they show that VLPO neurons can be driven to induce NREM by targeted injection of L-glutamate, while this action can be blocked by injecting bicuculline into the TMN. Conversely, L-glutamate injection into the TMN during lights-ON induced wakefulness that could be blocked by triprolidine injection in the VLPO.

Finally, we are beginning to understand how sleep-deprivation and sleep-rebound are perceived and encoded in the brain Wang H. et al. recorded an impairment of fine motor control alongside impaired functional connectivity, observed in fMRI, following sleep deprivation in healthy adult men In contrast to the human experience, Xu et al. found that sleep-deprivation at the circuit level in rats, by direct activation of the medial parabrachial nucleus (MPB), does not always result in sleep-rebound. This has parallels to the lesioning of ventral tegmental area (VTA) *Vgat*-Cre neurons in mice, that also do not exhibit rebound sleep following sleep deprivation, showing clearly that these intrinsic properties of sleep can be decoupled (Yu et al., 2021). Furthermore, if only certain wake-active populations can induce sleep-rebound, are the waking-behaviors linked to these neurons also more important for the function of sleep?

CONCLUSIONS AND PERSPECTIVE

This series has emphasized that molecular heterogeneity is an on-going challenge for understanding sleep circuitry. Common mouse lines expressing Cre recombinase (e.g., *Vgat*-Cre, *Vglut2*-cre) facilitate access to smaller, but still highly diverse group of neurons, complicating our interpretation (Moffitt et al., 2018). We should also carefully consider the role of neuropeptides, as well as potential neuromodulators such as nitric oxide, that may not be functioning within the normal synaptic cascade. These non-canonical pathways may underly the integration of permissive conditions to sleep such as warmth-seeking and satiety (Goldstein et al., 2018; Harding et al., 2018; Komagata et al., 2019).

Finally, general anesthetics and sedatives share many features with sleep but not all anesthetics use the same circuitry. This allows for the exciting possibility that some compounds may induce more natural sleep than others and further development may move us closer to a true sleep-inducing agent (Franks and Wisden, 2021).

AUTHOR CONTRIBUTIONS

EH wrote the manuscript with assistance from XY. XY initiated this article collection. ZZ and HD provided feedback on the manuscript. All authors were topic editors for this article series.

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The Interaction Between the Ventrolateral Preoptic Nucleus and the Tuberomammillary Nucleus in Regulating the Sleep-Wakefulness Cycle

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The ventrolateral preoptic nucleus (VLPO) in the anterior hypothalamus and the tuberomammillary nucleus (TMN) in the posterior hypothalamus are critical regions which involve the regulation of sleep-wakefulness flip-flop in the central nervous system. Most of the VLPO neurons are sleep-promoting neurons, which co-express γ-aminobutyric acid (GABA) and galanin, while TMN neurons express histamine (HA), a key wake-promoting neurotransmitter. Previous studies have shown that the two regions are innervated between each other, but how to regulate the sleep-wake cycle are not yet clear. Here, bicuculline (Bic), a $GABA_A$-receptor antagonist, L-glutamate (L-Glu), an excitatory neurotransmitter, and triprolidine (Trip), a HA_1 receptor (HRH_1) inhibitor, were bilaterally microinjected into TMN or VLPO after surgically implanting the electroencephalogram (EEG) and electromyography (EMG) electrode recording system. Microinjecting L-Glu into VLPO during the night significantly increased the NREM sleep time, and this phenomenon was weakened after selectively blocking $GABA_A$ receptors with Bic microinjected into TMN. Those results reveal that VLPO neurons activated, which may inhibit TMN neurons inducing sleep via $GABA_A$ receptors. On the contrary, exciting TMN neurons by L-Glu during the day, the wakefulness time was significantly increased. These phenomena were reversed by blocking HRH_1 with Trip microinjected into VLPO. Those results reveal that TMN neuron activating may manipulate VLPO neurons via HRH_1, and induce wakefulness. In conclusion, VLPO GABAergic neurons and TMN histaminergic neurons may interact with each other in regulating the sleep-wake cycle.

Keywords: VLPO, TMN, L-glutamate, bicuculline, $GABA_A$-receptor, HRH_1, sleep-wake circuitry

INTRODUCTION

The sleep-wake cycle is controlled by homeostasis and circadian rhythm, which regulates the amount, and the time of sleep, respectively (Borbely, 1982). The inhibitory relationship between sleep and wakefulness systems work as a trigger for the rapid conversion of sleep and wakefulness in the form of a positive feedback-loop (Wang et al., 2013). It is believed that GABAergic neurons in the ventral lateral hypothalamus (VLPO) and central preoptic region are the basis for the occurrence and maintenance of sleep (Sherin et al., 1996; Saper et al., 2010). Extracellularly electrophysiological recording results show that VLPO neurons have more activation during sleep, and the firing rate significantly increased during paradoxical sleep (Koyama and Hayaishi, 1994). Chemoactivating and photoactivating galanin-expressing neurons promoted total sleep time (TST), while photoinhibiting galanin-expressing neurons decreased NREM sleep (Kroeger et al., 2018). Lesions of the VLPO reduced sleep time and caused insomnia in cats and rats (Nauta, 1946; McGinty and Sterman, 1968; Lu et al., 2000). More than 85% of the neurons in the VLPO region are GABAergic neurons, which co-express the inhibitory neurotransmitters GABA and galanin (Sherin et al., 1996, 1998). VLPO neurons send axons to many regions that are implicated in the regulation of wakefulness, including the locus coeruleus (LC), median raphe nuclei, and the tuberomammillary nucleus (TMN) (Saper et al., 2010; Chung et al., 2017).

In the brain, the histaminergic (HAergic) neurons only gathered in the TMN. During the sleep-wake cycle, compared to non-rapid eye movement (NREM) sleep, the firing rate significantly increased during the awakening period, while they were almost silenced during rapid eye movement (REM) sleep (Sakai et al., 2010). TMN projects to almost the whole brain, and extraordinarily has highly dense innervation to the VLPO, the basal forebrain and the amygdala (Brown et al., 2001). Microinjection HA to VLPO can significant increase the locomotor activity of rats, also the electrophysiological experiments showed that HAs can inhibit the activity of VLPO neurons, the membrane potential hyperpolarized and firing rate were significantly decreased (Liu et al., 2010; Cheng et al., 2018).

Therefore, we predicted that the TMN and VLPO might inhibit each other, by which VLPO neurons release galanin and/or GABA at its terminal in TMN, and TMN neurons release HA and/or GABA in VLPO to maintain the balance of the sleep-wakefulness system. Here, we focus on the innervation of the two regions and the role of the neurotransmitters in the transition and maintenance of the sleep-wake cycle rhythm. In order to further explore and interpret the mechanisms in regulating sleep between VLPO and TMN, we inject cannula excitatory neurotransmitters L-glutamate (L-Glu), and $GABA_A$ receptors antagonist bicuculline (Bic), and HRH_1 antagonist triprolidine (Trip) in either VLPO or TMN, and *in vivo* recording by EEG and EMG observed the variations in rat sleep-wakefulness cycles. We found that the NREM sleep time was significantly increased after L-Glu was injected into VLPO, and this phenomenon was weakened after selectively blocking $GABA_A$ receptors in TMN. Furthermore, both REM and NREM sleep time decreased after the excited TMN neurons, and these phenomena were reversed by blocked HRH_1 in VLPO. Those results indicated that TMN histaminergic neurons and VLPO GABAergic neurons may interact with each other in regulating the sleep-wake cycle.

RESULTS

Activating VLPO Neurons Decreased Wakefulness and Increased NREM in Rats

A larger number of sleep-promoting neurons in VLPO were identified and innervated with the wake promoting system, and released inhibitory neurotransmitters (GABA) at its terminal, including TMN (Sherin et al., 1996, 1998). In order to investigate the effect of VLPO on the sleep-wake cycle, we microinjected aCSF (1 μl) into VLPO and TMN in a part of rats at 10:00–10:20 as the night control group, and microinjected L-Glu (L-Glu, 5 mmol/L with 1 μl) into VLPO and aCSF into TMN in another part of rats. Compared with the night control group (TMN + aCSF and VLPO + aCSF), the wakefulness time at the 2nd and 3rd h, after L-Glu was injected into VLPO, decreased about 54.0% ($p < 0.01$) and 37.6% ($p < 0.05$), respectively (**Figure 1B** top). The REM sleep time on the 2nd and the 3rd h after L-Glu was injected into VLPO increased about 73.1% and 37.6%, respectively (**Figure 1B** middle). However, the difference was not significant in REM sleep time. The NREM sleep time at the 2nd and 3rd h after L-Glu was injected into VLPO increased about 121.3% ($p < 0.01$) and 75.3% ($p < 0.01$), respectively (**Figure 1B** under).

The cumulative amount of wakefulness, REM sleep, NREM sleep, and TST in the next 5 h after injected L-Glu into VLPO were calculated. Compared with the night control group, the amount time of NREM sleep and TST after L-Glu was injected into VLPO increased about 56.4% (112.61 min ± 7.37 vs. 71.99 min ± 6.34, $p < 0.01$) and 49.5% (136.29 min ± 8.16 vs. 91.17 min ± 7.87, $p < 0.01$), meanwhile, the wakefulness time decreased by about 21.6% (163.71 min ± 8.17 vs. 208.83 min ± 7.89, $p < 0.01$), respectively (**Figure 2**). Those results indicate that excited VLPO neurons can decrease wakefulness and increase NREM at night in rats.

Activating VLPO Neurons and Blocking $GABA_A$ Receptors in TMN Significantly Affected the Sleep-Wakefulness Cycle in Rats

We designed to excite VLPO by L-Glu and inhibit TMN by Bic (0.1 mmol/L with 1 μl) in a rat, and analyzed the sleep-wakefulness cycle variation of rats. Compared with the night group of VLPO + L-Glu and TMN + aCSF, the wakefulness time at the 2nd and 3rd h after Bic injected into TMN was increased about 94.7% ($p < 0.01$) and 81.5%

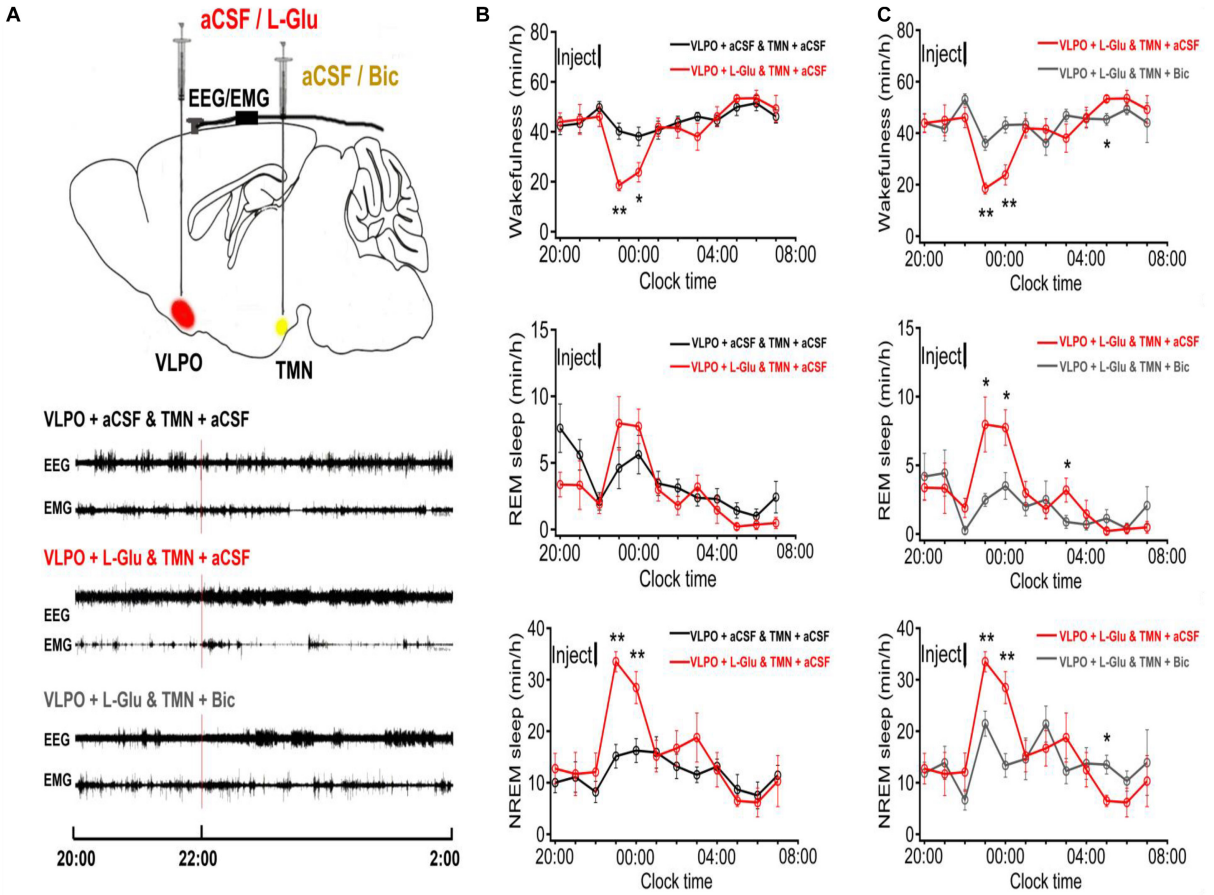

FIGURE 1 | The sleep-wakefulness cycle was influenced by injecting L-Glu into VLPO which could be blocked by injecting Bic into TMN. **(A) Top:** Schematic drawing of the location of drug injection, or aCSF into the VLPO/TMN of a rat (Red: aCSF/L-Glu into VLPO, Yellow: aCSF/Bic into TMN). **Bottom:** Representative traces of EEG and EMG with drug injected. Drugs were applied at 22:00 (red line), and PSG recording started from 20:00 and lasted for 24 h. **(B)** Time-course changes of the sleep-wakefulness cycle in rat administration with L-Glu into VLPO. **(C)** Time-course changes of the sleep-wakefulness cycle in rat administration with L-Glu into VLPO and Bic into TMN. Data were presented as mean ± *SEM*. The paired *t*-test was used in the statistical comparisons of two groups. $^{*}p < 0.05$, $^{**}p < 0.01$ is compared between two groups at the same time. The arrow shows the time of microinjection.

($p < 0.01$), respectively (**Figure 1C** top). The REM sleep time at the 2nd and 3rd h after Bic was injected into TMN was reduced by 69.0% ($p < 0.05$) and 54.8% ($p < 0.05$), respectively (**Figure 1C** middle). The NREM sleep time at the 2nd and 3rd h after Bic was injected into TMN was decreased about 36.0% ($p < 0.01$) and 53.1% ($p < 0.01$), respectively (**Figure 1C** under).

The 5 h cumulative amounts of wakefulness, REM and NREM sleep times were calculated. Compared with the night group of VLPO + L-Glu and TMN + aCSF, the amount of wakefulness after Bic was injected into TMN increased about 25.6% (163.71 min ± 8.17 vs. 205.56 min ± 10.12, $p < 0.01$). Meanwhile the amount of REM sleep, NREM sleep and TST after Bic was injected into TMN decreased about 52.1% (23.67 min ± 2.78 vs. 11.33 min ± 2.78, $P < 0.01$), 26.2% (112.61 min ± 7.37 vs. 85.09 min ± 8.09, $p < 0.05$), and 30.7% (136.29 min ± 8.16 vs. 94.42 min ± 10.12, $p < 0.01$), respectively (**Figure 2**). Those results indicate that the effect of L-Glu exciting VLPO neurons on sleep-wakefulness at night can be inhibited by blocking $GABA_A$ receptors in TMN in rats.

Excited TMN Neurons Increased Wakefulness Time and Decreased REM and NREM Sleep Time in Rats

In order to figure out whether the excited TMN neurons would reduce the sleep time and increase the wakefulness time, we stereotaxically implanted cannulas and microinjected excitatory neurotransmitters L-Glu to TMN at 10:00–10:20. The representative traces of EEG and EMG of the 4 day groups after either injection of aCSF or drugs are shown in **Figure 3B**. Microinjecting L-Glu (5 mmol/L with 1 μl) into TMN increased the wakefulness time of rats at the 2nd h and lasted 3 h (**Figure 4B**). Compared with the day group of VLPO + aCSF and TMN + aCSF group, the wakefulness time

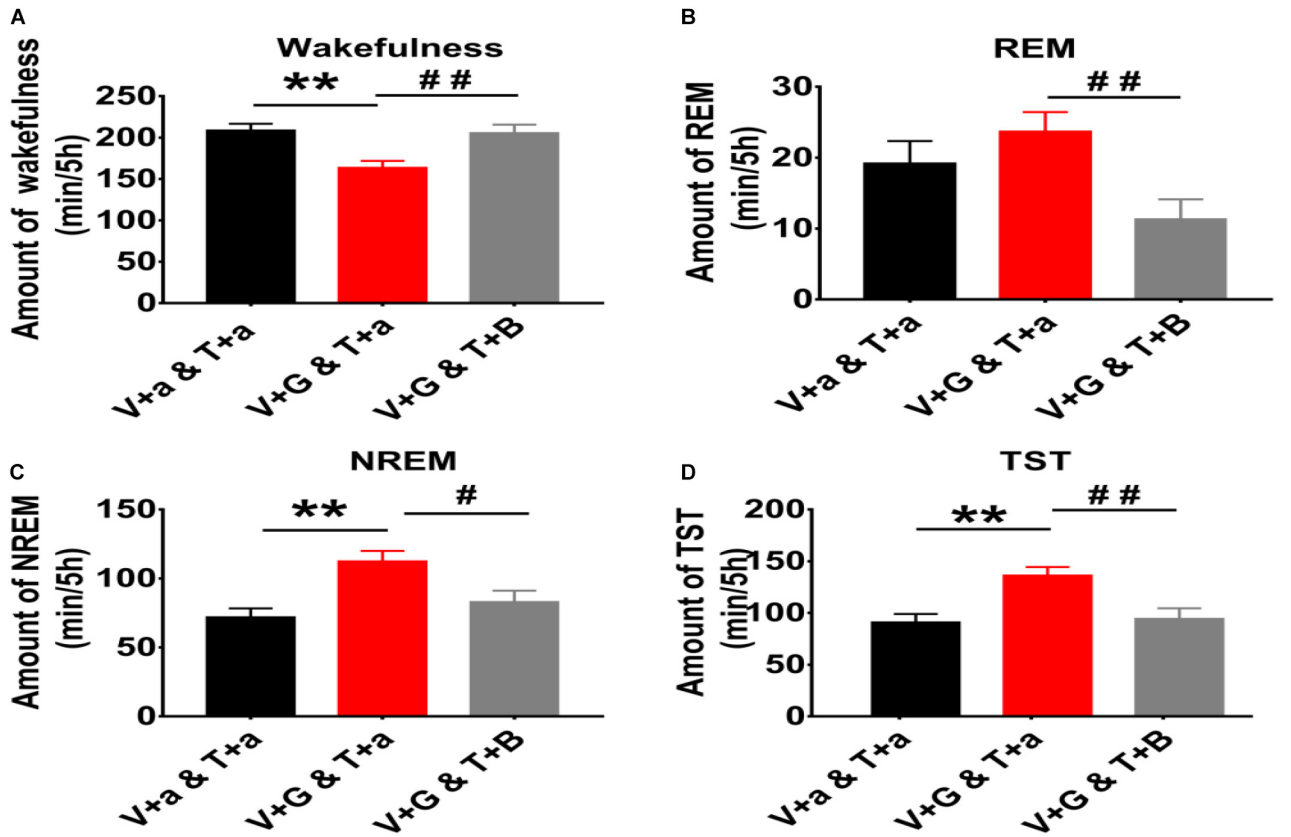

FIGURE 2 | The 5 h cumulative amount of wakefulness, REM and NREM sleep, and TST after administration of aCSF or drugs into the TMN and VLPO of rats. **(A)** The 5 h cumulative amount of wakefulness. **(B)** The 5 h cumulative amount of REM sleep. **(C)** The 5 h cumulative amount of NREM sleep. **(D)** The 5 h cumulative amount of TST. Data were presented as mean ± *SEM*. The paired *t*-test was used in the statistical comparisons of two groups. Compared with the night group of VLPO + aCSF and TMN + aCSF group, $^{**}p < 0.01$. Compared with the night group of VLPO + L-Glu and TMN + aCSF group, $^{\#}p < 0.05$, $^{\#\#}p < 0.01$. V, VLPO; T, TMN; G, L-Glu; B, Bic; a, aCSF.

after L-Glu was injected into TMN at the 2nd, 3rd, and 4th h was increased about 132.2% ($p < 0.01$), 54.3% ($p < 0.01$), and 64.6% ($p < 0.01$), respectively (**Figure 4B** upper). The REM sleep time after L-Glu was injected into TMN at the 2nd, 3rd, and 4th h decreased about 85.9% ($p < 0.05$), 55.9% and 60.6%, respectively. However, there were no significant differences after L-Glu was injected into TMN at the 3rd and 4th h (**Figure 4B** middle). The NREM sleep time after L-Glu was injected into TMN at the 2nd, 3rd, and 4th h was reduced to about 50.5% ($p < 0.01$), 40.6% ($p < 0.01$), and 34.5% ($p < 0.05$), respectively (**Figure 4B** under).

Compared with the day group of VLPO + aCSF and TMN + aCSF group, the 5 h cumulative amount of wakefulness time after L-Glu was injected into TMN was increased about 35.0% (113.35 min ± 4.32 vs. 179.00 min ± 13.91, $p < 0.01$). Meanwhile, the 5 h cumulative amount of REM sleep time, NREM sleep time and TST decreased about 55.5% (14.57 min ± 3.79 vs. 6.48 min ± 2.68, $p > 0.05$), 33.4% (172.05 min ± 3.57 vs. 114.51 min ± 13.07, $p < 0.01$) and 35.2% (186.63 min ± 4.32 vs. 120.99 min ± 13.91), respectively (**Figure 3**). Those results indicate that L-Glu excited TMN neurons which increased wakefulness and decreased NREM during the day.

Activating TMN Neurons and Blocking HRH_1 Receptors in VLPO Significantly Affected the Sleep-Wakefulness Cycle in Rats

It has been proven that the histaminergic neurons of TMN can project to the VLPO (Sakai et al., 2010; Chung et al., 2017). Here, we microinjected triprolidine (Trip, 0.5 μmol/L with 1 μl), a HRH_1 blocker, into VLPO to block the histaminergic afference from TMN. Compared with the day group of VLPO + aCSF and TMN + L-Glu, the wakefulness time at the 2nd, 3rd, and 4th h after Trip was injected into VLPO was reduced by about 54.9% ($p < 0.01$), 32.1% ($p < 0.01$), and 26.1% ($p < 0.05$), respectively (**Figure 4C** upper). The REM sleep time at the 2nd, 3rd, and 4th h after Trip was injected into VLPO increased about 1175.4% ($p < 0.01$), 196.5% ($p > 0.05$), and 89.7% ($p > 0.05$), respectively (**Figure 4C** middle). The NREM sleep time at the 2nd, 3rd, and 4th h after Trip was injected into VLPO increased by about 87.5% ($p < 0.01$), 58.8% ($p < 0.05$), and 36.0%($p > 0.05$), respectively (**Figure 4C** under).

Compared with the day group of VLPO + aCSF and TMN + L-Glu group, the 5 h cumulative amount of wakefulness time after Trip was injected into VLPO was decreased by 30.2%

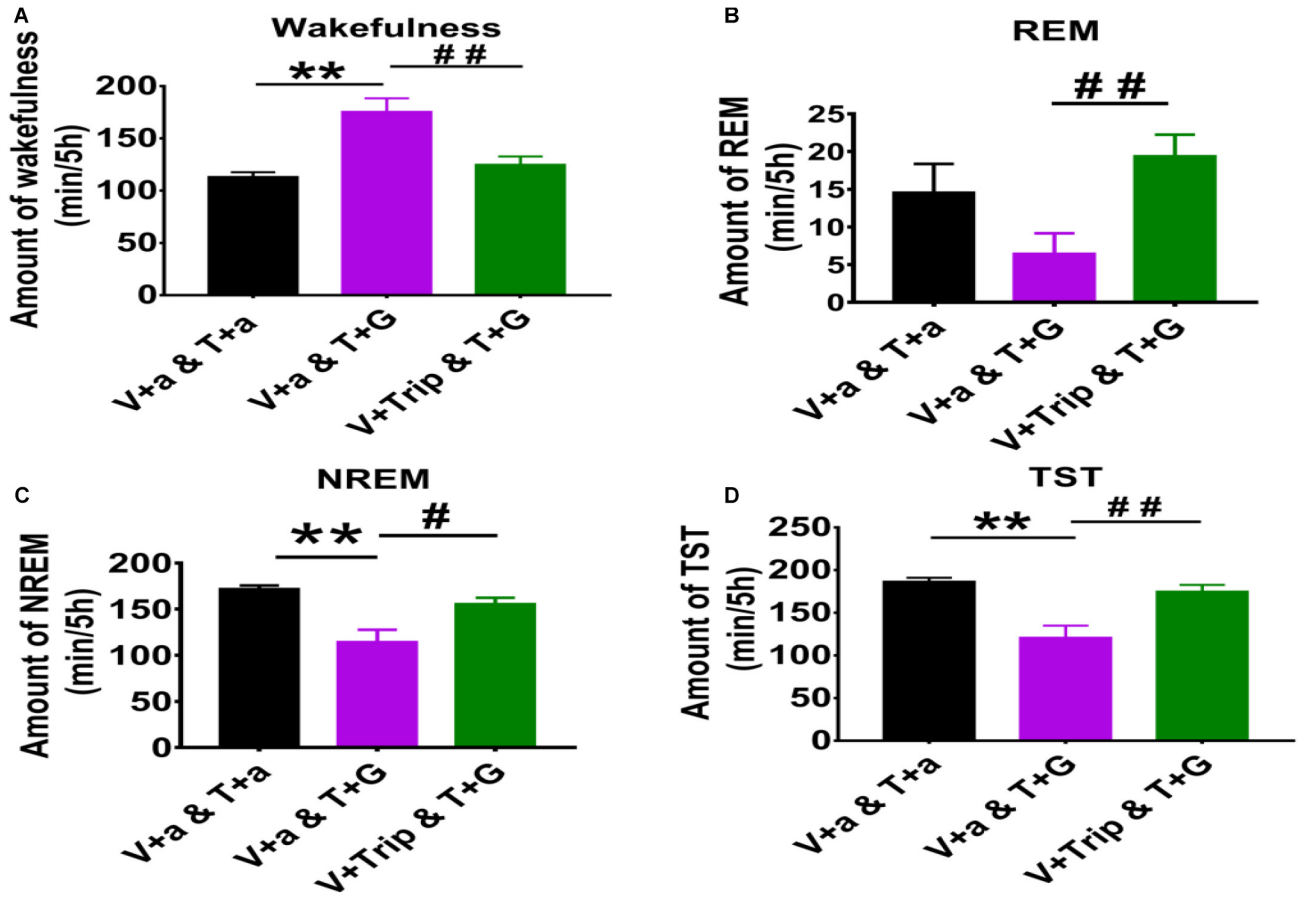

FIGURE 3 | The 5 h cumulative amounts of wakefulness, REM and NREM sleep, and TST after administration of aCSF or drugs into the TMN and VPO of rats. **(A)** The 5 h cumulative amount of wakefulness. **(B)** The 5 h cumulative amount of REM sleep. **(C)** The 5 h cumulative amount of NREM sleep. **(D)** The 5 h cumulative amount of TST. Data were presented as mean ± *SEM*. The paired *t*-test was used in the statistical comparisons of two groups. Compared with the day group of VLPO + aCSF and TMN + aCSF, $^{**}p < 0.01$; Compared with the day group of VLPO + aCSF and TMN + L-Glu, $^{\#}p < 0.05$, $^{\#\#}p < 0.01$. V, VLPO; T, TMN; G, L-Glu; B, Bic; a, aCSF.

(179.00 min ± 13.91 vs. 125.03 min ± 7.68, $p < 0.01$), meanwhile, the 5 h cumulative amount of REM sleep, NREM sleep, and TST were increased about 199.3% (6.48 min ± 2.68 vs. 19.40 min ± 2.85, $p < 0.01$), 35.9% (114.51 min ± 13.07 vs. 155.57 min ± 6.80, $p < 0.05$), and 44.6% (120.99 min ± 13.91 vs. 174.97 min ± 7.66, $p < 0.01$), respectively (**Figure 3**). Those results indicate that the effect of exciting TMN neurons on sleep-wakefulness at day can be inhibited by blocking HRH_1 receptors in VLPO in rats.

DISCUSSION

The sleep-promoting system is mainly composed of the preoptic area and adjacent basal forebrain. Sleep deprivation might induce the brain and cognitive function disorder, such as learning and memory. Recently, studies have shown that light-induced SWS (slow wave sleep) might have strongly enhanced the process in memory consolidation (Lu et al., 2018). Sleep inducing neurons in the preoptic area are mainly located in the VLPO, as many *c-fos*-positive neurons found after sleep to have recovered following sleep deprivation (Zhang et al., 2015). Neurons in the VLPO synthesized and released the inhibitory neurotransmitter GABA, and their fibers terminal can innervation to 5-HT neurons in dorsal raphe nucleus, blue-spot norepinephrine neurons, cholinergic neurons in lateral tegmental nucleus, and histaminergic neurons in TMN. The inhibitory effect of the sleep-promoting neurons innervated from VLPO can reduce the sleep-promoting effects of HA neurons and other key arousal regions, inducing and maintaining sleep (Sherin et al., 1996; Saper et al., 2010; Chung et al., 2017; Scammell et al., 2017).

For the arousal system, polysomnography, pharmacological, and tracing studies showed that different functional cell groups with different terminal projections might modulate the sleep-wake switch though various signaling pathways, such as histaminergic, glutamatergic, orexinergic, GABAergic pathways, and so on (Liu et al., 2010; Eban-Rothschild and de Lecea, 2017; Scammell et al., 2017; Schöne and Burdakov, 2017). Among them, TMN is an area where histaminergic neurons are concentrated in the cell body of the central nervous system, and can project the

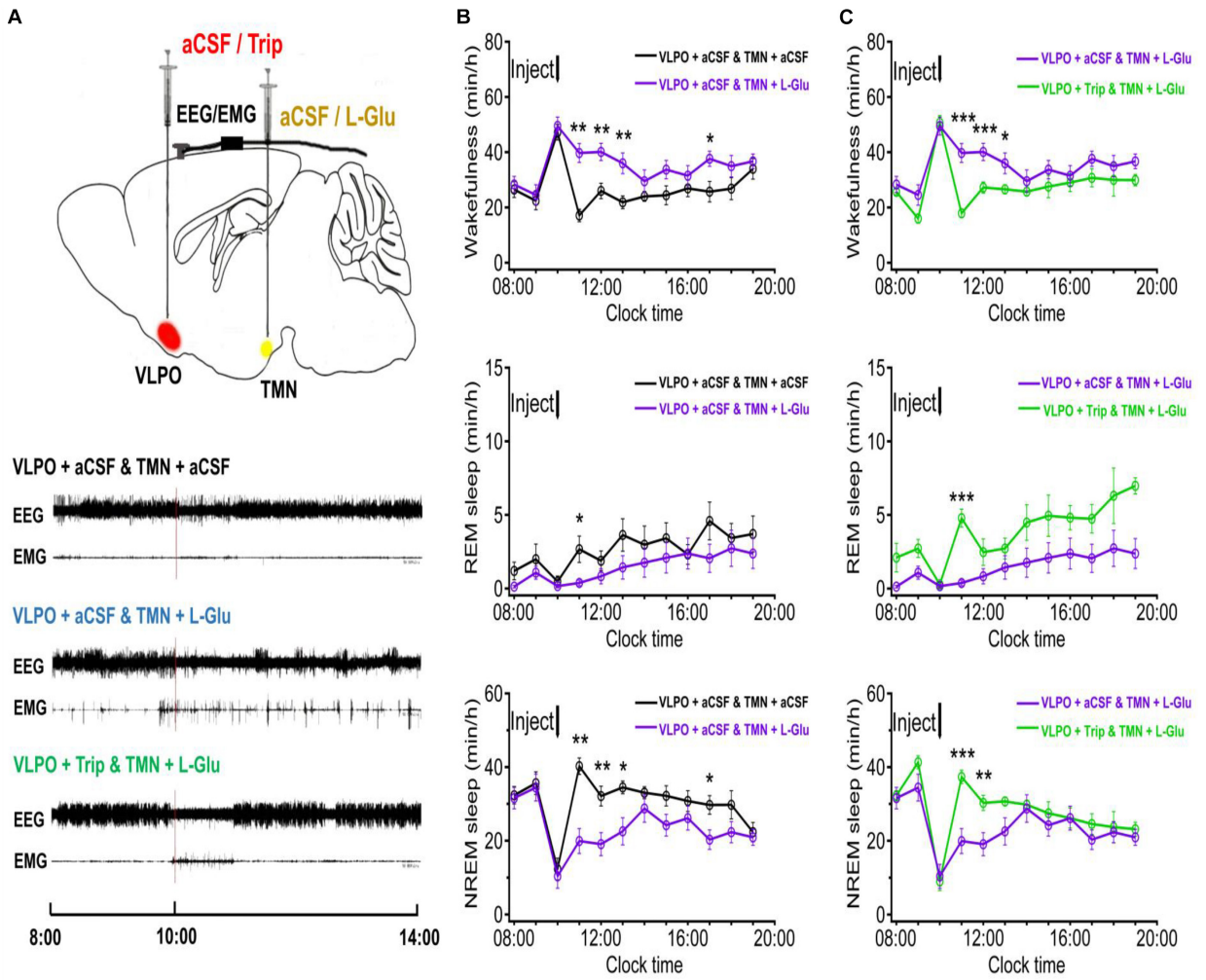

FIGURE 4 | The sleep-wakefulness cycle was influenced by injecting L-Glu into TMN which could be blocked by injecting Trip into VLPO. **(A)** Top: Schematic drawing of the location of drug injection, or aCSF into the VLPO/TMN of a rat (Red: aCSF/Trip into VLPO, Yellow: aCSF/L-Glu into TMN). Bottom: Representative traces of EEG and EMG with drug injected. Drugs were applied at 10:00 (red line), and polysomnography recording started from 08:00 and lasted for 24 h. **(B)** Time-course changes of the sleep-wakefulness cycle in rat administration with L-Glu into TMN. **(C)** Time-course changes of the sleep-wakefulness cycle in rat administration with L-Glu into TMN and Trip into VLPO. Data were presented as mean ± *SEM*. The paired *t*-test was used in the statistical comparisons of two groups. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ is compared between two groups at the same time. The arrow shows the time point of microinjection.

orexinergic neurons on the lateral hypothalamus and the basal forebrain (Eriksson et al., 2001; Schöne and Burdakov, 2017).

As an excitatory neurotransmitter, L-Glu can selectively excite neurons via N-methyl -D-aspartate (NMDA) receptors, which play important roles in synaptic plasticity, synaptic transmission and neuron degeneration (Swaminathan et al., 2019). The administration of kainite, a type of L-Glu receptor antagonist, into the nucleus ceruleus in the rat can induce a significant increase in REM sleep, however, damage to the preoptic area with kainite in the rat can decrease sleep and increase wakefulness (John et al., 1994; Onoe and Sakai, 1995; Vataev et al., 2013). In our study, we found that microinjecting L-Glu into VLPO can inhibit wakefulness and increase NREM sleep, but has no significant effect on REM sleep. Therefore, this result infers that the injection L-Glu in VLPO might excite sleep-promoting neurons and maintain NREM sleep.

We found the microinjection of Bic (Ramshini et al., 2019), a $GABA_A$ receptor specific antagonist, into TMN has no significant effect on the sleep-wakefulness cycle. Bic can directly work on histaminergic neurons to increase its firing rate (Haas et al., 2008), and the perfusion of Bic into the hypothalamus can increase the expression of HAs in the nucleus accumbens and the prefrontal cortex (Cenni et al., 2006; Giannoni et al., 2009). Thus, we microinjected Bic to TMN in the early night. During the wakefulness state, the release of inhibitory neurotransmitters at the terminal projection of VLPO were decreased, while histaminergic neurons in TMN are particularly activating, and the firing of histaminergic neurons is significantly higher than

that during the sleep phase (Zeitzer et al., 2012; Valko et al., 2013), with up-regulation of *c-fos*-protein expression (Ko et al., 2003).

In this study, L-Glu microinjected into VLPO during the night significantly increased the NREM sleep time and decreased the wakefulness time. Those phenomena were weakened after selectively blocking $GABA_A$ receptors by Bic microinjected into TMN. The phenomena were consistent with the previous results of injecting $GABA_A$ receptor blockers into TMN and adjacent areas, which can block the sleep-inducing effects caused by central sedatives and anesthetics, such as pentobarbital, muscarine, and propofol (Nelson et al., 2002). Thus, it may be deduced that activated VLPO neurons can induce sleep, which may mainly inhibit TMN neurons via $GABA_A$ receptors.

Bilateral microinjection HA into the basal forebrain of rats showed that dose-dependent wakefulness was increased and accompanied by the decreasing of NREM sleep, suggesting that HAs might induce wakefulness by relayed cholinergic neurons in the basal forebrain (Ramesh et al., 2004). Microinjection HAs into the VLPO can increase the activity rate of rats, and the electrophysiological experiment of isolated brain slices showed that HAs can inhibit the activity of VLPO neurons by over-polarizing the membrane potential (Liu et al., 2010). In this study, microinjection of L-Glu into TMN at day results in increasing wakefulness time and decreasing sleep time in rats, which was weakened when Trip, a HRH_1 blocker, was injected into VLPO at the same time. These results reveal that activated TMN neurons might manipulate VLPO neurons via HRH_1, and induce wakefulness.

In conclusion, our results indicate that activation of VLPO by L-Glu can promote sleep and weaken the transition to wakefulness. Moreover, inactivation of TMN by $GABA_A$ antagonist can turnover those phenomena. On the contrary, exciting TMN neurons by glutamate receptor antagonist can promote wakefulness and weaken the transition to sleep, and, these phenomena can be reversed by blocking HRH_1 with Trip microinjected into VLPO. This relationship between TMN arousal and VLPO sleep-promoting pathways may produce the conditions for a flip-flop switch, which can generate rapid and complete transitions between waking and sleeping states, but certain types of neurons in VLPO and TMN participate and modulate the transition between REM, NREM, and wakefulness need to be further studied.

MATERIALS AND METHODS

Animal Model

Adult male Sprague Dawley rats (SPF grade) weighing 270–290 g were used. All rats were housed in a free moving environment kept at room temperature (22–24°C), with the humidity maintained at 55% and 12 h of light/dark (light on 8:00–20:00 h, illumination intensity ≈ 100 lx). The sound insulation shielding and ventilated environment was kept separately, free to water and feeding. The animals in the experiment were kept strictly in accordance with the regulations of the People's Republic of China on the management of experimental animals and the methods for quality management of experimental animals.

Surgery and Implantations for *in vivo* Polysomnographic Recording

After anesthetized by pentobarbital (50 mg/kg, i.p.), EEG and EMG electrodes were implanted for polysomnographic recording (MP150, Data acquisition and analysis system, Biopac Ltd., United States), and two guide cannulas were bilaterally inserted into VLPO (AP: −0.36 mm; R: 1.00 mm; H: −7.50 mm) and TMN (AP: −4.20 mm; R: 1.10 mm; H: −7.70 mm) for drug application in rats. The microinjection cannulas for drugs were embedded at 2 mm above the VLPO or TMN regions in the brain. The recording electrodes for EEG recording were embedded at 1 mm in front of the coronal suture and herringbone stitch before 1 mm and side open 1 mm node installed on both sides of the midline skull, and recording electrodes for EMG were inserted into the bilateral neck muscle. Guide cannulas and recording electrodes were fixed to the skull surface with dental cement. Each animal needed 7 days for recovery in a sound proof recording room after surgery, then they were connected to an EEG/EMG recording cable and habituated for 3 days before polysomnographic recording.

Grouping

Rats were randomly divided into day and night groups: the night group was composed of (1) VLPO + aCSF (artificial cerebrospinal fluid) and TMN + aCSF group (both TMN and VLPO are microinjected with aCSF (Cheng et al., 2018) containing (mM): 125 NaCl, 1.25 KCl, 25 $NaHCO_3$, 1.25 KH_2PO_4, 25 D-Glucose, 2 $CaCl_2$, 1 $MgCl_2$, supplemented with 400 Na-pyruvate and 80 L-ascorbic acid, $n = 8$), The pH was adjusted to 7.25 with D-gluconic acid and osmolarity was adjusted to 290–300 mOsm with D-Glucose as necessary; (2) VLPO + L-Glu and TMN + aCSF group (Microinjection of aCSF and L-Glu into TMN and VLPO, respectively, $n = 7$); (3) VLPO + L-Glu and TMN + Bic group [microinjection of Bic (Sigma, St. Louis, MO, United States) and L-Glu (Sigma, St. Louis, MO, United States) into TMN and VLPO, respectively, $n = 8$]. The day group was composed of (1) VLPO + aCSF and TMN + aCSF group (both VLPO and TMN are microinjected with aCSF, $n = 7$); (2) VLPO + aCSF and TMN + L-Glu group (microinjection of aCSF and L-Glu into VLPO and TMN, respectively, $n = 8$); (3) VLPO + Trip and TMN + L-Glu group [microinjection of Trip (Sigma, St. Louis, MO, United States) and L-Glu into the VLPO and TMN, respectively, $n = 7$].

Microinjection

The drugs (L-Glu, Bic, and Trip) of 1 μl were dissolved in aCSF. Each administration was 5 mmol/L L-Glu, 0.1 mmol/L Bic or 0.5 μmol/L Trip. Control groups were microinjected in aCSF. The microinjection was through a stainless steel guide cannula at an injection rate of 1 μl per min, and the needle was kept in the cannula for 1 min to prevent the physic liquor overflow. For the night group, the administration was performed at 22:00–22:20 for polysomnography (PSG) recording. For the day group, the administration was performed at 10:00–10:20 for PSG recording. **Figures 1A**, **4A** present the schematic drawing of the location of drug or aCSF injection into the VLPO/TMN of the rat.

Polysomnography Recording

Polysomnography recording (including EEG and EMG) was started 2 h before drug application at 20:00 or 08:00, and was sustained for 24 h. According to the PSG results, every 10 s were regarded as one epoch. The sleep-wake cycle is divided into wakefulness (W), non-rapid eye movement sleep (NREM), and rapid eye movement sleep (REM), each of which have distinct characteristics. W was characterized by high frequency and low amplitude waves of EEG and relatively high tone EMG; NREM sleep was characterized by low frequency, spindles, high amplitude and slow waves of EEG with significantly decreased EMG tone, and REM sleep was characterized by high frequency and low amplitude waves of EEG with a lack of EMG tone, except for occasional muscle twitches. The representative traces of EEG and EMG of the four night groups after either injection of aCSF or drugs were shown in **Figure 1A**. In our study, we counted the total sleep time (TST),which was composed of NREM sleep and REM sleep time.

Statistical Analyses

GraphPad Prism 7 was used for statistical analyses. The experimental data was presented as mean ± *SEM*. The paired *t*-test was used in the statistical comparisons of the experimental data between the two groups, the line charts were performed in Igor pro (WaveMetrics, Portland, OR, United States), and those at $P < 0.05$ were considered as the level of significance.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The animal study was reviewed and approved by the Institutional Animal Care Unit Committee of Anhui Medical University. Written informed consent was obtained from the owners for the participation of their animals in this study.

AUTHOR CONTRIBUTIONS

LW and JZ designed and supervised the research work. JC wrote the manuscript and revised the manuscript. JC, FW, and GC performed and analyzed the polysomnographic recording experiments. MZ, DD, and SF set up the recording system and breed rats. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fnins.2020.615854/full#supplementary-material

Supplementary Figure 1 | VLPO and TMN injection sites in all the animals used in the experiments. **(A)** An example brain slice was obtained by frozen section which showing cannulae locations in the VLPO. **(B)** An example brain slice was obtained by frozen section which showing cannulae locations in the TMN. Scale bar = 100 μm.

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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REVIEW
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Preoptic Area Modulation of Arousal in Natural and Drug Induced Unconscious States

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The role of the hypothalamic preoptic area (POA) in arousal state regulation has been studied since Constantin von Economo first recognized its importance in the early twentieth century. Over the intervening decades, the POA has been shown to modulate arousal in both natural (sleep and wake) as well as drug-induced (anesthetic-induced unconsciousness) states. While the POA is well known for its role in sleep promotion, populations of wake-promoting neurons within the region have also been identified. However, the complexity and molecular heterogeneity of the POA has made distinguishing these two populations difficult. Though multiple lines of evidence demonstrate that general anesthetics modulate the activity of the POA, the region's heterogeneity has also made it challenging to determine whether the same neurons involved in sleep/wake regulation also modulate arousal in response to general anesthetics. While a number of studies show that sleep-promoting POA neurons are activated by various anesthetics, recent work suggests this is not universal to all arousal-regulating POA neurons. Technical innovations are making it increasingly possible to classify and distinguish the molecular identities of neurons involved in sleep/wake regulation as well as anesthetic-induced unconsciousness. Here, we review the current understanding of the POA's role in arousal state regulation of both natural and drug-induced forms of unconsciousness, including its molecular organization and connectivity to other known sleep and wake promoting regions. Further insights into the molecular identities and connectivity of arousal-regulating POA neurons will be critical in fully understanding how this complex region regulates arousal states.

Keywords: preoptic area, sleep, anesthesia, sedation, hypothalamus

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INTRODUCTION

Prior to the twentieth century, sleep was considered to be a passive process, caused not by specific neural circuits but rather by reduced sensory input that led to low levels of brain activity. This thinking shifted in the early twentieth century during a viral pandemic of encephalitis lethargica. In some of the earliest examinations into the neurobiology of sleep and wake regulation, neurologist Constantin von Economo noted lesions in the posterior hypothalamus of his patients with excessive sleepiness. Conversely, others exhibiting lesions in the anterior hypothalamus, suffered from severe

insomnia. This led him to propose the existence of a "sleep center" in the anterior hypothalamus and a corresponding "wake center" in the posterior hypothalamus that act in opposition to actively regulate arousal state (von Economo, 1930). Since these original findings, the existence of hypothalamic circuits involved in regulating arousal state has been repeatedly confirmed across a variety of mammalian species.

Since these early investigations, the hypothalamus has been increasingly recognized as a loose confederation of autonomous neurons that regulate many essential social and homeostatic functions (Sternson, 2013; Wu et al., 2014; Scott et al., 2015; Tan et al., 2016; Allen et al., 2017; Leib et al., 2017), including sleep and wake (Szymusiak et al., 2007). More specifically, the preoptic area of the hypothalamus (POA) is known to modulate arousal in both natural (sleep and wake) (Gallopin et al., 2000; Lu et al., 2000, 2002; McGinty and Szymusiak, 2001; Gong et al., 2004; Chung et al., 2017) as well as drug-induced (anesthetic-induced unconsciousness) states (Nelson et al., 2002; Lu et al., 2008; Li et al., 2009; Moore et al., 2012; Liu et al., 2013; Han et al., 2014; McCarren et al., 2014; Zhang Y. et al., 2015; Yatziv et al., 2020). However, the degree to which the same population of neurons within the POA modulates arousal in both sleep and anesthesia is unclear. Failure to properly regulate arousal state can have serious costs, including increased risk of obesity, cardiovascular disease, and impaired cognition from improper sleep/wake regulation (Everson, 1993; Taheri, 2006; Gallicchio and Kalesan, 2009; Vgontzas et al., 2009; Buxton and Marcelli, 2010; Cappuccio et al., 2010; Besedovsky et al., 2012)., as well as intraoperative awareness and delayed emergence from anesthesia (Mesa et al., 2000; Sebel et al., 2004; Mashour and Avidan, 2015; Sanders et al., 2017). Given these consequences of improper arousal state regulation—both in natural sleep/wake and in response to general anesthesia—untangling the circuits by which the brain coordinates arousal state is critical.

In this review, we summarize the current understanding of the POA's involvement in regulating arousal states, both in natural sleep and wake, and under anesthesia, including a growing body of literature that suggests the POA is not strictly a somnogenic node. We also review the shared circuitry hypothesis of anesthesia, and examine the evidence for and against a shared population of sleep- and anesthesia-modulating neurons in the POA. Further, we discuss the obstacles facing investigations into arousal state regulation by the POA, focusing on the functional and molecular heterogeneity of the region. New technical innovations are also highlighted that should enable more refined targeting of POA neuronal subtypes and greatly enhance our understanding of how this complex region regulates arousal states.

POA REGULATION OF UNCONSCIOUSNESS ACCOMPANYING NATURAL SLEEP

Regulating the timing and stability of the states of consciousness and unconsciousness is critical for an individual's health and survival. A complete and extended period of sleep deprivation can result in death, while both total and partial sleep deprivation cause neurobehavioral deficits including diminished cognitive performance, increased risk of obesity and cardiovascular disease, and impaired immune system function, among other effects (Everson, 1993; Taheri, 2006; Vgontzas et al., 2009; Besedovsky et al., 2012; Irwin et al., 2016; Al Khatib et al., 2017; Gaine et al., 2018; Hudson and Van Dongen, 2019; Frau et al., 2020). On the other hand, excessive sleep is also associated with pathology, including obesity, diabetes, heart disease, and increased mortality (Gallicchio and Kalesan, 2009; Buxton and Marcelli, 2010; Cappuccio et al., 2010; Barateau et al., 2017). Thus, regulating arousal to ensure the proper timing and amount of sleep is crucial for normal physiological function. While essential for survival, sleep confers a period of extreme vulnerability, as the unconscious individual is unaware of its surroundings. Thus, the ability to rapidly transition from sleep to wake is crucial in order to defend against external threats and respond to the surrounding environment. Given the importance of these states, an understanding of how the brain properly regulates arousal state is essential. Although the POA was one of the earliest studied regions in regards to sleep and wake regulation, it remains one of the most difficult to untangle.

POA Involvement in Sleep

The POA can be divided into four anatomically defined regions: the median preoptic area (MnPO), medial preoptic area (MPO), lateral preoptic area (LPO), and ventrolateral preoptic area (VLPO). Early investigations into the role of the POA in arousal state regulation found that broad activation of the POA results in the rapid onset of sleep (Sterman and Clemente, 1962), while lesions of the area significantly decrease sleep (Nauta, 1946; McGinty and Sterman, 1968; John and Kumar, 1998; Lu et al., 2000). Subsequent recordings from individual neurons revealed that, while sleep-active neurons are scattered across the POA, higher densities of these neurons exist in the VLPO and MnPO (Sherin et al., 1996, 1998; Szymusiak et al., 1998; Takahashi et al., 2009). For this reason, the majority of studies examining POA regulation of arousal state has focused on these two subregions, though a small number of more recent studies have investigated the wider POA as well, which will also be discussed. Although GABAergic neurons in the MnPO show increases in activity just prior to the onset of sleep (Suntsova et al., 2007), suggesting a role in sleep initiation, their activity has been shown to be more strongly correlated with sleep pressure, rather than sleep *per se*. Thus, this region will be discussed further in a later section of this review.

VLPO

The VLPO contains a small cluster of largely GABAergic neurons that are most active during NREM and REM sleep (Sherin et al., 1996; Szymusiak et al., 1998; Gong et al., 2000, 2004; Alam et al., 2014). These GABAergic VLPO neurons also express galanin, an inhibitory neuropeptide (Sherin et al., 1998). VLPO activity correlates with sleep amount, with the average number of c-Fos-expressing VLPO neurons increasing with more time spent asleep (Sherin et al., 1996). In addition to being sleep-active, VLPO neurons are also sleep-promoting.

Chemogenetic and optogenetic activation of galaninergic VLPO neurons significantly increases NREM sleep (Kroeger et al., 2018). While the VLPO is typically associated with NREM sleep, a cluster of GABAergic/galaninergic neurons in the extended VLPO is active during REM sleep and reduces REM when lesioned (Lu et al., 2002).

Investigations into the VLPO's connectivity support the flip-flop switch theory of sleep regulation. The VLPO projects to many members of the arousal-promoting circuitry, including the cholinergic basal forebrain (BF), the lateral hypothalamus (LH), tuberomammillary nucleus (TMN), raphe nuclei (RN), parabrachial nucleus (PB), and locus coeruleus (LC) (**Figure 1**). It is in turn reciprocally innervated by these same regions (**Figure 2**; Sherin et al., 1998; Steininger et al., 2001; Chou et al., 2002; Yoshida et al., 2006). VLPO neurons with projections to the LH, RN and ventral periaqueductal gray (vPAG) express c-Fos during sleep (Uschakov et al., 2006, 2009; Hsieh et al., 2011). GABAergic VLPO neurons are directly inhibited by acetylcholine, norepinephrine, and serotonin (Gallopin et al., 2000), providing a mechanism by which the release of wake-promoting neurotransmitters can inhibit sleep-active VLPO neurons to reinforce the waking state. Conversely, the combination of increasing GABA levels in the LC and RN during sleep (Nitz and Siegel, 1997a,b) and enhanced inhibitory galaninergic signaling in the TMN and LC (Schönrock et al., 1991; Pieribone et al., 1995) reduce activity of wake-promoting neurons and further stabilize states of sleep. Furthermore, activation of the VLPO in *ex vivo* brain slices produces GABA-mediated inhibitory postsynaptic potentials in histaminergic TMN neurons (Yang and Hatton, 1997), supporting a role for GABAergic/galaninergic VLPO neurons in promoting sleep. Thus, not only are the VLPO and many wake-promoting regions reciprocally connected, they also mutually inhibit each other, providing support for the flip-flop switch theory of arousal regulation.

Wider POA

Lesioning neurons within the POA, including those within the MPO, MnPO, LPO, and VLPO all have been shown to cause insomnia (Szymusiak and McGinty, 1986b; John and Kumar, 1998; Lu et al., 2000; Srividya et al., 2006; Lortkipanidze et al., 2009). This insomnia has been partially reversed by transplantation of fetal preoptic neurons into the lesioned MPO preoptic area (John et al., 1998). Single cell recordings of 128 LPO neurons show that ∼38% are wake/REM-active, ∼43% are sleep-active, and ∼19% are state-indifferent (Alam et al., 2014). Given this evidence that arousal state regulating neurons exist throughout the POA, not just in the VLPO and MnPO, more recent studies have begun investigating the wider POA, including the LPO. A majority of recent studies have focused on the GABAergic/galaninergic population in the region. These populations project to many of the same wake-promoting centers as VLPO and MnPO, including the LH and TMN (Saito et al., 2013; Chung et al., 2017). Activation of GABAergic POA projections to the LH directly inhibits orexinergic neurons in the area (Saito et al., 2013). Furthermore, optogenetic activation of GABAergic POA projections to the TMN promotes sleep, while inhibition promotes wake (Chung et al., 2017). From this population of TMN-projecting, GABAergic POA neurons, they identified 3 subpopulations labeled by neuropeptide markers (cholecystokinin, corticotropin-releasing hormone, and tachykinin 1) that, when optogenetically activated, promote sleep (Chung et al., 2017). Within the LPO, activation of galaninergic neurons promotes NREM sleep, while ablation of this population fragments NREM sleep during the active phase, increasing the number of transitions between the wake and NREM sleep states (Ma et al., 2019). This suggests that galaninergic LPO neurons are essential for consolidated sleep, and are sufficient, but not necessary, for NREM sleep.

POA Involvement in Wake

While many optogenetic and chemogenetic stimulation and lesion/inhibition studies demonstrate a sleep-promoting role for the POA, a growing body of evidence suggests that the region also plays an important role in promoting wakefulness. Single cell *in vivo* recordings illustrate that the POA is much more heterogeneous than originally thought. In addition to sleep-active neurons, the POA contains wake-active and arousal state-indifferent neurons scattered among the sleep-active population (**Figure 3**; Kaitin, 1984; Szymusiak and McGinty, 1989; Takahashi et al., 2009).

Furthermore, optogenetic activation of GABAergic POA neuronal cell bodies, or glutamatergic POA projections to TMN neurons promotes wakefulness (Chung et al., 2017). A recent study also demonstrated that chemogenetic activation of glutamatergic neurons in the ventral half of the POA increases time spent awake (Vanini et al., 2020). Additionally, we recently showed that chemogenetic activation of tachykinin 1-expressing POA neurons strongly stabilizes and consolidates the waking state, decreasing the number of transitions between sleep and wake, while greatly increasing the average length of wake bouts (Reitz et al., 2020). With this growing evidence supporting a dual role in sleep and wake, it is clear that more work is needed in order to more accurately characterize and understand the POA's roles in arousal state regulation.

Homeostatic and Adaptive Arousal State Regulation by the POA

While sleep and wake cycles are strongly regulated by circadian rhythms, a core feature of sleep is that it is also subject to homeostatic regulation. Total or partial sleep deprivation increases sleep drive, ultimately resulting in a period of recovery sleep that is longer and deeper (characterized by enhanced delta power in the EEG) than sleep under unrestricted conditions. The mechanisms by which the brain senses and responds to this homeostatic sleep pressure are not fully understood, though the evidence discussed below points toward the involvement of the POA.

In addition to increasing activity during sleep, both VLPO and MnPO neurons exhibit higher activity in response to sleep deprivation, prior to recovery sleep, suggesting a role in tracking sleep debt (Alam et al., 2014). It was previously thought that VLPO had no role in sensing or responding to sleep pressure, as c-Fos levels were not increased unless animals experienced

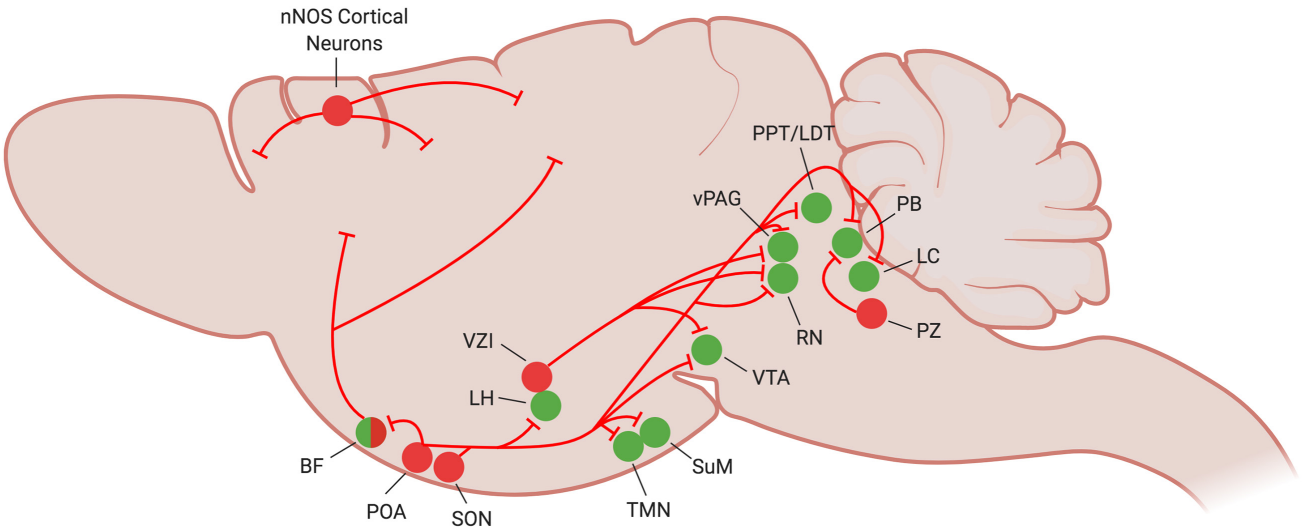

FIGURE 1 | Current understanding of NREM sleep circuitry in the mouse brain. At the onset of NREM sleep, sleep-promoting neurons (red circles) become active and inhibit many of the wake-promoting nuclei of the brain (green circles) to reinforce and stabilize the sleep state. Figure created with BioRender.com.

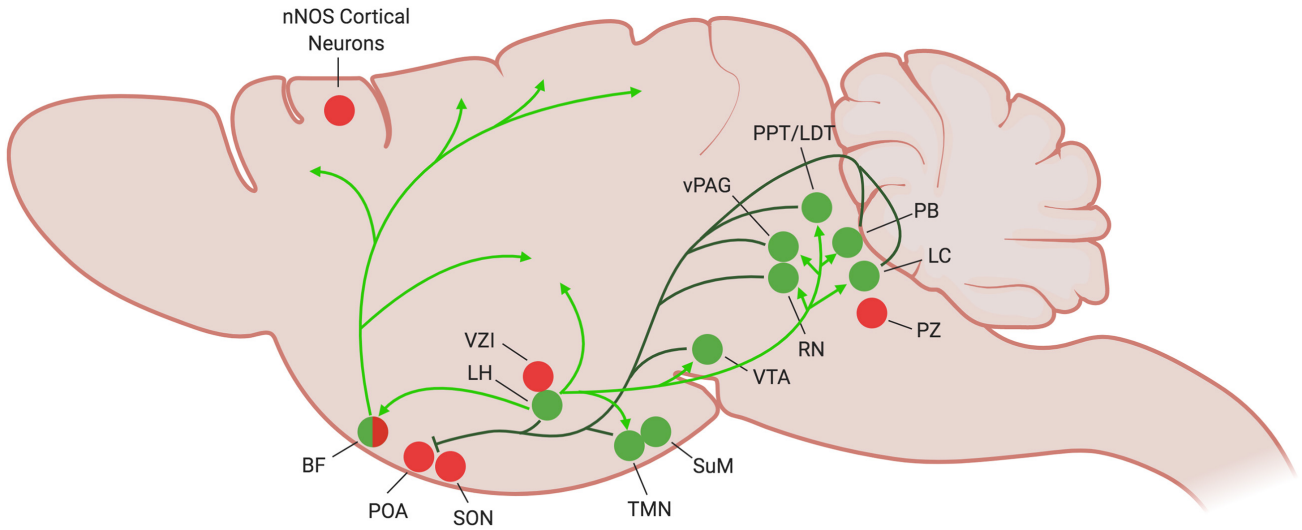

FIGURE 2 | Current understanding of wake-promoting circuitry in the mouse brain. During periods of wake, the wake-promoting nuclei (green circles) become active and inhibit sleep-promoting regions (dark green lines), particularly the POA. Inhibitory projections to other sleep-promoting regions have yet to be established. The orexinergic LH stabilizes the wake state by exciting many of the wake-promoting areas (light green arrows). Many of the wake-promoting regions (LH, TMN, SuM, RN, vPAG, LC) also promote wakefulness by activating the cortex, as illustrated by the BF (light green arrows). Figure created with BioRender.com.

recovery sleep following sleep deprivation (Sherin et al., 1996; Gvilia et al., 2006). However, more recent studies found that the VLPO exhibits increased c-Fos expression and higher firing rates during sleep deprivation, prior to recovery sleep, suggesting at least a minor role for VLPO in sleep homeostasis as well (Gong et al., 2004; Alam et al., 2014). These may represent two distinct subpopulations within VLPO: one that promotes sleep in response to sleep pressure, and one that maintains sleep (Gallopin et al., 2005).

Similar to VLPO, the MnPO consists largely of GABAergic neurons that are most active during NREM and REM sleep compared to baseline wake (Gong et al., 2000; McGinty et al., 2004; Alam et al., 2014). These neurons are also sleep-promoting, as chemogenetic activation of the GABAergic MnPO promotes sleep (Vanini et al., 2020). However, MnPO activity appears to correlate with sleep pressure rather than sleep amount (Suntsova et al., 2002). The number of c-Fos positive, GAD-expressing MnPO neurons is highest following sleep deprivation but prior to recovery sleep (Gvilia et al., 2006). Additionally, sleep-active MnPO neurons exhibit increased firing rates as sleep pressure builds during sleep deprivation, ultimately firing twice as frequently after 2 h of sleep deprivation compared to baseline

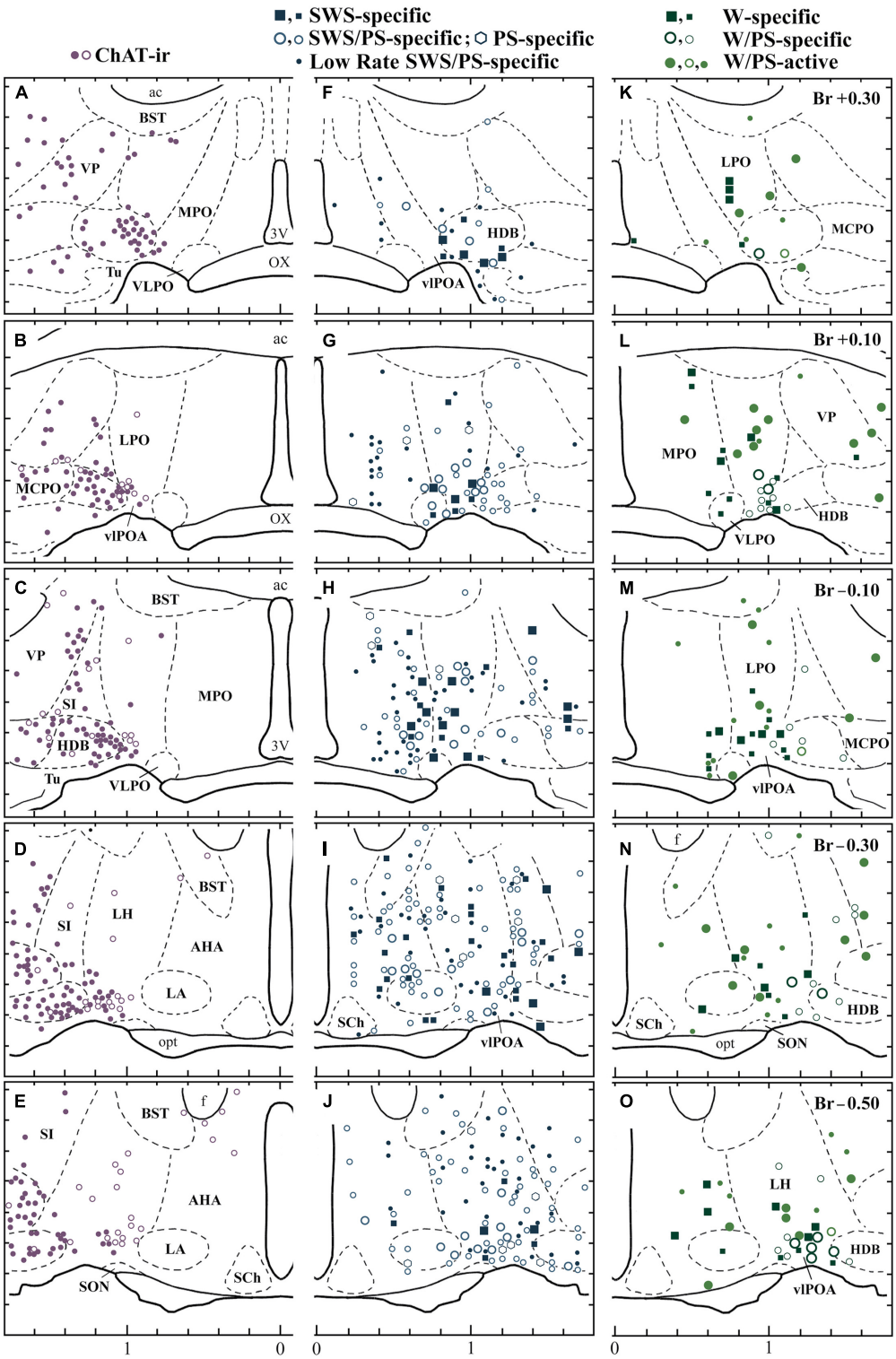

FIGURE 3 | Distribution of sleep-active and wake-active neurons in the POA. Camera lucida drawings of frontal sections (five different planes at 0.2 mm intervals rostral to caudal). **(A–E)** Distribution of ChAT-immunoreactive neurons (dots and circles). **(F–J)** The four groups of sleep-active neurons (squares, circles, circles with a central dot, and dots). **(K–O)** Distribution of waking-specific (squares), waking/PS-specific (circles), and waking/PS-active (dots and thick-lined circles) neurons. The dots and circles in **(A–E)** indicate heavily and faintly stained ChAT-immunoreactive neurons, respectively. The large and small symbols indicate rapidly firing (fast) and slowly firing (slow) neurons, respectively. The thick-lined circles in **(K,M,O)** indicate W/PS-active neurons discharging in close relation to theta waves. 3V, third ventricle; ac, anterior commissure; BST, bed nucleus of the stria terminalis; f, fornix; LA, lateroanterior hypothalamic nucleus; opt, optic tract; OX, optic chiasma; SCh., suprachiasmatic nucleus; SON, supraoptic nucleus; VP, ventral pallidum. Modified from Takahashi et al. (2009). Reprinted with permission from Elsevier.

sleep. This firing returns to baseline levels as the animal is allowed to sleep (Alam et al., 2014). Together, this suggests a role for the MnPO in tracking sleep debt and maintaining sleep homeostasis.

The MnPO may contribute to sleep homeostasis via inhibition of numerous wake-promoting regions. Like the VLPO, the MnPO is also reciprocally connected to many members of the arousal-promoting circuitry, including the cholinergic BF, LH, TMN, RN, PB, and LC (Steininger et al., 2001; Yoshida et al., 2006; Uschakov et al., 2007). MnPO neurons with projections to the RN and vPAG exhibit increased expression of c-Fos during sleep (Uschakov et al., 2009; Hsieh et al., 2011). Furthermore, activation of MnPO neurons suppresses activity in the wake-active LH, while inhibition of the MnPO had the opposite effect (Suntsova et al., 2007). Similarly, inhibition of MnPO neurons increases c-Fos in the orexinergic LH neurons and serotonergic RN neurons (Kumar et al., 2008), further suggesting functional inhibition of these two wake centers by the MnPO. In contrast to the MnPO and VLPO, sleep-active neurons in the LPO do not show increased activity in response to sleep deprivation (Alam et al., 2014), suggesting this population is not involved in sleep homeostasis.

One possible mechanism by which the POA senses sleep pressure is via a buildup of sleep-generating small molecules, called somnogens, in the brain (Benington and Craig Heller, 1995; Porkka-Heiskanen et al., 1997; Scammell et al., 2001; Basheer et al., 2004). One of the best studied somnogens is adenosine. Adenosine is a byproduct of metabolism in the brain, and brain levels of this molecule increase across waking and during sleep deprivation, decreasing during recovery sleep (Porkka-Heiskanen et al., 1997, 2000; Basheer et al., 2004; Kalinchuk et al., 2011). Application of adenosine to VLPO neurons in *ex vivo* rat brain slices suppresses spontaneous IPSPs (Chamberlin et al., 2003). Furthermore, administration of adenosine A_{2A} receptor agonists into the POA directly activates VLPO neurons and promotes sleep in rats (Ticho and Radulovacki, 1991; Gallopin et al., 2005; Kumar et al., 2013). Additionally, local administration of A_{2A} receptor antagonists into the VLPO reduces sleep deprivation- and recovery sleep-induced firing of VLPO neurons (Alam et al., 2014). Finally, the aforementioned cycles of adenosine levels in the POA that correspond to sleep and wake are present in rats at post-natal day 30 (P30), but not at P22, suggesting that the development of this homeostatic response to sleep loss coincides with the functional emergence of adenosine signaling in the brain. This study also found that sleep-active MnPO neurons are more responsive to sleep deprivation at P30 compared to P22 (Gvilia et al., 2017).

Another small molecule linked to sleep pressure is prostaglandin D_2 (PGD_2). PGD_2 is generated in the leptomeninges and choroid plexus, and is found circulating in the cerebrospinal fluid, where it fluctuates in parallel with the sleep-wake cycle (Huang et al., 2007). Like adenosine, PGD_2 levels also increase during sleep deprivation (Ram et al., 1997). Administration of PGD_2 in the subarachnoid space just anterior to the MnPO and VLPO promotes sleep and increases c-Fos expression in VLPO neurons (Scammell et al., 1998; Hayaishi and Urade, 2002). This effect is likely mediated by adenosine, as infusion of PGD_2 into the subarachnoid space dose-dependently increases extracellular adenosine (Mizoguchi et al., 2001).

As emphasized earlier, regulating the timing and stability of sleep and wake is critical for health and survival, as life-sustaining activities such as eating, seeking shelter, copulating, and escaping from danger all depend upon proper control of arousal. Multiple lines of evidence suggest the POA is capable of integrating diverse inputs, such as temperature and energy status, to produce the most appropriate arousal state response. For instance, local administration of glucose in the VLPO, simulating the "well-fed" state, activates VLPO neurons and promotes NREM sleep, thus providing a potential link between metabolism/energy status and arousal state regulation in the POA (Varin et al., 2015).

Under more extreme conditions, when faced with resource scarcity, some mammals will adapt by initiating energy-conserving survival strategies, such as hibernation or torpor. Recent work highlights that the MPO and LPO are capable of overriding homeostatic setpoints to coordinate profound reductions in metabolism, body temperature, and caloric need to enhance survival (Hrvatin et al., 2020). Work in hibernating ground squirrels has shown increases in c-Fos expression in MPO neurons during entry into hibernation (Bratincsák et al., 2007). Additionally, microinjections of opioid receptor antagonists into the POA of hibernating ground squirrels increased the squirrels' body temperature and induced arousal from hibernation, suggesting a role for opioid signaling in the POA in hibernation (Yu and Cai, 1993).

Even under less extreme conditions, alterations in body temperature are known to correlate with arousal state. The onset of sleep coincides with a decline in body temperature, and entry into REM is accompanied by near total inhibition of thermoregulatory responses in many species (Krueger and Takahashi, 1997). The POA is poised to be the link between sleep and thermoregulation, as the POA is known to contain thermosensitive and thermoregulatory neurons (Zhao et al., 2017; Ma et al., 2019), with many of the warm-sensitive POA neurons also exhibiting sleep-active firing, while cold-sensitive POA neurons show increased activity during wake (Alam et al., 1995, 1997). Further supporting this link is evidence that local warming of the broad POA or the GABAergic MPO neurons promotes sleep (Roberts and Robinson, 1969; Harding et al., 2018), while local cooling promotes wakefulness (Sakaguchi et al., 1979; McGinty and Szymusiak, 1990).

Recent studies using activity-dependent tagging and reactivation of neurons further reveal a link between arousal state and body temperature. Reactivation of neuronal nitric oxide synthase (*Nos1)*-expressing MnPO/MPO neurons activated during external warming induces both sleep and hypothermia in mice, while reactivation of warming-tagged GABAergic MPO neurons produces NREM sleep (Harding et al., 2018). Further investigations revealed that reactivation of MPO or LPO neurons that were activated during recovery sleep produces profound drops in body temperature (Zhang Z. et al., 2015). Additionally, chemogenetic activation of galaninergic VLPO neurons reduces core body temperature by 4–6°C (Kroeger et al., 2018). Moreover, activation of those same neurons at warmer temperatures (29 and 36°C) decreases latency to NREM

and increases NREM duration compared to activation at 22°C (Kroeger et al., 2018). The extreme drop in body temperature resulting from galaninergic VLPO activation also suggests a role for the VLPO in torpor, another state of unconsciousness accompanied by hypothermia, decreased metabolism, and slow wave EEG activity (Berger, 1984).

POA REGULATION OF DRUG-INDUCED UNCONSCIOUSNESS

While sleep is a universal, natural form of unconsciousness, unconsciousness also occurs under general anesthesia. However, despite the use of anesthetics for over 170 years and in over 300 million surgeries annually (Weiser et al., 2016), the precise molecular and neuronal mechanisms underlying their hypnotic actions remain poorly understood.

The molecular mechanisms of anesthetic-induced unconsciousness remain unknown due in part to the transient interactions and promiscuous number of general anesthetic binding partners (Eckenhoff, 2001; Urban, 2002), yet all produce an apparently similar behavioral endpoint. While a variety of ion channels are affected by anesthetics, the net effect of anesthetic binding is the hyperpolarization of resting membrane potentials, enhancement of inhibitory neurotransmission, and inhibition of excitatory neurotransmission (Rudolph and Antkowiak, 2004). With the knowledge that anesthetics act on a diverse range of ion channels yet all enhance inhibition and/or inhibit excitation, more recent research has examined the hypothesis that anesthetics may exert their effects not by acting at identical molecular targets, but rather by differentially affecting neurons in a common neural pathway. However, because these receptors are widely expressed throughout the brain, identifying the exact neural circuits critical for producing a state of anesthesia has been difficult.

The Shared Circuitry Hypothesis

One target that has emerged as a likely mediator of anesthetic hypnosis is the neural circuitry governing sleep and arousal discussed earlier. Although sleep and anesthesia are undoubtedly two distinct states, they share a number of similar traits (Lydic and Biebuyck, 1994). For example, both NREM sleep and anesthetic hypnosis show EEG patterns that include spindles and slow waves (Murphy et al., 2011). Neuroimaging studies have also shown reduced activity in brain regions involved in arousal (Alkire et al., 2000; Vahle-hinz et al., 2001; Detsch et al., 2002) as well as cortical regions involved in association and integration in both states of unconsciousness (Fiset et al., 1999; Franks, 2008).

In addition to these phenotypic similarities, multiple lines of evidence demonstrate a functional relationship between sleep and anesthetic hypnosis. Sleep deprivation reduces the amount of anesthetic required to enter the hypnotic state (Tung et al., 2002), while administration of barbiturates during the waking phase results in a shorter duration of hypnosis (Einon et al., 1976). Furthermore, administration of select anesthetics for prolonged periods does not incur new sleep debt and may actually relieve preexisting sleep debt (Tung et al., 2004; Nelson et al., 2010; Pal et al., 2011; Pick et al., 2011). These findings have led to what is known as the "shared circuitry hypothesis" of anesthesia, which posits that anesthetics exert their hypnotic effects in part by acting on the neural circuitry that regulates endogenous sleep and wake. More specifically, this theory hypothesizes that anesthetics cause unconsciousness via activation of sleep-promoting populations and/or inhibition of wake-promoting populations, rather than by the wet-blanket theory of non-specific, global disruption of CNS function (Lydic and Biebuyck, 1994; Yatziv et al., 2020).

Although a number of studies have implicated sleep- and wake-regulating brain areas in anesthetic hypnosis, controversy remains as to whether the neural circuits, and more specifically, the same neurons shaping sleep and wakefulness actually do influence the anesthetic state *in vivo*. Past work has demonstrated that the POA, in addition to modulating sleep and wake, is also capable of modulating anesthetic-induced unconsciousness (Nelson et al., 2002; Lu et al., 2008; Li et al., 2009; Moore et al., 2012; Liu et al., 2013; Han et al., 2014; McCarren et al., 2014; Zhang Y. et al., 2015; Yatziv et al., 2020). However, the degree to which the same population of neurons within the POA modulates arousal in both sleep and anesthesia is unclear.

POA Involvement in General Anesthesia

Because the VLPO and MnPO contain the highest densities of sleep-active neurons, the majority of work investigating the role of the POA in anesthetic hypnosis has focused on these two regions, particularly the VLPO.

VLPO

Exposure to hypnotic doses of all anesthetics except for ketamine increases c-Fos expression in VLPO (Nelson et al., 2002; Lu et al., 2008; Li et al., 2009; Moore et al., 2012; Han et al., 2014), positioning this region as a potential common mediator of anesthetic hypnosis. Furthermore, c-Fos expression in VLPO is positively correlated with isoflurane dose, suggesting that isoflurane may dose-dependently activate VLPO neurons (Moore et al., 2012). Activation of VLPO under anesthesia may arise from either disinhibition or from direct excitation. This has not been examined for many anesthetics, though isoflurane is known to directly depolarize putative sleep-active VLPO neurons (Moore et al., 2012). However, not every VLPO neuron is activated by isoflurane. Single cell recordings within VLPO reveal two distinct subpopulations: isoflurane-activated VLPO neurons, and isoflurane-inhibited VLPO neurons (Moore et al., 2012; McCarren et al., 2014). The isoflurane-activated neurons are considered to be putative sleep-active as well since they match the *ex vivo* neurochemical phenotype of low-threshold spiking neurons that are inhibited by norepinephrine (Moore et al., 2012); however, formal *in vivo* proof of this potential convergence was not obtained.

On a functional level, VLPO lesions increase resistance to propofol, significantly increasing the time to loss of righting reflex after administration and decreasing the duration of loss of righting reflex (Zhang Y. et al., 2015). Lesions of VLPO neurons also increase wakefulness and decrease isoflurane

sensitivity, though only acutely following the lesion. This decreased sensitivity to isoflurane subsequently transitioned to enhanced sensitivity at later timeponts (Eikermann et al., 2011; Moore et al., 2012). The enhanced sensitivity to isoflurane observed at these later timepoints is thought to be the result of sleep deprivation caused by the VLPO lesions, which increases sensitivity to anesthetics as discussed earlier (Tung et al., 2002). Conversely, inhibition of VLPO neurons, via activation of α2 adrenergic receptors, increases behavioral arousal under isoflurane anesthesia (McCarren et al., 2014). Together, these results support a role of VLPO in regulating arousal under anesthesia. However, these studies did not confirm their VLPO modulations also affected sleep/wake activity. Thus, though evidence supports a role for the VLPO in arousal state regulation under sleep/wake and anesthesia, to what degree these two populations converge remains an open question. Recent work supports the existence of two distinct populations within the VLPO, demonstrating that chemogenetic activation of GABAergic VLPO neurons alters sleep-wake architecture without affecting anesthetic sensitivity or recovery time in the same mice (Eikermann et al., 2011; Vanini et al., 2020).

MnPO

Evidence for a role of the MnPO in anesthetic hypnosis is much less clear than VLPO. Exposure to isoflurane increases c-Fos expression in MnPO neurons, however, exposure to halothane, pentobarbital, and chloral hydrate do not (Lu et al., 2008; Han et al., 2014). This activation by isoflurane likely results from either disinhibition or secondary activation via VLPO or another anesthetic-activated region, as isoflurane-induced activation of MnPO does not occur in the presence of tetrodotoxin (Han et al., 2014). Additionally, reactivation of MnPO neurons that were active during dexmedetomidine-induced sedation does not promote sleep (Zhang Z. et al., 2015) and activation of GABAergic MnPO neurons that promote sleep do not alter anesthetic state transitions (Vanini et al., 2020), suggesting that anesthetic-activated MnPO neurons are not the same neurons that promote sleep.

Broader POA

POA Tac1 neurons that promote and consolidate wakefulness also enhance resistance to isoflurane and sevoflurane anesthesia (Reitz et al., 2020). This increase in resistance is more pronounced on emergence from the anesthetic state compared to induction. Thus, the potent effects of Tac1 activation work to support the waking state over both endogenous as well as anesthetic-induced impairment of arousal. However, chemogenetic inhibition of this population had no effect on either sleep or anesthetic sensitivity. Whether this is due to a technological limitation, a relative quiescence of Tac1 neurons at baseline, or a true indication that POA Tac1 neurons are sufficient but not necessary for promoting wakefulness remains to be seen. Also unclear is whether POA Tac1 neurons play any endogenous role in circuits regulating natural sleep/wake and those regulating anesthetic-induced unconsciousness, or whether POA Tac1 neurons increase resistance to anesthesia via a neural pathway independent from that utilized by general anesthetics.

POA Involvement in Sedation

Distinct from the deep unconsciousness achieved by general anesthetics such as propofol or isoflurane, dexmedetomidine produces a state of moderate sedation. This moderate sedation shares many properties with natural sleep, with both states characterized by a loss of consciousness, but intact ability to be aroused by external stimuli.

A recent study examined the relationship between sleep and dexmedetomidine-induced sedation in the LPO. Zhang and colleagues show that the same neurons active during dexmedetomidine-induced sedation promote NREM sleep when subsequently re-activated. Zhang's clever use of the TetTag system provides causal support for the shared circuitry hypothesis in the LPO. Whether these results are true for general anesthetics such as isoflurane or propofol remain unknown. Additionally, re-activation of these dexmedetomidine-active LPO neurons produces a drop in body temperature, linking the POA to thermoregulation in both natural and drug-induced forms of unconsciousness. Finally, deletion of the *Vgat* gene from LPO neurons increases resistance to dexmedetomidine-induced sedation (Zhang Z. et al., 2015), suggesting that GABAergic LPO neurons specifically are required for dexmedetomidine-induced sedation.

CHALLENGES IN STUDYING AROUSAL REGULATION BY THE POA

Molecular Heterogeneity

Perhaps the largest challenge facing the study of arousal regulation by the POA is the immense heterogeneity of the region (**Figure 4**; Moffitt et al., 2018). In addition to regulating arousal state, the POA is involved in many essential social and homeostatic functions, including thermoregulation, thirst and osmotic homeostasis, parenting, and social play behaviors (Sternson, 2013; Wu et al., 2014; Scott et al., 2015; Tan et al., 2016; Allen et al., 2017; Leib et al., 2017; Zhao et al., 2020). As mentioned earlier, heterogeneity exists even among arousal state regulating neurons in the POA, with sleep-active neurons interspersed among wake-active and arousal state-indifferent neurons (Kaitin, 1984; Szymusiak and McGinty, 1986a; Szymusiak et al., 1998). Recordings in the LPO and MPO found 21% of recorded neurons to be wake/REM-active, 66% to be sleep-active, and 13% to be state-indifferent (**Figure 3**; Takahashi et al., 2009). Additional recordings of 128 LPO neurons show that roughly 38% are wake/REM-active, 43% are sleep-active, and 19% are state-indifferent (Alam et al., 2014). Similarly, although the VLPO and MnPO are more densely populated by sleep-active neurons, roughly 20% of neurons in each area are estimated to be wake-active (Gaus et al., 2002; Alam et al., 2014).

Because of this heterogeneity, the majority of studies investigating arousal state regulation by the POA have focused on two broad classes of neurons: the inhibitory GABAergic/galaninergic neurons typically shown to be sleep-active, and the excitatory glutamatergic neurons, typically

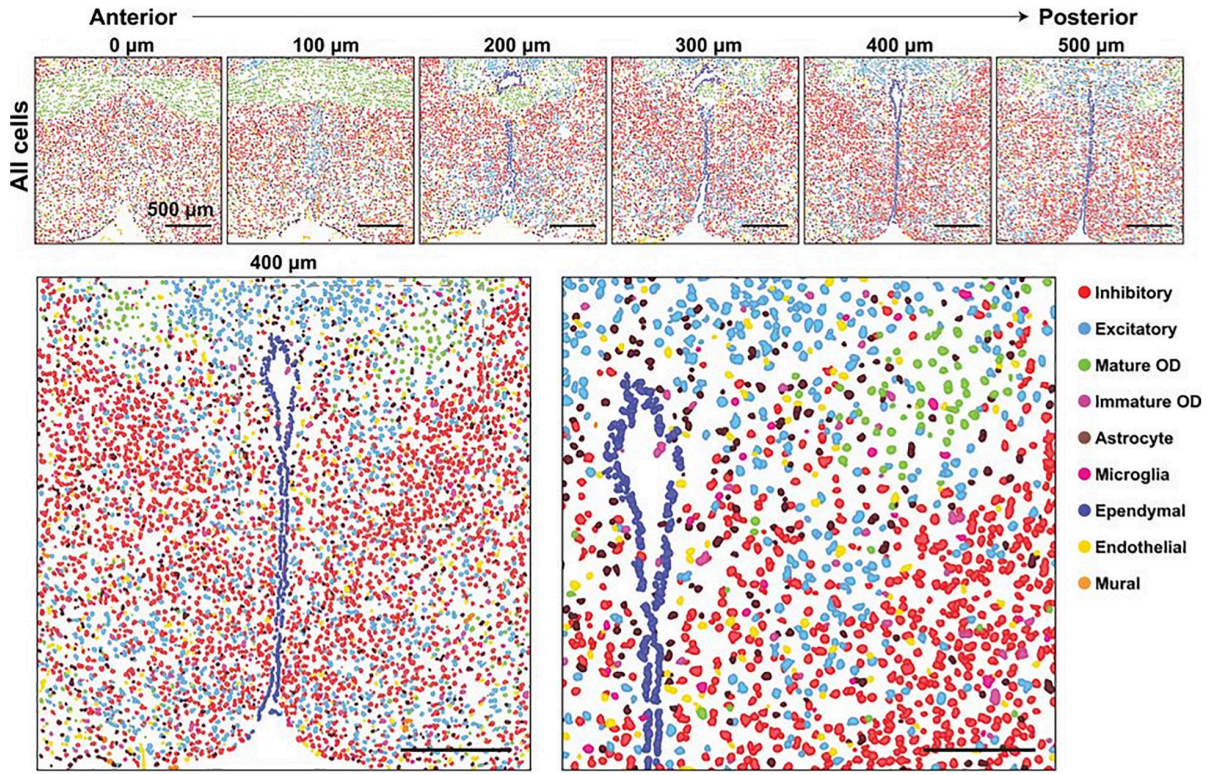

FIGURE 4 | Major cell classes and their spatial organizations in the POA as revealed with MERFISH. **(Top)** Spatial distribution of all major cell classes across sections at different anterior–posterior positions from a single female mouse. Cells are marked with cell segmentation boundaries and colored by cell classes as indicated. Six of the twelve 1.8- by 1.8-mm imaged slices are shown. The 0, 100, 200, 300, 400, and 500 mm labels indicate the distance from the anterior position (Bregma + 0.26). **(Bottom)** Enlarged image of the slice at 400 mm from the anterior position (left) and a further magnified image of the region shown in the gray dashed box (right). Scale bars, 500 mm (left), 250 mm (right). From Moffitt et al. (2018). Reprinted with permission from AAAS.

associated with wake. However, the assumption that inhibitory neurons are sleep-active and excitatory neurons are wake-active is not as straightforward as is often assumed. Though the majority of galaninergic VLPO neurons are sleep-active, roughly 20% of the population is actually wake-active (Gaus et al., 2002). Further, of the POA neurons activated during recovery sleep, roughly 15% are glutamatergic (Zhang Z. et al., 2015). These functional differences within the broad class of excitatory POA neurons is further illustrated by work showing that activation of glutamatergic VLPO neurons promotes wake (Vanini et al., 2020), while activation of largely glutamatergic NOS1-expressing MnPO neurons causes entry into NREM sleep (Harding et al., 2018). While activation of inhibitory POA neurons promotes sleep in some studies (Chung et al., 2017; Kroeger et al., 2018; Ma et al., 2019), yet have no effect on sleep or wake in others (Vanini et al., 2020).

As a result, investigations into the role of inhibitory POA neurons in anesthetic-induced unconsciousness have also produced sometimes-opposing results. For instance, isoflurane-induced unconsciousness directly depolarizes and increases expression of c-Fos in putative sleep-active GABAergic neurons within the VLPO (Moore et al., 2012), yet broad activation of this GABAergic population alters sleep-wake architecture (Saito et al., 2013; Kroeger et al., 2018) without affecting the time to anesthetic induction or time required for emergence (Vanini et al., 2020). Together, this emphasizes that these molecular markers traditionally used to distinguish the sleep-active and wake-active populations within the POA are not accurate enough, and that a more refined targeting of POA cell types is needed when investigating arousal state regulation.

The immense heterogeneity of these two neuronal subtypes may underlie these differing results. Recent studies have shown that the molecular marker chosen to access the inhibitory population is one important consideration. The GABA transporter, VGAT, is rarely expressed alongside Vglut2, the glutamatergic transporter. However, GABA synthesis genes *gad1* and *gad2* are sometimes expressed in the same neurons as Vglut2, representing a population of POA neurons capable of coreleasing GABA and glutamate (Romanov et al., 2016; Moffitt et al., 2018). Thus, studies using Gad as a marker of inhibitory neurons in the POA may unintentionally also modulate glutamatergic signaling to an unknown degree. Furthermore, recent single-cell RNA-sequencing of GABAergic and glutamatergic neurons within the POA has revealed an enormous level of molecular diversity within these two groups, consisting of nearly 70 subpopulations clustered based on gene expression (Moffitt et al., 2018). Even more selective neuropeptide markers may be inadequate to conclusively distinguish sleep-from wake-promoting neuronal

populations within the POA, as activation of POA Tac1 neurons has been shown to increase sleep in one investigation (Chung et al., 2017), yet strongly enhance and stabilize wakefulness in another (Reitz et al., 2020).

Together, this suggests that a single molecular marker may not be sufficient to accurately distinguish sleep and wake population within the POA. Molecular markers combined with projection-specific labeling may be one method to more accurately identify these populations, as past work has shown that optogenetic activation of GABAergic POA neuronal cell bodies or glutamatergic POA projections to the TMN produces wake, while activation of GABAergic POA terminals in the TMN produces sleep (Chung et al., 2017). However, optogenetic stimulation of GABAergic POA projections to either the habenula or the dorsomedial hypothalamus have no effect on arousal state (Chung et al., 2017), further emphasizing the importance of examination specific axonal targets.

Methodological Limitations and Considerations

Another set of challenges when studying arousal state regulation by the POA arise from methodological limitations, both from an experimental design standpoint and inherent limitations with the methods themselves. Studies investigating the shared circuitry hypothesis in the POA often use GABA or galanin as markers of the sleep-active neuronal population, yet many fail to confirm that these same anesthetic-activated neurons are also involved in arousal regulation. Thus, though multiple lines of evidence demonstrate that general anesthetics and sedatives modulate the activity of the POA, and even the GABAergic/galaninergic POA, it is still unclear whether the same population of neurons involved in sleep/wake regulation also modulate arousal in response to general anesthetics, or whether these are two separate populations that exist in the same region. Given the uncertainty of the exact role of GABAergic and glutamatergic POA neurons in sleep and wake, it is clear that modulations of sleep and anesthesia must be examined in the same cohort without assuming that neurons expressing a particular molecular marker represent an arousal state-regulating population.

Another important consideration when studying arousal state regulation in the POA is the method of activation used. We recently demonstrated that chemogenetic activation of POA Tac1 neurons using designer receptors exclusively activated by designer drugs (DREADDs) strongly stabilizes the wake state, decreasing the number of transitions between sleep and wake while greatly increasing the average length of wake bouts (Reitz et al., 2020). This is in contrast to previously published results describing NREM-promoting effects when POA Tac1 neurons are optogenetically activated (Chung et al., 2017). It is possible that fundamental differences between the neuronal activation achieved by DREADDs compared to that achieved by optogenetics underlie these contrasting findings. While we will briefly discuss important considerations to take into account when utilizing either method, more detailed comparisons of these techniques can be found in a number of reviews specifically discussing this topic (Aston-Jones and Deisseroth, 2013; Krook-Magnuson and Soltesz, 2015; Vlasov et al., 2018).

When utilizing optogenetic activation, the stimulation frequency is a critical concern. This has been highlighted in recent work demonstrating optogenetic activation of GABAergic POA neurons promotes wakefulness when stimulated at 10 Hz (Chung et al., 2017). However, chemogenetic activation or optogenetic activation at lower frequencies (0.5–4 Hz), which more closely match the endogenous firing rate during NREM sleep, have been shown to promote NREM sleep (Kroeger et al., 2018). This discrepancy is likely due to a conduction block resulting from stimulation above 8 Hz, functionally inhibiting the neurons (Kroeger et al., 2018). Thus, in addition to emphasizing the importance of identifying endogenous firing rates of the targeted neuronal population, these results demonstrate that care should be given to stimulate the neurons at a frequency that matches their endogenous firing rate during the behavioral state of interest.

The interpretation of results from chemogenetic or optogenetic inhibition presents another set of challenges. Inhibition using hM4Di DREADDs is known to have variable efficacy, with incomplete suppression of activity of hM4Di-expressing neurons occurring whether CNO is administered locally or systemically (Mahler et al., 2014; Chang et al., 2015; Cichon and Gan, 2015). Our own hM4Di results reflect this, with the number of c-Fos-expressing POA Tac1 neurons decreasing by only 30% after systemic administration of 3 mg/kg CNO. Incomplete inhibition may not be enough to alter behavior, given that many of the neurons of interest remain active. While optogenetic inhibition with halorhodopsin or archaerhodopsin may be more effective at silencing neuronal activity than chemogenetic inhibition, the effect of photoinhibition is more spatially limited, only to the area illuminated by the laser. Thus, the laser penetration may not be enough to effectively silence the entire POA.

Ultimately, the benefits and drawbacks of each method must be weighed carefully in order to select the most appropriate technique for a given experiment, and comparisons of studies using each technique when investigating POA regulation of arousal state will be important to fully untangle its role.

TECHNICAL INNOVATIONS

New innovations in biomedical science and equipment are bringing the field ever closer to untangling the role of the POA in natural and anesthetic-induced arousal state regulation. The use of more temporally specific measurements of neuronal activity, such as calcium imaging and *in vivo* electrode recordings in freely behaving animals, now allows for examinations of POA neuronal activity across sleep stages as well as anesthetic induction, maintenance, and emergence. Given evidence that the neural circuitry involved in anesthetic induction may not be identical to that involved in emergence (Kelz et al., 2008; Dong et al., 2009; Gompf et al., 2009; Friedman et al., 2010; Zhang et al., 2012, 2016), the ability to record neuronal activity across each of these phases will

be an invaluable contribution to understanding anesthetic mechanisms. Additionally, advancements in neural circuit mapping such as channel rhodopsin-assisted circuit mapping (Petreanu et al., 2007) will aid in distinguishing functional projections to and from the POA that may help mediate the region's effects on arousal state.

One exciting area of technical innovation lies in techniques linking immediate early gene expression to an effector molecule, controlled by pharmacological treatment. These have been discussed in detail in a recent review (Franceschini et al., 2020), so we will only highlight a selection here. One technique that has already been utilized to study the POA is the TetTag system (Reijmers et al., 2007). This system uses the *Fos* promoter to drive the expression of a tetracycline transactivator in the absence of doxycycline. Thus, this system is switched off in the presence of doxycycline, which can be administered in a number of ways. Once doxycycline is removed and a c-Fos activating stimulus occurs, the tetracycline transactivator is expressed, ultimately driving the expression of a downstream effector molecule, which can include optogenetic or chemogenetic tools (Reijmers et al., 2007).

This TetTag system has been used to study the relationship between sleep and dexmedetomidine-induced sedation in the POA. Neurons tagged with excitatory DREADDs during dexmedetomidine sedation and later reactivated promote sleep, demonstrating that an identical population of neurons is involved in both states (Zhang Z. et al., 2015). Given that dexmedetomidine produces a type of sedation distinct from other general anesthetics, the degree to which this result can be generalized to other anesthetics is not clear given the distinct types of unconscious induced by each. Still, these results highlight the utility of this technique when investigating arousal state regulation.

Another drug-dependent immediate early gene-linked technique is *targeted recombination in active populations* (TRAP), which shows improved temporal resolution compared to TetTag (Guenthner et al., 2013). This technique places a tamoxifen-inducible recombinase under control of an immediate early gene reporter such as c-Fos. Thus, the recombinase is only active in the presence of tamoxifen, the administration of which is controlled by the experimenter. By coupling this technique with optogenetic and chemogenetic effector molecules, neuronal populations active during a specific task or time can be TRAPed and later reactivated (Franceschini et al., 2020). Additionally, neuronal populations can be TRAPed with a fluorescent molecule during one stimulus and this fluorescent pattern can be compared to a c-Fos signal induced by a later, second type of stimulus. A new version of TRAP (TRAP2) was also recently developed that exhibits enhanced effector expression and improved penetration in many brain regions (DeNardo et al., 2019).

Finally, another recently developed immediate early gene-linked tool has already been used to study sleep and anesthetic mechanisms. This technique, called *capturing activated neuronal ensembles with engineered mice and viruses* (CANE), inserts a destabilized avian tumor virus receptor A (TVA) under the control of the Fos promoter (Sakurai et al., 2016). Thus, the TVA is only expressed in activated neurons, and only for a window of a few hours until the TVA is degraded. A virus pseudotyped with the ligand of TVA, EnvA, is injected into the brain region of interest and infects neurons that express TVA during the injection window. This virus can carry fluorescent proteins to label the active neurons, or chemogenetic or optogenetic effector molecules to enable subsequent activation or inhibition of this tagged population (Sakurai et al., 2016; Jiang-Xie et al., 2019). Though not in the POA, this technique identified a population of anesthetic-activated neurons in the neighboring supraoptic nucleus that promote NREM sleep when reactivated at a later time (Jiang-Xie et al., 2019), highlighting the power of this technique in investigations of the shared circuitry hypothesis in the POA and other regions.

CONCLUSION

Understanding the role of the POA in regulating arousal state is a critically important topic, given the range of consequences that result from improper arousal state regulation. Each year a small but nevertheless significant number of patients experience undesirable arousal state transitions in response to general anesthesia. Such patients may regain consciousness during surgery (Sebel et al., 2004; Mashour and Avidan, 2015; Sanders et al., 2017) or exhibit delayed emergence from the anesthetic state (Mesa et al., 2000; Cascella et al., 2018). Thus, understanding the mechanisms by which general anesthetics alter the arousal state of an organism, producing a state of unconsciousness, is an important medical question to ultimately reduce or prevent these inappropriate arousal state transitions from occurring. Additionally, insights into the mechanisms of anesthetic-induced unconsciousness will have important implications for our understanding of the neural basis of consciousness and natural arousal state regulation itself, as well as disorders of consciousness such as coma and sleep disorders. With the continuous improvement and development of technical methods and an improved ability to distinguish and target arousal state-regulating neurons, our understanding of the exact role that POA plays in regulating arousal states under natural sleep and wake as well as general anesthesia is closer than ever.

AUTHOR CONTRIBUTIONS

SR contributed to the conceptualization of the manuscript, wrote the initial draft, contributed to editing, and prepared the figures. MK contributed to the conceptualization, reviewed, and edited the manuscript. Both authors approved the final manuscript.

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The Inert Brain: Explaining Neural Inertia as Post-anaesthetic Sleep Inertia

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"Neural inertia" is the brain's tendency to resist changes in its arousal state: it is manifested as emergence from anaesthesia occurring at lower drug doses than those required for anaesthetic induction, a phenomenon observed across very different species, from invertebrates to mammals. However, the brain is also subject to another form of inertia, familiar to most people: sleep inertia, the feeling of grogginess, confusion and impaired performance that typically follows awakening. Here, we propose a novel account of neural inertia, as the result of sleep inertia taking place after the artificial sleep induced by anaesthetics. We argue that the orexinergic and noradrenergic systems may be key mechanisms for the control of these transition states, with the orexinergic system exerting a stabilising effect through the noradrenergic system. This effect may be reflected at the macroscale in terms of altered functional anticorrelations between default mode and executive control networks of the human brain. The hypothesised link between neural inertia and sleep inertia could explain why different anaesthetic drugs induce different levels of neural inertia, and why elderly individuals and narcoleptic patients are more susceptible to neural inertia. This novel hypothesis also enables us to generate several empirically testable predictions at both the behavioural and neural levels, with potential implications for clinical practice.

Keywords: neural inertia, sleep inertia, anaesthesia, orexin, noradrenaline, anticorrelations, aging

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INTRODUCTION

Anaesthesia and Sleep

General anaesthesia refers to a pharmacological intervention designed to produce a state of controlled and reversible unconsciousness and unresponsiveness to sensory stimulation. Its discovery is among the greatest in medical history: it allows surgeons to perform millions of life-saving interventions every year, which would be otherwise impossible or extremely distressing.

However, the mechanisms of anaesthetic action in the brain remain incompletely understood—especially since multiple anaesthetic drugs exist, with different pharmacological profiles (Scharf and Kelz, 2013). Nevertheless, anaesthesia is not the only way in which one can become unconscious: the brain exhibits a strong need for periodic unconsciousness in the form of sleep, with the average human spending about a third of their life in this state. A sleep-like state of rapidly reversible

physical quiescence, with elevated thresholds to sensory stimulation, has been identified in most species, including even insects (Shaw, 2000) and nematodes (Raizen et al., 2008).

In addition to behavioural similarities with sleep, several anaesthetic drugs generate EEG rhythms that resemble those observed during different stages of sleep: halothane and isoflurane produce a theta rhythm (5–9 Hz) reminiscent of rapid eye movement (REM) sleep (Pang et al., 2009), whereas the GABA-ergic agent propofol and the α2-adrenoreceptor agonist dexmedetomidine induce slow-wave activity (<4 Hz) analogous to what is observed during non-REM (NREM) sleep (Gent and Adamantidis, 2017). Given the behavioural and electrophysiological similarities between sleep and the effects of several anaesthetic agents, the neuronal circuitry underlying sleep may provide critical insights into the mechanisms of anaesthetic action (Karan et al., 2007), with evidence that at least some anaesthetics do in fact intervene on sleep-wake regulating neurons, especially in hypothalamic areas (Franks, 2008; Zecharia et al., 2009; Zhang et al., 2015; Gent and Adamantidis, 2017)—although it should be noted that this similarity is not universal: some other anaesthetics produce desynchronised EEG with little resemblance to sleep EEG, e.g., ketamine, benzodiazepines (Gent and Adamantidis, 2017). The function of sleep is only partly understood, and several different theories have been put forward to explain the existence of this peculiar state (Vyazovskiy, 2015; Joiner, 2016; Krueger et al., 2016), including energy restoration (Berger and Phillips, 1995; Schmidt, 2014) memory consolidation (Abel et al., 2013) and synaptic homeostasis (Tononi and Cirelli, 2014, 2016). Nevertheless, the brain circuits that control sleep are relatively well understood: a wake-promoting and a sleep-promoting system interact in the brain (**Figure 1**; Saper et al., 2005; Luppi, 2010; Weber and Dan, 2016).

The ascending reticular activating system (Moruzzi and Magoun, 1949) comprises cholinergic, monoaminergic (serotonin, noradrenaline, histamine) and orexinergic nuclei in the brainstem, basal forebrain, and hypothalamus—with wide-ranging projections throughout the entire brain (Luppi, 2010). The hypothalamus also contains key sleep-promoting neuronal populations; in particular, the ventrolateral preoptic area (VLPO) and median preoptic area (MNPO) primarily express the inhibitory neurotransmitters γ-aminobutyric acid (GABA) and galanin, and project to all major hypothalamic and brainstem nuclei of the wake-promoting system (Sherin et al., 1996). Homeostatically arranged, the sleep-active neurons of the preoptic hypothalamus are in turn inhibited by the wake-active nuclei they target, especially those of predominantly noradrenergic and serotonergic transmitter phenotype (Gallopin et al., 2000; Chou et al., 2002). This architecture of mutually inhibitory wake-promoting and sleep-promoting circuits constitutes what is known as a "flip-flop switch" (Saper et al., 2001, 2005, 2010): a bistable system characterised by sharp transitions between its two possible states. Damage to the wake-promoting system causes excessive sleep, while insomnia results from damage to the VLPO (Economo, 1930; Lu et al., 2000). In addition to their sleep-promoting effects, VLPO neurons have also been implicated in the mechanisms of action of anaesthetic drugs (Moore et al., 2012; Zhang et al., 2015). Of note, recent evidence also indicates a common role of hypothalamic neuroendocrine cells of the mouse in sleep generation and general anaesthesia induced by several different anaesthetics, with opto- or chemo-genetic activation of these cells promoting both slow-wave sleep and anaesthesia, and the opposite result obtained by inhibiting them (Jiang-Xie et al., 2019).

Current theories propose that at least some anaesthetic drugs may exert their effect by recruiting the brain's endogenous mechanisms for the production of unconsciousness (Franks, 2008; Alkire et al., 2009; Scharf and Kelz, 2013; Van Swinderen and Kottler, 2014; but see Vanini et al., 2020, for a recent suggestion that this may not be the case, for isoflurane). This may occur through activation of the sleep-promoting pathways, inhibition of the wake-promoting ones, or both [especially since, given their mutually inhibitory nature, activating one will also result in inhibition of the other (Pace-Schott and Hobson, 2002)].

Neural Inertia and Sleep Inertia

Neural Inertia

"Neural inertia" refers to the brain's tendency to resist changes in its arousal state: it is manifested as emergence from anaesthesia (recovery of responsiveness, ROR) occurring at lower drug doses than those required for anaesthetic induction (loss of responsiveness, LOR) (Friedman et al., 2010). Thus, for intermediate dosages between those required for ROR and LOR, a given individual may be anaesthetised or awake, depending on their previous state. This "path dependence" (referred to as hysteresis in physics; **Figure 2**) is in contrast with pharmacokinetic-pharmacodynamic accounts, which assume that anaesthetic state is fully determined by current effect-site concentration of anaesthetic (McKay et al., 2006).

Rather, evidence of hysteresis between anaesthetic induction and emergence obtained in mice and *Drosophila* led to the proposal that the brain has a tendency to resist transitions in its arousal state, called "neural inertia" (Friedman et al., 2010). Indeed, bistable systems—of which the brain appears to be one, with respect to its sleep-wake states (Saper et al., 2001)—tend to show distinct non-overlapping paths between their states, indicating hysteresis (Chatterjee et al., 2008). Consistent with theoretical work on anaesthesia (Steyn-Ross et al., 2004), this is precisely what Friedman and colleagues observed with regard to anaesthetic induction and emergence in both mammals and invertebrates (Friedman et al., 2010). However, evidence for neural inertia in humans is less clear-cut (Sepúlveda et al., 2019) since it is not possible to measure anaesthetic concentration in the brain in the same way this is commonly done in animal models. Sepulveda and colleagues (Sepúlveda et al., 2018) found that LOR occurred at greater propofol concentrations than ROR, but noted that this result may be alternatively explained by incomplete equilibration between plasma and effect-site concentrations. A different team of researchers (Kuizenga et al., 2018) did not find evidence of neural inertia with propofol, whereas they did observe it with sevoflurane, when combined with the opioid remifentanil. These authors also observed that the choice of marker (behavioural endpoint) with respect to which to compute differences in drug concentration at induction and emergence

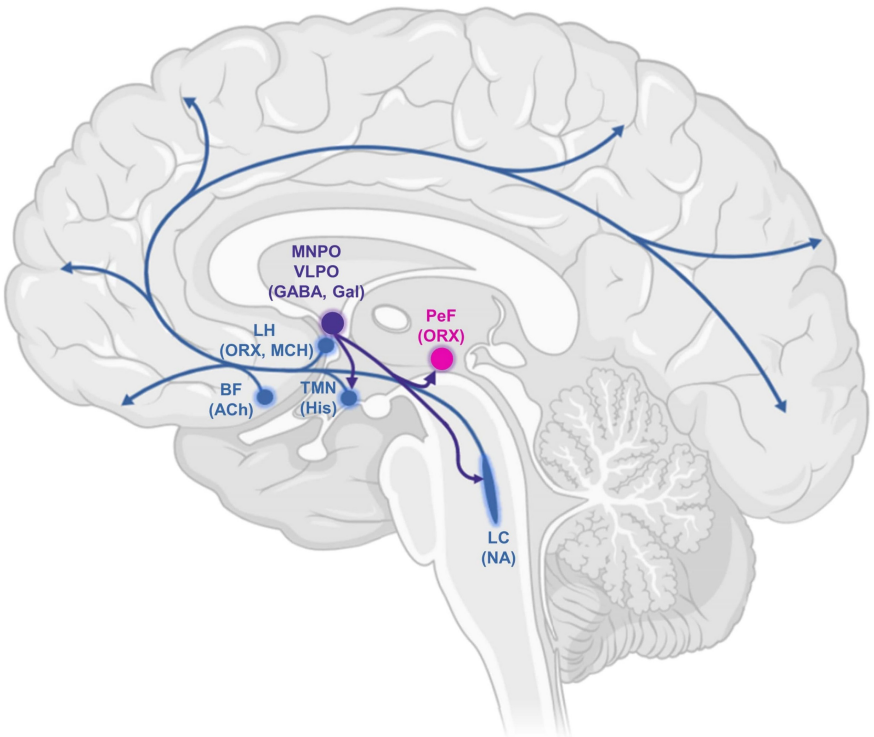

FIGURE 1 | Schematic drawing of some key components of the ascending arousal system, highlighting projections of the ventrolateral preoptic area. This comprises cortical projection neurons originating from the basal forebrain (BF); the recently characterised orexin/hypocretin neurons in the lateral hypothalamus (LH); perifornical orexin neurons (PeF); and several monoaminergic nuclei: the noradrenergic locus coeruleus (LC), the histaminergic tuberomammillary nucleus (TMN) and the ventrolateral preoptic area (VLPO) and median preoptic area (MNPO). Serotonergic and dopaminergic components are not shown. MCH, melanin-concentrating hormone; Gal, galanin; Ach, acetylcholine; ORX, orexin; His, histamine; NA, noradrenaline.

(e.g., loss and recovery of responsiveness, or EEG features) may also make a difference in investigators' ability to detect evidence of neural inertia (Kuizenga et al., 2018).

In line with this observation, Warnaby et al. (2017) reported hysteresis for the prevalence of slow-wave EEG activity for both propofol and sevoflurane, with or without addition of opioids; slow-wave persistence was therefore proposed as a marker of neural inertia in humans. While some authors (Colin et al., 2018) criticised this study by arguing that the hysteresis observed by Warnaby and colleagues can be collapsed if a different effect-site equilibration model is assumed, recent modelling work by Proekt and Kelz (2020) demonstrated that—since effect-site concentration is a theoretical construct that cannot be measured directly—it is experimentally impossible to distinguish between an equilibration model that collapses hysteresis and one that does not, even when hysteresis is in part attributable to genuine neuronal dynamics. Therefore, although it is clear that improved methodologies will be required (Proekt and Kelz, 2020), there is reason to believe that humans may also be subject to neural inertia—a postulation consistent with the unequivocal evidence that neural inertia is a widespread phenomenon observed in species as diverse as fruit flies, zebrafish, and rodents (Sepúlveda et al., 2019; Wasilczuk et al., 2020). As Proekt and Kelz observe: *"whereas going from the structured to the unstructured state is trivial, the restoration of structure is not generically expected after a dramatic perturbation"* (Proekt and Kelz, 2020). Thus, emergence may be an active rather than passive phenomenon, the understanding of which will likely need to invoke specific and distinct neurobiological mechanisms beyond a mere reversal of the induction process.

Sleep Inertia

Transitions in the brain's arousal state do not occur only after anaesthesia, but also after sleep. Familiar to many people, this state of transition between sleep and wakefulness, characterised by low levels of arousal and vigilance, sleepiness, confusion, and a temporary reduction in performance, is called sleep inertia (SI) (Tassi and Muzet, 2000; Voss, 2010; Trotti, 2017). Sleep inertia dissipates with time awake, with estimates of its typical duration ranging from 20 to 30 min (Dinges et al., 1987; Tassi et al., 1992) to 1–2 h post-awakening (Jewett et al., 1999). Although sleep inertia occurs even in the absence of sleep debt (Akerstedt and Folkard, 1997), its effects are more profound and long-lasting after a period of sleep deprivation (Ferrara and De Gennaro, 2000). Finally, waking up from slow-wave sleep appears to have the most profound negative impact on subsequent vigilance and performance (Dinges, 1990; Bonnet, 1993; Matchock and Mordkoff, 2014).

From a behavioural perspective, sleep inertia affects performance in the same way as sleepiness

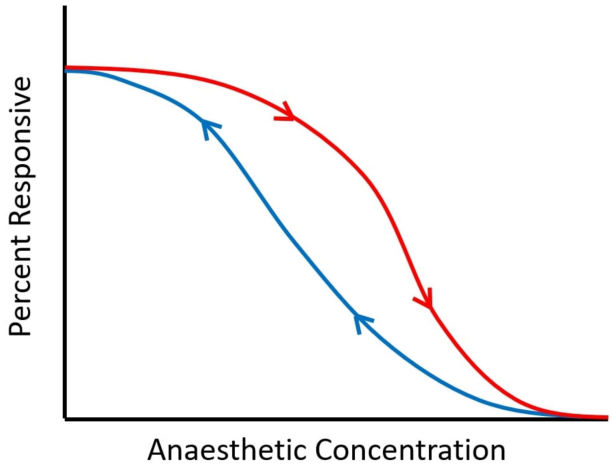

FIGURE 2 | Schematic of neural inertia. As anaesthetic dose is increased, responsiveness is diminished. However, the dose at which a certain proportion of responses is observed is not the same for induction (downward arrow, in red) and emergence (upward arrow, in blue), indicating path-dependence (hysteresis). Between the two curves, subjects may be awake or anaesthetised, depending on whether the drug concentration is being increased or decreased. The wider the gap between the two curves, the greater the hysteresis.

(Balkin and Badia, 1988). The human electroencephalographic (EEG) signatures of sleep inertia are also analogous to what is observed at increased levels of sleepiness (Voss, 2010). For approximately 10 min post-awakening, EEG is characterised by elevated low-frequency (1–9 Hz) and reduced beta (18–25 Hz) power (Ogilvie and Simons, 1992; Ferrara et al., 2006; Marzano et al., 2011). Analogous results have been obtained in rodents using intracranial recordings during the first 10 min post-sleep: neuronal activity was low upon awakening, with brief periods of neuronal silence (Vyazovskiy et al., 2014). Crucially, such population OFF periods are typically observed not only during sleep, but also after prolonged wake, as revealed by intracranial recordings in rats (Vyazovskiy et al., 2011). Likewise, recordings in monkeys transitioning from wake to sleep show sleep-like patterns of activity in their visual cortex, even while performing a visual task (Pigarev et al., 1997). Thus, across species sleep inertia appears to be the post-sleep counterpart of pre-sleep sleepiness, with both states characterised by similar behavioural changes and EEG signatures, as well as local sleep-like OFF periods.

Neural Inertia as the Effect of Sleep Inertia

Single-gene mutations that increase or decrease neural inertia also affect the sleep-wake cycle, pointing to a connection between anaesthesia, neural inertia and sleep in both invertebrates and mammals (Friedman et al., 2010; Joiner et al., 2013). Here, we propose that neural inertia—the reduction in anaesthetic dose required for emergence compared to induction—may be an effect of the sleep inertia that follows anaesthetic-induced sleep. Specifically, GABA-ergic anaesthetics such as propofol and the inhalational agents sevoflurane, isoflurane, and halothane are believed to induce a state of artificial sleep (Brown et al., 2010; Van Swinderen and Kottler, 2014; but see Vanini et al., 2020). Like natural sleep, this artificial sleep should then be followed by sleep inertia—especially for intravenous drugs such as propofol that induce an artificial sleep characterised by high levels of slow-wave activity (SWA) (Brown et al., 2011; Murphy et al., 2011; Gent and Adamantidis, 2017), since sleep inertia is particularly pronounced upon awakening from slow-wave sleep (Dinges, 1990).

Thus, in the process of emerging from anaesthesia the brain would find itself in the state of sleep inertia, which is behaviourally and neurally equivalent to sleepiness. Since sleepiness is known to increase susceptibility to anaesthesia with propofol, isoflurane, and sevoflurane by lowering the dose that is required for induction, as indicated by rodent studies (Tung et al., 2002; Pal et al., 2011; Scharf and Kelz, 2013), this could explain neural inertia: due to being in a state equivalent to sleepiness, the brain during emergence is more susceptible to anaesthetics than it was at induction, and a smaller dose is sufficient to maintain unconsciousness—producing the hysteresis characteristic of neural inertia.

If this hypothesis is correct, then we predict that neural inertia should be larger when awakening from "recovery sleep" after sleep deprivation, since sleep deprivation increases the sleep inertia that is observed after awakening (Ferrara and De Gennaro, 2000). This is precisely what is observed empirically, with higher neural inertia in previously sleep-deprived animals (Joiner et al., 2013). Moreover, this hypothesis could explain why Friedman and colleagues (Friedman et al., 2010) observed greater neural inertia with halothane than with isoflurane—a result that was recently replicated in mice exposed to equipotent doses of isoflurane, sevoflurane, and halothane, demonstrating that different anaesthetics have different effects on neural inertia, distinct from their potency (Wasilczuk et al., 2020). Specifically, to explain these results we note that unlike isoflurane, halothane does not reduce NREM sleep-debt in rodents (Pick et al., 2011; Scharf and Kelz, 2013). Thus, higher levels of NREM sleep debt would be present upon emergence from halothane than isoflurane, leading to stronger sleep inertia, and hence stronger neural inertia, as observed.

Thus, we have proposed that anaesthesia causes artificial, SWA-rich sleep, which in turn induces sleep inertia. The latter's effects resemble those of sleepiness, which increases sensitivity to anaesthetics. Therefore, a lower dose of anaesthetic will suffice to keep the brain anaesthetised, resulting in neural inertia at emergence (**Figure 3**). This hypothesis for the origin of neural inertia could be tested by inducing anaesthesia during the state of sleep inertia, and assessing the prediction that the induction dose will be lower than usual and comparable to the drug level at which emergence typically occurs.

Furthermore, our hypothesis predicts that in the presence of neural inertia, neural activity during emergence should resemble the patterns of sleep-like activity characteristic of sleepiness and sleep inertia—and indeed, there is evidence that slow-wave activity reminiscent of sleep dominates human EEG at the beginning of emergence from anaesthesia, before most patients transition to non-slow-wave activity and subsequent waking

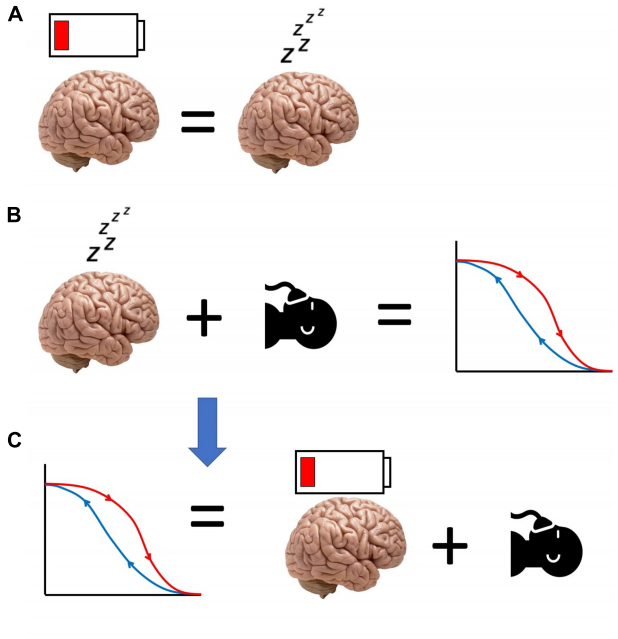

FIGURE 3 | Schematic of our hypothesis equating neural inertia with post-anaesthetic sleep inertia. **(A)** Sleep inertia (represented by the depleted battery icon over the brain) is neurally and behaviourally equivalent to sleepiness. **(B)** Sleepiness reduces the need for anaesthetic, and increases post-anaesthetic neural inertia. Therefore, **(C)** neural inertia may be seen as the manifestation of sleep inertia occurring after anaesthesia, reducing the amount of anaesthetic that is needed for the brain to be unresponsive.

(Chander et al., 2014). Additionally, individual measures of susceptibility to sleep inertia could be used to predict individual susceptibility to neural inertia, such as the recently developed Sleep Inertia Questionnaire (Kanady and Harvey, 2015). Indeed, there is already evidence that state-dependent EEG markers at baseline can predict individual susceptibility to anaesthetic induction with propofol (Chennu et al., 2016; Zhang et al., 2020), and future research may seek to determine whether such markers are related to sleep inertia.

We also note that our hypothesis would likely not apply to the dissociative anaesthesia induced by ketamine, whose molecular mechanisms of action and neurophysiological effects at the micro- and macroscale are very different from other known anaesthetics, and do not appear to resemble sleep (Hemmings et al., 2019). Although we are not aware of tests of neural inertia with ketamine, our hypothesis leads us to predict that little should be observed, since sleep does not seem to be involved in the context of dissociative anaesthesia. Testing this prediction in humans is not straightforward, for the same reason that complicates existing attempts to identify neural inertia in humans (Sepúlveda et al., 2019): namely Proekt and Kelz (2020) demonstrated that since effect-site concentration cannot be measured directly, effect-site models could be constructed to collapse hysteresis even when it would actually be attributable to genuine neuronal dynamics. However, the hypothesis is not specific to humans and could be tested in other species for which neural inertia has already been demonstrated with other anaesthetics (Friedman et al., 2010; Joiner et al., 2013; McKinstry-Wu et al., 2019; Wasilczuk et al., 2020), with the prediction being that little hysteresis should be observed. Additionally, we reported above that if neural inertia is due to the increased susceptibility to anaesthetics that occurs during post-anaesthetic sleep inertia, then our hypothesis predicts that higher susceptibility to anaesthesia should be observed during sleep inertia (e.g., as induced by awakening from slow-wave sleep). We expect that ketamine would constitute an exception to this general prediction—which should be testable in humans.

Recently, a modelling study observed that neural inertia is compatible with an account of the brain as a bistable system, stochastically switching between two states (Proekt and Hudson, 2018). If the states are seen as wells in an energy landscape, the system can be conceptualised as transitioning between them whenever noise-driven (stochastic) fluctuations are large enough to overcome the energy differential between the wells. Under conditions of low noise, the system is therefore more likely to remain trapped in whatever state it is currently occupying, and therefore inertia (resistance to state transitions) will be observed (Proekt and Hudson, 2018). It is important to note that our hypothesis of neural inertia as the effects of sleep inertia arising from anaesthetic-induced "artificial sleep" is not incompatible with this account of neural inertia: the two operate at different levels of explanation (Marr, 2010). In fact, if our hypothesis is correct, then it suggests that the account of Proekt and Hudson (2018) could also be invoked to understand sleep inertia.

If corroborated, the hypothesis presented here could have direct relevance for clinical practice: anaesthetists could use tools such as the recently developed Sleep Inertia Questionnaire (Kanady and Harvey, 2015) to evaluate each patient's individual susceptibility to sleep inertia, which we expect should predict (together with their current amount of sleep debt) their individual likelihood of experiencing neural inertia.

Neuroimaging Evidence: Diminished Anticorrelations in the Inert Human Brain

At the macroscale, there is additional recent evidence to suggest that anaesthesia resembles the state of sleep inertia. Under conditions of normal restfulness, it is well known from functional MRI that the human brain self-organises into distinct sets of brain regions, known as resting-state networks (Yeo et al., 2011; Smith et al., 2012). In particular, a "default mode" network (DMN) of medial frontal and parietal regions, and a set of "task-positive" networks such as the "executive control" network of lateral fronto-parietal regions (FPN) and the "dorsal attention network" (DAN) tend to exhibit anticorrelated patterns of activation (Raichle et al., 2001; Fox et al., 2005) (but note that the DMN can also be recruited by tasks, especially pertaining to self-referential cognition, "mental time travel," or automated processing (Vatansever et al., 2015a,b, 2017; Buckner and DiNicola, 2019) (**Figure 4**).

Intriguingly, recent EEG-fMRI evidence indicates that loss of DMN-FPN/DAN anticorrelations is a neural correlate of sleep inertia itself in humans (Vallat et al., 2018; but see Chen et al.,

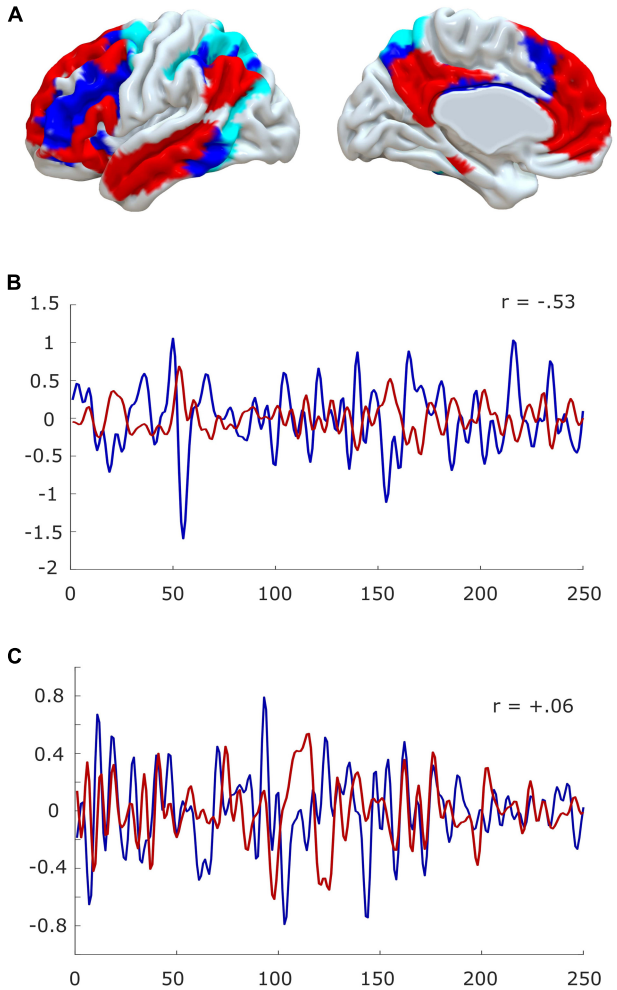

FIGURE 4 | Anticorrelations in the human brain. **(A)** Surface projection of the default mode network (red) and fronto-parietal (blue)/dorsal attention networks (cyan) onto medial and lateral surfaces of a standard brain (left hemisphere). **(B)** The timecourses of default mode and fronto-parietal networks are anti-correlated during quiet wakefulness. **(C)** Anticorrelations are reduced or even abolished in the anaesthetised brain. Data from one representative subject, before and during propofol anaesthesia; for experimental details, see Stamatakis et al. (2010) and Varley et al. (2020).

2020). Indeed, earlier work had also demonstrated, by employing positron emission tomography (PET) that for a short period of time after awakening (5–20 min, compatible with the duration of sleep inertia; Trotti, 2017), there is a gradual increase of cerebral blood flow in heteromodal areas, especially lateral prefrontal cortex (lPFC), a core component of the executive control network (Balkin et al., 2002). Additionally, as previously mentioned, both awakening from deep sleep and previous sleep deprivation intensify subsequent sleep inertia upon awakening. And indeed, a loss of DMN-FPN/DAN anticorrelations is also observed during sleep in humans (Sämann et al., 2011), as well as in the awake but sleep-deprived human brain (De Havas et al., 2012). Thus, sleep inertia and conditions that favour it, share a common neural substrate in the reduction of DMN-FPN/DAN anticorrelations. Conversely, caffeine consumption, perhaps the most widely adopted countermeasure to sleep inertia (Van Dongen et al., 2001) is known to have the opposite effect: it increases the anticorrelations between DMN and FPN/DAN in the human brain (Wong et al., 2012).

This suggests that sleep inertia, at least in the human brain, may correspond to a carry-over of diminished DMN-FPN/DAN anticorrelations. Remarkably, perturbed DMN-FPN/DAN interactions are also one of the most robustly observed neural markers of human loss of consciousness induced by a variety of anaesthetics (Boveroux et al., 2010; Guldenmund et al., 2013; Golkowski et al., 2019; Luppi et al., 2019, 2020; Huang et al., 2020) (**Figure 4**), and the anticorrelations are even diminished one hour after emergence from sevoflurane anaesthesia (Nir et al., 2020). Thus, we propose that neural inertia may be the effect of anaesthetic-induced sleep inertia, which corresponds to a carry-over of diminished anticorrelations between DMN and FPN/DAN. In other words, we propose that the inert brain is a brain that has lost its characteristic anticorrelations. This specific hypothesis could be empirically tested, since it predicts that humans experiencing higher neural inertia after anaesthesia should exhibit more prominent loss of anticorrelations.

Inertia in the Aging Brain

Intriguingly, the hypothesis presented here may also explain why older adults are more susceptible to neural inertia (Warnaby et al., 2017). Namely, according to the present view, this is because they are more susceptible to sleep inertia. Reduced and fragmented sleep is common among the elderly, and especially patients with Alzheimer's disease (Bonanni et al., 2005; Guarnieri et al., 2012). Since fragmented sleep tends to increase subsequent slow-wave activity (Bonnet, 1987), awakening from which causes higher levels of sleep inertia (Dinges, 1990), as does sleep deprivation, the elderly should show higher levels of sleep inertia. This is indeed the case (Silva and Duffy, 2008).

Additionally, if the hypothesis proposed here about the link between sleep inertia and neural inertia is correct, these populations should also suffer from higher levels of neural inertia. Again, this is precisely what is observed: rat studies indicate that ageing increases sensitivity to anaesthetics, and prolongs their effect (Chemali et al., 2015); likewise, older humans are also more susceptible to anaesthesia (Kanonidou and Karystianou, 2007). Furthermore, recent evidence indicates that age influences the newly discovered EEG marker of neural inertia in humans, slow wave activity saturation (SWAS): SWAS is more likely to cease abruptly rather than gradually in older patients, predicting their likelihood of post-operative delirium (Warnaby et al., 2017).

Neuroimaging evidence in older adults further supports the link between sleep and neural inertia and loss of anticorrelations between DMN and FPN/DAN: it is well established that aging corresponds to a reduction of anticorrelations between these networks (Keller et al., 2015; Siman-Tov et al., 2017), even in the absence of concomitant psychiatric conditions (Kobuti Ferreira et al., 2015) and more so in those with mild cognitive impairment (Esposito et al., 2018). Thus, older brains are intrinsically more

prone to loss of anticorrelations, and suffer from higher sleep inertia and higher neural inertia.

Molecular Mechanisms of Sleep and Neural Inertia

Orexin/Hypocretin

One candidate system for the control of sleep inertia—and hence, we have argued, neural inertia—is the orexinergic system. Located exclusively in the lateral hypothalamus (De Lecea et al., 1998; Sakurai et al., 1998), orexin/hypocretin neurons are wake-active (Lee et al., 2005; de Lecea and Huerta, 2014), and innervate the wake-promoting monoaminergic and cholinergic nuclei (Carter et al., 2012). And indeed, using channelrhodopsin-2 to selectively stimulate orexin neurons promotes awakening from sleep in mice (Adamantidis et al., 2007), and increased wakefulness is reported in rodents after orexin-A administration, either intracerebroventricular or directly into monoaminergic and cholinergic wake-promoting nuclei (Hagan et al., 1999; Sakurai and Mieda, 2011).

Conversely, optogenetic suppression of orexin neurons with archaerhodopsin has sleep-promoting effects in mice (Tsunematsu et al., 2011, 2013); and in humans, orexin blockers are now available as medication against insomnia (Bennett et al., 2014). These effects were confirmed using Designer Receptors Exclusively Activated by Designer Drugs (DREADDs) to chemogenetically activate or silence orexin neurons, resulting in increased wakefulness or sleep in rodents, respectively (Sasaki et al., 2011). Loss of orexin neurons causes the sleep disorder narcolepsy in dogs (Lin et al., 1999) and humans (Nishino et al., 2000; Thannickal et al., 2000), and the same is obtained by selective orexin knock-out in mice (Chemelli et al., 1999; Mochizuki et al., 2004), as well as pharmacological lesions in rats (Gerashchenko et al., 2001). Crucially, narcolepsy is characterised by an unstable and fragmented sleep-wake cycle, and difficulty in becoming awake (i.e., high sleep inertia) (Scammell, 2003). Indeed, sleep inertia is often present in narcoleptic children (Wise, 1998).

Thus, there is ample evidence, in both humans and other animals, that orexin and orexinergic neurons play a crucial role in sleep-wake regulation (Mieda, 2017). A recent computational study indicates that the specific role of orexin may be to stabilise the transitions between sleep and wake (Fulcher et al., 2014). According to the model, a bistable region of state-space exists when the inputs to the sleep-promoting and wake-promoting systems are balanced, and state transitions are easy (**Figure 5**). By increasing the activity of wake-promoting monoaminergic nuclei upon awakening, orexin pushes the system out of the bistable region, stabilising it. Indeed, simulating orexin loss in the model lowered transition thresholds, resulting in frequent wake-sleep transitions and sleep fragmentation, analogous to what is observed in orexin-deficient narcoleptic patients.

Intriguingly, recent neuroimaging work using a variant of functional MRI called MR encephalography, which has high temporal resolution (100 ms), determined that human narcoleptic patients have aberrant interactions between DMN and FPN/DAN, characterised by delayed and monotonic

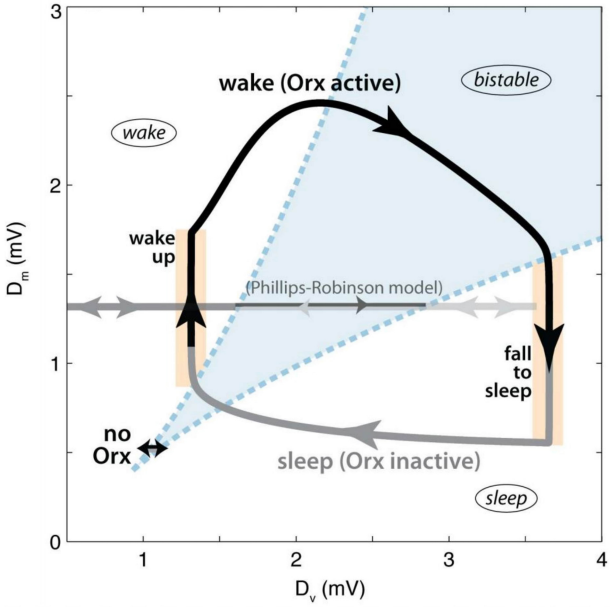

FIGURE 5 | Dynamics of orexin stabilisation of state transitions according to the model of Fulcher et al. (2014). The axes represent the net drives to the wake-promoting (D_M) and sleep-promoting (D_V) circuits. Regions are labelled, with the bistable region shown in blue. The black arrow represents the waking period, while the grey arrow represents sleep. The trajectory marked "no Orx" represents the dynamics of the model in the absence of orexin input. Figure adapted from Figure 3 of Fulcher et al. (2014), published under CC-BY licence.

interactions, which the authors interpreted as a compromised ability of task-positive networks to suppress the DMN (Järvelä et al., 2020); Once again, this observation is in line with our proposed macroscale identification of sleep inertia with abnormal anticorrelations between large-scale networks of the brain.

Thus, evidence suggests that low orexin levels lead to high levels of sleep inertia, and its associated neural signatures. According to the hypothesis developed here, such high sleep inertia should be accompanied by high levels of neural inertia. This is indeed the case: case reports suggest high neural inertia in at least some narcoleptic human patients (Mesa et al., 2000; Burrow et al., 2005), confirmed by the increased neural inertia observed in rodents with narcolepsy arising from genetic ablation of orexin neurons (Hara et al., 2001; Kelz et al., 2008). Moreover, orexin is known to be involved in anaesthetic action: the activity of orexin neurons is reduced by propofol, sevoflurane and isoflurane, as indicated by a reduced number of c-Fos-immuno-reactive orexinergic neurons in rodents (Kelz et al., 2008; Zhang et al., 2012; Scharf and Kelz, 2013). Moreover, rodent studies show that reduced activation of orexin neurons during anaesthesia is exacerbated when the anaesthesia is administered under conditions of sleep deprivation (Ran et al., 2015). Conversely, intracerebroventricular administration of orexin-A (though not orexin-B) causes emergence from propofol, isoflurane and sevoflurane anaesthesia in rats (Dong et al., 2009; Shirasaka et al., 2011; Zhang et al., 2012, 2016), and similar results have also been obtained in mice, whereby activation of orexin

neurons with DREADDs facilitated emergence from isoflurane anaesthesia (Zhou et al., 2018). Thus, orexin appears to play a major role in anaesthesia and the sleep-wake cycle, with its absence increasing both sleep inertia and neural inertia.

Noradrenaline

The action of orexin neurons is believed to occur mainly through excitation of monoaminergic wake-promoting nuclei, which they innervate (Sakurai and Mieda, 2011). In particular, orexinergic neurons may exert their effects on sleep-wake transitions through the noradrenergic *locus coeruleus* (LC) (Carter et al., 2012). Orexin neurons send strong excitatory projections to the LC, and the wake-inducing effect of orexin infusion involves activation of the LC (Hagan et al., 1999).

Indeed, the fragmented sleep-wake cycle of narcolepsy was reconsolidated by restoring orexin receptors in the LC of mice, and equivalent results were achieved by chemogenetically activating these neurons with DREADDs (Hasegawa et al., 2014). Furthermore, optogenetic inactivation of LC prevents the arousal-promoting effect of optogenetically activating orexin neurons; conversely, the latter is potentiated by concomitant stimulation of LC neurons (Carter et al., 2012). Thus, there is strong evidence that noradrenergic system activity is one of the primary routes through which orexin neurons perform their regulatory role (De Lecea, 2015).

Specifically supporting a role for noradrenaline in neural inertia, previous work (Friedman et al., 2010) established that genetic deletion of dopamine-ß-hydroxylase (DBH) in mice to remove noradrenergic signalling resulted in hypersensitivity to isoflurane anaesthesia, as well as increased neural inertia. This could be reversed by pharmacologic CNS-specific rescue of adrenergic signalling, achieved by providing the amino acid L-DOPS so that it would be converted into noradrenaline by L-amino acid decarboxylase (Friedman et al., 2010). In humans, Kuizenga et al. (2018) reported evidence of neural inertia when sevoflurane was supplemented with remifentanil, which is believed to influence sleep-wake regulation through adrenergic neurotransmission (McCormick and Bal, 1997; Samuels and Szabadi, 2008).

Indeed, implication of orexin and noradrenaline in neural inertia has been considered before (Sepúlveda et al., 2019; Wasilczuk et al., 2020). Wasilczuk et al. (2020) observed that halothane does not suppress hypothalamic orexinergic neurons and LC noradrenergic neurons (Gompf et al., 2009), whereas isoflurane does suppress them (Kelz et al., 2008). Thus, these authors proposed that this difference may underlie the increased neural inertia induced by halothane compared with isoflurane (Friedman et al., 2010; Wasilczuk et al., 2020) due to non-abolished orexinergic activity. As mentioned above, our own explanation of the same phenomenon is in terms of halothane failing to reduce sleep debt, unlike isoflurane (Pick et al., 2011), thereby producing more sleep inertia (and hence neural inertia, according to our account). These two explanations are not in contrast: indeed, they suggest that a fruitful avenue for future research may be to seek a connection between persistent orexinergic activity and halothane's failure to discharge sleep debt.

On the other hand, studies providing a direct link between noradrenaline and sleep inertia are presently lacking; nevertheless, several indirect lines of evidence suggest that low levels of noradrenaline may be related to sleep inertia. Behaviourally, noradrenaline is implicated in cognitive functions such as sustained attention and working memory (Chamberlain and Robbins, 2013; Spencer et al., 2015), which are especially vulnerable to sleep deprivation (Goel et al., 2009; Killgore, 2010)—of which sleep inertia is a post-awakening counterpart, we have argued here. Noradrenaline is also increased following consumption of coffee (Papadelis et al., 2003), and caffeine consumption can reverse many of the cognitive adverse effects of clonidine (Smith et al., 2003), which mimics the state of reduced arousal observed as a result of sleep deprivation by reducing turnover of central noradrenaline, by binding to autoreceptors (Nutt and Glue, 1988).

Recently, Bellesi et al. (2016) used *in vivo* microdialysis to demonstrate decreasing levels of prefrontal noradrenaline in rodents undergoing sleep deprivation, correlating with an increase in low EEG frequencies tracking the need to sleep. Thus, low levels of prefrontal noradrenaline could contribute to explain the cognitive deficits observed during sleepiness induced by prolonged wakefulness. Crucially, noradrenaline restoration to baseline levels post-awakening was slower in prefrontal cortex than in other areas, such as M1—and in humans, prefrontal regions are those that were found to have reduced cerebral blood flow upon awakening in the PET study of Balkin et al. (2002). Thus, evidence suggests that decreased prefrontal noradrenaline could also explain the confusion and cognitive deficits observed during sleep inertia—especially since this state is very similar to sleepiness, as we have shown. This evidence also suggests that, if our hypothesis is correct, then we should expect noradrenaline to modulate the prevalence of anticorrelations between DMN and FPN/DAN in the human brain, since anticorrelations are also enhanced by caffeine and decreased by sleepiness (De Havas et al., 2012), sleep (Sämann et al., 2011), sleep inertia (Vallat et al., 2018), and anaesthesia (Boveroux et al., 2010; Golkowski et al., 2019; Luppi et al., 2019; Huang et al., 2020). Interestingly, recent studies indicate that caffeine infusion can accelerate emergence from isoflurane anaesthesia in both rodents and humans (Fong et al., 2018; Fox et al., 2020), and future research may seek to determine whether this effect corresponds to faster recovery of anticorrelations in the brain after anaesthesia (Nir et al., 2020) and whether it is specifically attributable to caffeine's action on noradrenergic neuromodulation (Papadelis et al., 2003; Smith et al., 2003).

Indeed, as major wake- and alertness-promoting neurotransmitter, noradrenaline is modulated by both sleep and anaesthesia—just as we should expect if noradrenaline were involved in both sleep and neural inertia, as we propose here. Noradrenaline levels are highest during wake and drop during sleep (Léna et al., 2005) and stimulation of the noradrenergic LC of mice induces waking (Carter et al., 2010, 2013; Berridge et al., 2012); activity of the LC is inhibited by GABA during sleep (Gervasoni et al., 1998), as well as during propofol and isoflurane anaesthesia in mice (Zecharia et al., 2009). Administration of noradrenaline

by microinjection into the central medial nucleus of the thalamus accelerates emergence from propofol anaesthesia in rodents, and reverses the local physiological effects of propofol (Fu et al., 2016). Likewise, pharmacogenetic activation of noradrenergic neurons in the LC with virally delivered DREADDs promotes EEG markers of neural arousal and accelerates emergence from isoflurane anaesthesia in rats, an effect that can be prevented by application of noradrenergic antagonists (Vazey and Aston-Jones, 2014). The anaesthetic dexmedetomidine also operates on noradrenergic transmission: as an adrenergic α-2 receptor agonist, it decreases the firing of LC neurons (Nelson et al., 2003), and indeed α-2A receptor activation inhibits noradrenergic LC neurons (Lakhlani et al., 1997).

Although it was originally thought that dexmedetomidine would induce sedation by inhibiting the LC (Sanders and Maze, 2012) thereby removing the noradrenergic inhibition on the sleep-promoting VLPO neurons (Nelson et al., 2003), recent evidence suggests a more intricate picture: acute inhibition of LC neurons does not induce strong sleep in mice (Carter et al., 2010), and LC inhibition is not required for low doses of dexmedetomidine to produce their sedative effects, since knockdown of LC α2A adrenergic receptors in mice does not prevent sedation, even though loss of the righting reflex is still observed at high doses (Zhang et al., 2015). Intriguingly, the same hypothalamic neurons in the mouse are involved in inducing recovery sleep and dexmedetomidine-induced sedation, by locally exciting neurons in the preoptic area (Zhang et al., 2015).

Other studies also indicate a more complicated picture: microdialysis of noradrenaline into rat prefrontal or parietal cortex under constant levels of sevoflurane anaesthesia failed to produce wake-like behaviour—although it did produce wake-like EEG (Pal et al., 2018). Similar failure to awaken rats from continuous sevoflurane anaesthesia was also reported after pharmacological blockade of noradrenaline reuptake (Kenny et al., 2015). Since cholinergic stimulation of prefrontal cortex did induce wake-like behaviour in the rats studies by Pal et al. (2018), this evidence suggests that a full picture will likely need to also take additional neuromodulatory systems into account. Dopamine in particular has been implicated, largely in rodent studies. Lesions to the wake-active dopaminergic ventral tegmental area in the brainstem shorten the induction time of anaesthesia, and lengthen the time taken for recovery—whereas both electrical and optogenetic stimulation of the VTA can reverse the anaesthetic effects of propofol in rats and mice (Solt et al., 2014; Taylor et al., 2016). These contributions of dopaminergic signalling have recently also been extended to a dopaminergic population in the ventral periaqueductal grey (Li et al., 2018; Liu et al., 2020). Given the shared pathways of dopaminergic and noradrenergic transmitter production, it seems plausible that these transmitters and their nuclei in the brainstem may act in-concert to produce wakefulness, and to counter the effects of sleep inertia and neural inertia, as evidenced by their influences on recovery and induction times. Likewise, the recent discovery that hypothalamic neuroendocrine cells are involved in both slow-wave sleep and general anaesthesia induced by multiple classes of anaesthetic drugs (Jiang-Xie et al., 2019) suggests that a fuller understanding of the link between sleep and neural inertia may benefit from taking into account neuroendocrine involvement.

DISCUSSION

Overall, there is converging human and animal evidence that neural inertia strongly resembles sleep inertia, in terms of both behavioural manifestations and microscale and macroscale neural markers. Both phenomena are influenced by orexin neurons, which seem to perform a state-stabilising function via noradrenergic transmission. Loss of orexin neurons in narcolepsy, results in fragmented sleep-wake cycles and increases in both sleep inertia and neural inertia. Therefore, we have argued here that neural inertia may in fact be a manifestation of sleep inertia, as it occurs after the artificial slow-wave sleep induced by anaesthetics. Of note, this hypothesis can account for phenomena as diverse as the higher inertia-inducing properties of halothane vs. isoflurane (Friedman et al., 2010; Wasilczuk et al., 2020), and the increased susceptibility to neural inertia in the elderly and in narcoleptic patients.

If our hypothesis is correct, then it could have implications for clinical practice: by assessing each patient's individual susceptibility to sleep inertia and current sleep debt, anaesthetists may be able to estimate individual likelihood of their patient experiencing neural inertia. In turn, this may better equip them to counteract adverse effects such as post-anaesthetic delirium (Warnaby et al., 2017; Sepúlveda et al., 2019).

Multiple sources of evidence—behavioural and neurophysiological, in animals and humans—suggest that orexin may play a stabilising effect between states of sleep and wakefulness, possibly (though likely not exclusively) through its effects on locus coeruleus noradrenergic neurons. Together, these neuromodulatory systems may be key in determining sleepiness, sleep inertia and what we have argued is its post-anaesthetic counterpart: neural inertia. Nevertheless, direct evidence explicitly linking all pieces of this puzzle together is still lacking, and even evidence of a link between noradrenergic modulation and sleep inertia is at present only indirect. Further studies explicitly investigating involvement of noradrenaline and other neuromodulators in relation to sleep inertia remain necessary, as a test of the hypothesis presented here.

Of course, the brain is a remarkably complex system. There are other components of the sleep- and wake-promoting circuits beyond orexin and noradrenaline, and they are likely to play some direct or indirect role in the phenomena of sleep inertia and neural inertia, and the stabilisation of arousal states more broadly. All these circuits are intricately interconnected, and changes in one are likely to have multiple repercussions. Indeed, investigating such interactions will be required to further elucidate the hypothesis proposed here. Nevertheless, here we have provided a number of predictions that are testable with current scientific techniques, and we hope that these predictions

will stimulate fruitful avenues for further research—whether or not they ultimately support our hypothesis.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article, further inquiries can be directed to the corresponding author.

AUTHOR CONTRIBUTIONS

AL: conceptualisation and writing—original draft. LS and ES: writing—editing. ES: supervision. ES and DM: project administration and funding acquisition. All authors contributed to the article and approved the submitted version.

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The Impacts of Age and Sex in a Mouse Model of Childhood Narcolepsy

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Narcolepsy is a sleep disorder caused by selective death of the orexin neurons that often begins in childhood. Orexin neuron loss disinhibits REM sleep during the active period and produces cataplexy, episodes of paralysis during wakefulness. Cataplexy is often worse when narcolepsy develops in children compared to adults, but the reason for this difference remains unknown. We used *orexin-tTA; TetO DTA* mice to model narcolepsy at different ages. When doxycycline is removed from the diet, the orexin neurons of these mice express diphtheria toxin A and die within 2–3 weeks. We removed doxycycline at 4 weeks (young-onset) or 14 weeks (adult-onset) of age in male and female mice. We implanted electroencephalography (EEG) and electromyography (EMG) electrodes for sleep recordings two weeks later and then recorded EEG/EMG/video for 24 h at 3 and 13 weeks after removal of doxycycline. Age-matched controls had access to doxycycline diet for the entire experiment. Three weeks after doxycycline removal, both young-onset and adult-onset mice developed severe cataplexy and the sleep-wake fragmentation characteristic of narcolepsy. Cataplexy and maintenance of wake were no worse in young-onset compared to adult-onset mice, but female mice had more bouts of cataplexy than males. Orexin neuron loss was similarly rapid in both young- and adult-onset mice. As age of orexin neuron loss does not impact the severity of narcolepsy symptoms in mice, the worse symptoms in children with narcolepsy may be due to more rapid orexin neuron loss than in adults.

Keywords: orexin, narcolepsy, cataplexy, age, sex, mice, pediatric, childhood

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INTRODUCTION

Orexins are wake-promoting neuropeptides necessary for the maintenance of long periods of wakefulness and the regulation of REM sleep (Saper et al., 2001; Lu et al., 2006; Branch et al., 2016; Chowdhury et al., 2019). Narcolepsy is caused by severe loss of the orexin-producing neurons in the hypothalamus, and the resulting symptoms of narcolepsy include excessive daytime sleepiness, the occurrence of REM sleep at any time of day, and cataplexy – episodes of muscle atonia during wakefulness that are likely produced by some of the same neural mechanisms that produce atonia during REM sleep (Mahoney et al., 2019). Most patients develop narcolepsy before the age of 25,

Abbreviations: DOX, doxycycline; DOX−, mice removed from doxycycline; DOX+, mice maintained on doxycycline.

most commonly between age 10 and 20 (Yoss and Daly, 1960; Dauvilliers et al., 2001; Ohayon et al., 2005; Longstreth et al., 2009).

The symptoms of narcolepsy are usually more severe when the disease begins in childhood compared to adults. Children with narcolepsy are often sleepier than adults with narcolepsy, as indicated by shorter sleep latencies on the Multiple Sleep Latency Test (Young et al., 1988) and more total sleep over 24 h (Pizza et al., 2013). In adults, cataplexy is usually triggered by strong, positive emotions, but children can have spontaneous cataplexy (Serra et al., 2008; Overeem et al., 2011; Plazzi et al., 2011). Cataplexy typically lasts only 1–2 min in adults (Overeem et al., 2011), but children with narcolepsy can have status cataplecticus, periods of muscle weakness lasting hours which is extremely rare in adults (Quinto et al., 2005; Simon et al., 2004; Calabro et al., 2007; Ping et al., 2007; Panda, 2014; Antelmi et al., 2017). Nearly half of young patients report that cataplexy is their most disruptive symptom, yet even with treatment, more than 40% have cataplexy every day (Maski et al., 2017). Though longitudinal studies are sparse, it appears that this severe sleepiness and cataplexy with childhood-onset narcolepsy lessens over a few years, developing into the pattern typical of adults (Plazzi et al., 2011; Pizza et al., 2013). Narcolepsy onset in younger children is particularly disruptive because it is associated not only with more severe symptoms but also with precocious puberty and obesity, indicating multisystem disruption (Kotagal et al., 1990, 2004; Plazzi et al., 2006; Vendrame et al., 2008; Poli et al., 2013; Ponziani et al., 2016).

It is currently unknown why symptoms of narcolepsy are so severe with disease onset in childhood. To address this question, we compared the effects of orexin neuron loss in young mice vs. adult mice using *orexin-tTA; TetO DTA* mice, a novel mouse model that enables control over of the timing of orexin neuron loss (Tabuchi et al., 2014).

MATERIALS AND METHODS

Animals

All experiments were approved by the Institutional Animal Care and Use Committee of Beth Israel Deaconess Medical Center and Harvard Medical School and were performed in accordance with the National Institutes of Health *Guide for the Care and Use of Laboratory Animals*.

To induce orexin neuron death, we used male and female *orexin-tTA; TetO DTA* mice (Tabuchi et al., 2014). These mice express diphtheria toxin A (DTA) specifically in the orexin neurons under a Tet-off system. When mice have access to chow containing doxycycline (DOX), the orexin neurons are healthy and sleep/wake behavior is normal. However, after DOX removal, the orexin neurons express DTA and die within 2–3 weeks. Cataplexy begins around 3 weeks after DOX removal and increases until it plateaus 8–10 weeks later (Tabuchi et al., 2014).

All animals were housed in a temperature-controlled (22 ± 1.4°C) vivarium on a 12:12 h light:dark cycle with regular mouse chow or DOX chow (100 mg/kg by weight, Envigo) and water available *ad libitum*. DOX chow was stored at 4°C and changed weekly (as per vendor instructions) to prevent degradation of the DOX at room temperature. Genotyping was performed using real-time PCR (Transnetyx).

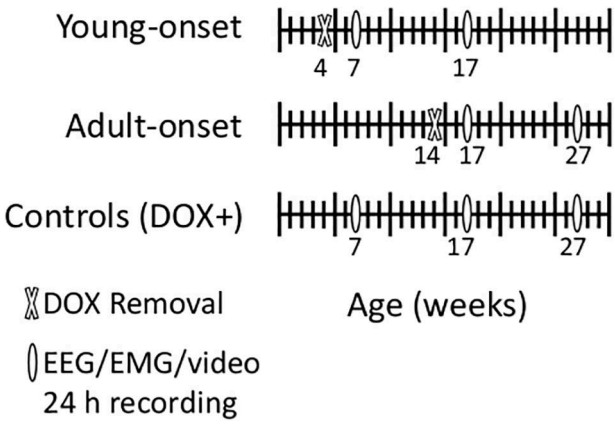

FIGURE 1 | Schematic of experimental design. We removed doxycycline (DOX) from the chow of young-onset mice at 4 weeks of age and of adult-onset mice at 14 weeks of age. We recorded EEG, EMG and video for characterization of sleep/wake/cataplexy 3 and 13 weeks later. We also recorded from age-matched control mice that were maintained on doxycycline the entire experiment (DOX+).

Orexin Neuron Loss

Mating pairs of *orexin-tTA; TetO DTA* mice were fed DOX chow so that litters would receive DOX *in utero* via maternal circulation and postnatally via lactation. We removed DOX from the diet (DOX−) of *orexin-tTA; TetO DTA* mice at age 4 weeks (young-onset group) or 14 weeks (adult-onset group) (**Figure 1**). These ages were chosen because a 4-week-old mouse is about the same developmental age as an 11–12 years old child, and a 14-week old mouse is roughly equivalent to an early-20s human (Flurkey et al., 2007).

Study Design

We implanted mice with EEG/EMG electrodes at 6 or 16 weeks of age to record cataplexy and sleep/wake behavior. We recorded brain activity using electroencephalography (EEG), muscle activity using electromyography (EMG), and general behavior using infrared video to aid in identification of sleep/wake behavior and cataplexy detection.

In adult *orexin-tTA; TetO DTA* mice, cataplexy begins about 3 weeks after DOX removal and plateaus 11–13 weeks after DOX removal (Tabuchi et al., 2014). Therefore, for young-onset mice (DOX removed at 4 weeks of age), we recorded sleep/wake behavior at 7 and 17 weeks of age. For adult-onset mice (DOX removal at 14 weeks of age), we recorded behavior at 17 and 27 weeks of age to control for time since DOX removal. In addition, we recorded from age-matched *orexin-tTA; TetO DTA* controls maintained on DOX chow for the entire experiment (DOX+ with recordings at age 7, 17, and 27 weeks) to control for any age-related changes in sleep/wake behavior. Each group contained male and female mice. Some

longitudinal recordings were disrupted by the 2020 COVID-19 lab shutdown, so this experiment includes both longitudinal (repeated recordings in the same mouse) and cross-sectional (only one recording from a mouse) data. The total numbers of mice were: DOX+ 7 weeks (seven males, seven females); DOX+ 17 weeks (seven males, including three longitudinal recordings; nine females, including four longitudinal recordings); DOX+ 27 weeks (five males, including four longitudinal recordings; 10 females including seven longitudinal recordings); young-onset 7 weeks (seven males, seven females); young-onset 17 weeks (eight males, including four longitudinal recordings; six females including two longitudinal recordings); adult-onset 17 weeks (eight males, six females); adult-onset 27 weeks (10 males, including five longitudinal recordings; nine females including three longitudinal recordings).

Surgery

We anesthetized mice using ketamine/xylazine (100/10 mg/kg, i.p.) and placed them into a stereotaxic frame. We soldered leads made from multistranded stainless steel wire (Cooner Wire, part number AS633) to two stainless steel screws which were implanted into the skull (1 mm lateral and 1 mm rostral to bregma, 1 mm lateral to bregma, and 1 mm rostral to lambda). We implanted two EMG electrodes made from Cooner wire into the neck extensor muscles. All leads were soldered to a 2 × 2 pin microstrip connector which we secured to the skull using dental cement. We treated each mouse with Meloxicam SR (4 mg/kg, s.c.) immediately after surgery.

EEG/EMG/Video Recordings and Analysis

After at least 1 week of recovery, we moved mice into the recording chambers to allow at least 5 days of acclimation to the recording cage and EEG/EMG cable. During recordings, EEG/EMG signals were amplified, filtered (EEG: 0.3–35 Hz; EMG: 100–300 Hz; Grass Amplifier 6SS, Grass Instruments), and digitized at a sampling rate of 256 Hz (VitalRecorder, Kissei Comtec) with simultaneous infrared video recordings. We scored sleep/wake signals in 10 s epochs semiautomatically using SleepSign (Kissei Comtec, band pass filter settings: EEG, 0.25–64 Hz; EMG, 10–60 Hz, with a notch filter at 60 Hz for each) and performed manual corrections as needed. We scored cataplexy manually using EEG/EMG and video according to a consensus definition (Scammell et al., 2009). Specifically, we scored an event as cataplexy if four criteria were met: the episode was (1) an event of nuchal atonia lasting at least 10 s, (2) the mouse was not asleep during the 40 s preceding the episode, (3) the mouse was immobile for the duration of the event, and (4) the EEG was dominated by theta activity (Scammell et al., 2009).

Confirmation of Orexin Neuron Loss

After the recordings, we perfused all mice and immunostained brains as outlined below to confirm orexin neuron loss in DOX− mice and to confirm that the genetic construct of the *orexin-tTA; TetO DTA* mice did not cause any unexpected orexin neuron loss in the DOX + control mice. In addition, we immunostained brains from young-onset and adult-onset mice perfused 0, 1, 2, and 3 weeks after DOX removal (n = 6–11 mice per group, including males and females) to test whether any differences in cataplexy could be due to different rates of orexin neuron loss.

Immunohistochemistry and Neuron Counting

We anesthetized mice with ketamine/xylazine (150/15 mg/kg i.p.) and transcardially perfused them with 30 mL phosphate-buffered saline (PBS, pH = 7.4) and 30 mL of 10% buffered formalin (pH = 7). We then harvested brains and post-fixed them in 10% formalin for 24–48 h. After fixation, we transferred brains to a 30% sucrose solution in PBS-azide for 48–72 h.

The orexin field spans the lateral and posterior hypothalamus (De Lecea et al., 1998; Sakurai et al., 1998), so we collected 30 μm sections in a 1:3 series from bregma −0.94 mm to −2.80 mm to capture the full orexin field across ∼20 sections in each series.

We immunostained one series for orexin-A to compare the number of orexin neurons at different time points across groups. We first rinsed sections three times with PBS for 5 min before incubating them for 30 min in 0.3% hydrogen peroxide in PBS with Triton (0.25% Triton X-100 in PBS) to quench endogenous peroxidases. Next, we rinsed sections again in PBS three times for 5 min before blocking them with 3% normal horse serum (NHS) for 2 h. We followed blocking with a primary overnight incubation in goat anti-orexin-A antibody (1:5,000; Santa Cruz SC-8070, Lot: C0512) in 0.02% sodium azide in PBT and 3% NHS.

The next day, we began by rinsing sections six times for 5 min in PBS and then incubated them in biotinylated donkey anti-goat IgG secondary antiserum (1:500; Jackson ImmunoResearch 705-065-147, lots: 129472, 150417) in 3% NHS in PBT for 2 h. After secondary incubation, we again rinsed sections three times for 10 min in PBS followed by a 1 h incubation in avidin-biotin complex in PBS (Vector Laboratories PK-6100, lot: ZF1218). To stain orexin-A brown, we then placed sections in 3,3′-diaminobenzidine (DAB) (Vector SK-4100, Lot: SLCD1660) in tris-buffered saline (TBS) and 0.024% hydrogen peroxide for 6 min. After the reaction, we again rinsed sections three times for 5 min in PBS before mounting them on Superfrost Plus slides and letting them dry overnight. All incubations and washes were carried out at room temperature on a shaker. We dehydrated the sections using graded ethanol for 3 min per step, followed by clearing with xylenes, and then coverslipped the slides using Cytoseal 68 mounting media (Thermo Fisher Scientific, 23-244256).

We imaged sections using bright field microscopy under the 5× lens of an Axioplan2 microscope (Zeiss) and captured images using AxioCam HRC (Zeiss). Finally, we analyzed the images and counted orexin-A immunoreactive neurons using ImageJ's Multi-Point tool. All immunostaining and cell counting were performed by AJ for consistency.

Statistical Analysis

We performed statistical analysis in R version 4.0.2 using the following packages: nlme, sjPlot, sjmisc, ggplot2, and plyr

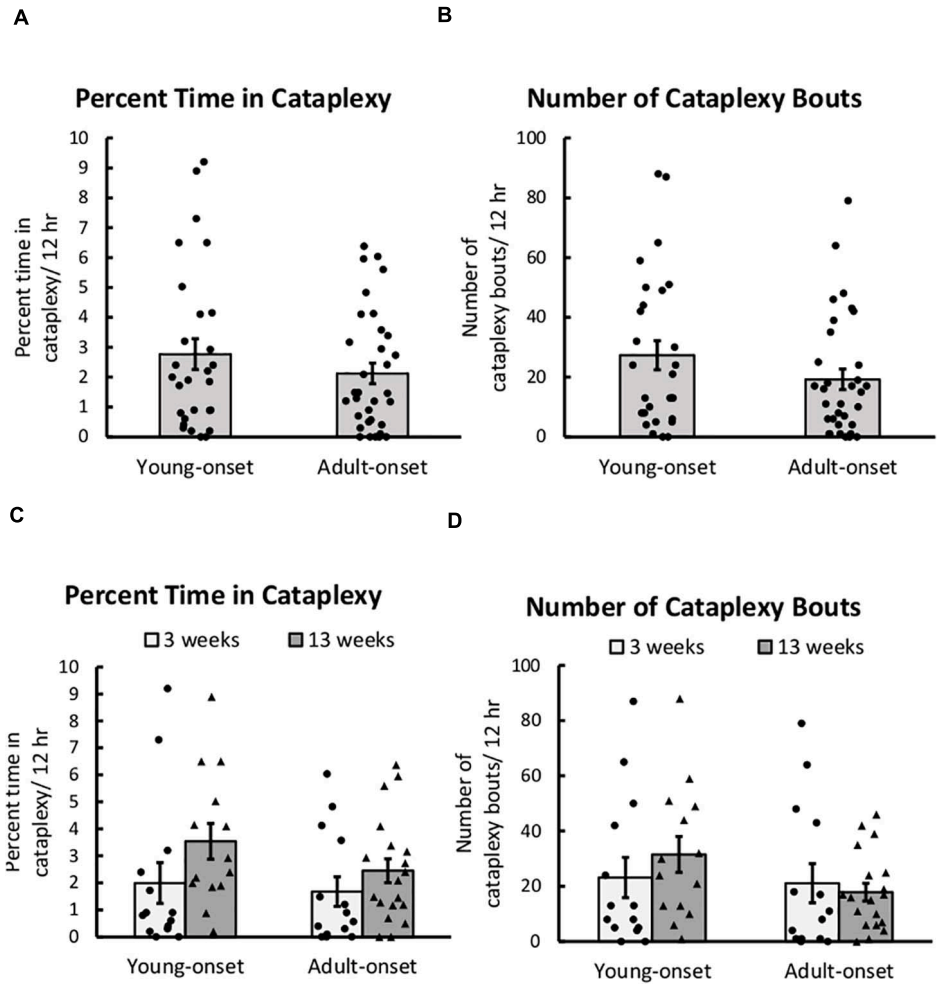

FIGURE 2 | Cataplexy was unaffected by age of orexin neuron loss. **(A)** Average percent of time young-onset and adult-onset mice spent in cataplexy during the 12 h dark period, collapsed across age. **(B)** Average number of cataplexy bouts during the 12 h dark period in young-onset and adult-onset mice, collapsed across age. **(C)** Average percent time spent in cataplexy bouts during the dark period in young-onset and adult-onset mice at 3 or 13 weeks after doxycycline removal. **(D)** Average number of cataplexy bouts during the dark period in young-onset and adult-onset mice at 3 or 13 weeks after doxycycline removal. All data are presented as mean ± SEM with individual data points overlaid.

(Wickham, 2011, 2016; Lüdecke, 2018, 2020; Pinheiro et al., 2020; R Core Team, 2020). We performed separate linear mixed multilevel regressions for each dependent variable of interest: percent time in cataplexy (during the dark period, light period, and over 24 h), number of cataplexy bouts (during the dark period, light period, and over 24 h), duration of cataplexy bouts (during the dark period), percent time awake (during the dark period, light period, and over 24 h), number of wake bouts (during the dark period), duration of wake bouts (during the dark period), percent time in NREM sleep (during the dark period, light period, and over 24 h), and percent time in REM sleep (during the dark period, light period, and over 24 h). The DOX+ control group was excluded from the cataplexy regressions because no control mice had cataplexy and including the group would violate the homoscedasticity assumption of the regression. Independent variables for the cataplexy regressions included: group (young-onset and adult-onset), sex (female and male), time since DOX removal, and all interaction terms (group × sex, sex × time, group × time, group × sex × time). Independent variables for the sleep/wake regressions included: group (young-onset, adult-onset, and DOX+), sex (female and male), age, and all interaction terms (group × sex, sex × age, group × age, group × sex × age). There were two potential sources of correlation in our data: mice within a litter and observations within a mouse (we recorded from some mice multiple times). To account for these, we included a litter-specific random intercept and an observation-specific random intercept in each model. We assumed a normal distribution and an unknown standard error for these random intercepts and the residual error. We log-transformed (base 10) bout durations of wake and cataplexy for each mouse to prevent violation of the normality assumption of the regressions.

For the orexin neuron loss confirmation experiment, we ran a 2 × 2 × 4 analysis of variance (ANOVA) including orexin

neuron count as the dependent variable and group (young-onset and adult-onset), sex (male and female), time since DOX removal (0, 1, 2, 3 week), and the relevant interaction terms (group × time, sex × time, group × sex × time) as the independent variables.

RESULTS

Age of Orexin Neuron Loss Did Not Impact Cataplexy

Young-onset and adult-onset mice had similar amounts of severe cataplexy. The main effect of group (young-onset vs. adult-onset) was not significant for either the percentage of time spent in cataplexy or the number of cataplexy bouts during the dark period (**Figure 2**). The group × sex, group × time since DOX removal, and group × sex × time since DOX removal interactions were all non-significant for the percentage of time spent in cataplexy and for the number of cataplexy bouts. Still, the number of bouts tended to increase over time since DOX removal in young-onset mice but not in adult-onset mice, $t(10) = -1.89$, $p = 0.087$ (**Figure 2D**). Cataplexy mainly occurs in the dark period, so we present all analyses on cataplexy during the dark period. Cataplexy was much less common in the light period, and results were similar for cataplexy during the light period or across the 24 h period. As cataplexy occurs only during wakefulness, cataplexy amounts could differ due to differences in time spent awake. For this reason, we also analyzed the amount of cataplexy as a percent of time awake and the number of cataplexy bouts per hour of wakefulness in all mice, but the conclusions were the same, indicating that differences in cataplexy were unrelated to differences in wake time were unrelated to differences in wake time. For representative EEG/EMG traces and video during a cataplexy bout in example young-onset and adult-onset mice, see **Supplementary Videos**.

There were no group differences in the duration of cataplexy bouts, but bouts of cataplexy tended to lengthen over time since DOX removal, $t(8) = 2.01$, $p = 0.079$ (**Figure 3**). On average, for every day since DOX removal, cataplexy bouts were about 1 s longer.

Female Mice Had More Cataplexy Than Male Mice

Female mice had more bouts of cataplexy during the dark period than male mice. The main effect of sex was significant for the number of cataplexy bouts in the dark period, $t(21) = -2.20$, $p = 0.039$, indicating that male mice have fewer bouts of cataplexy than female mice (**Figure 4**). The main effect of sex was not significant for percent of time spent in cataplexy. No interaction effects were significant for either percent time or number of cataplexy bouts during the dark period.

Female mice also spent more time in cataplexy during the light period and across the 24 h period than male mice. The main effect of sex was significant for the percentage of time spent in cataplexy during the light period, $t(21) = -2.85$, $p = 0.0095$, and across the 24 h period, $t(21) = -2.23$, $p = 0.037$. The main effect of sex was also significant for the number of cataplexy bouts during the light period, $t(21) = -2.63$, $p = 0.016$, and across the 24 h period, $t(21) = -2.59$, $p = 0.017$. Interestingly, the age × sex interaction for percent time spent in cataplexy during the light period was nearly significant, $t(10) = 2.199$, $p = 0.052$. While female mice spent more time in cataplexy during the light period at 3 weeks after DOX removal, male and female mice had similar amounts of cataplexy by 13 weeks after DOX removal. The same trend was seen in the number of cataplexy bouts during the light period, age × sex interaction, $t(10) = 1.96$, $p = 0.078$.

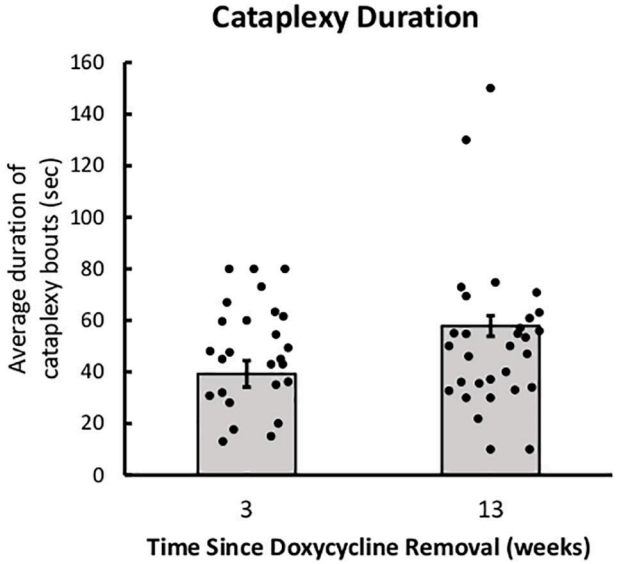

FIGURE 3 | Cataplexy duration increases across time since DOX removal. Average duration of cataplexy bouts during the dark period in both young-onset and adult-onset mice at 3 and 13 weeks after doxycycline removal.

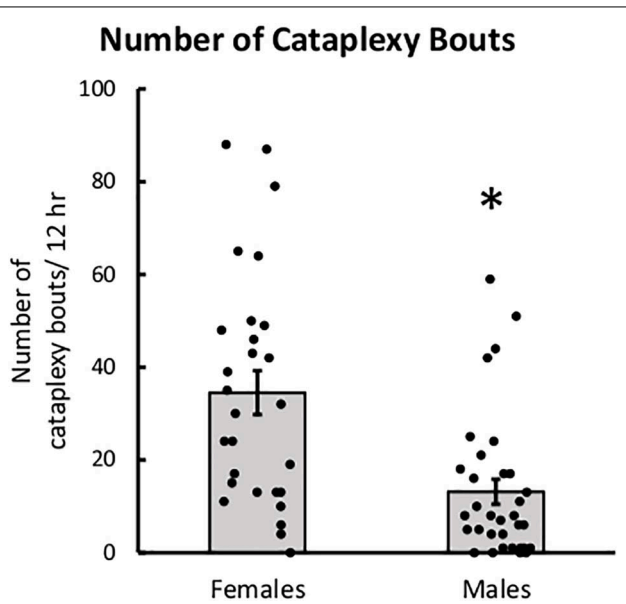

FIGURE 4 | Female mice had more cataplexy than males. Average number of cataplexy bouts during the 12-h dark period in female and male mice, collapsed across age. $^*p < 0.05$.

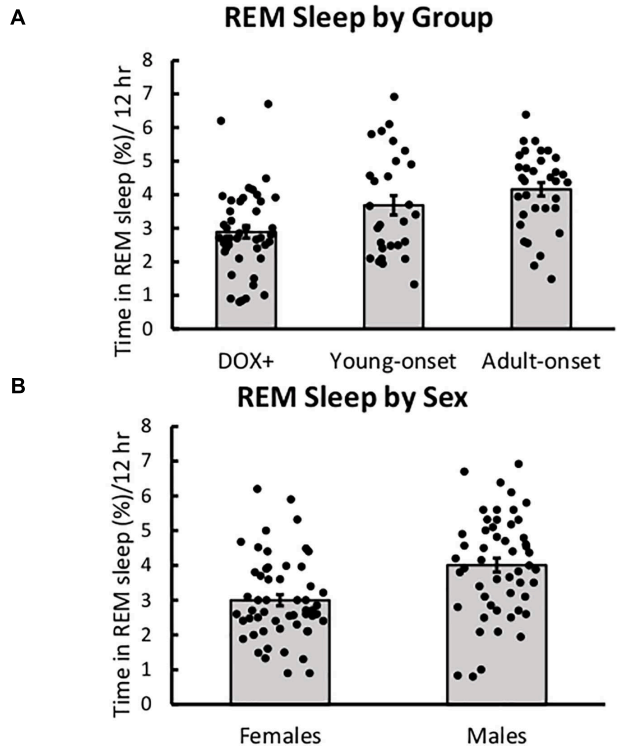

FIGURE 5 | REM sleep was unaffected by age of orexin neuron loss. **(A)** Average percent time spent in REM sleep during the 12-h dark period in DOX+ controls, young-onset mice, and adult-onset mice, collapsed across age. **(B)** Average percent time spent in REM sleep during the 12-h dark period in female and male mice.

Age of Orexin Neuron Loss Did Not Impact REM Sleep

There were no significant main or interaction effects on the percentage of time spent in REM sleep during the dark period (**Figure 5**). Still, young-onset mice tended to spend more time in REM sleep during the dark period than DOX+ controls, $t(43) = 1.86$, $p = 0.07$, and male mice tended to spend more time in REM sleep than females, $t(43) = 1.94$, $p = 0.059$.

Young-onset female mice spent less time in REM during the light period than DOX+ female mice, but DOX+ and young-onset males spent similar amounts of time in REM. Group × sex interaction, $t(42) = 2.25$, $p = 0.029$.

Orexin Neuron Loss Resulted in Poor Maintenance of Wake

Young-onset and adult-onset mice had significantly shorter wake bouts during the dark period than DOX+ controls, $t(43) = -3.90$, $p = 0.0003$ and $t(43) = -2.88$, $p = 0.0062$, respectively (**Figure 6A**). Wake durations were shorter in male DOX+ controls than females but the same between adult-onset males and females so the reduction in wake bout durations was steeper in female adult-onset mice than males [group × sex interaction, $t(43) = 2.13$, $p = 0.039$]. This interaction was not seen in young-onset mice.

Young-onset and adult-onset mice also had more wake bouts during the dark period than DOX+ controls [$t(43) = 3.61$, $p = 0.0008$, and $t(43) = 3.90$, $p = 0.0003$, respectively] (**Figure 6B**). DOX+ male mice had more bouts of wake than DOX+ female mice, but adult-onset male and female mice had similar numbers of wake bouts so the increase in number of wake bouts was steeper in female adult-onset mice than males [group × sex interaction, $t(43) = -3.26$, $p = 0.0022$].

Young-onset mice spent less time awake during the dark period than DOX+ controls, $t(43) = -2.53$, $p = 0.015$, and tended to spend more time in NREM sleep during the dark period than DOX+ controls, $t(43) = 1.96$, $p = 0.056$, but this effect was not significant (**Table 1**). Cataplexy in the dark period in young-onset mice likely explains this difference in wake time. No such differences were seen in the adult-onset mice.

Age of orexin neuron loss did not affect the percentage of time spent awake, in NREM sleep, or in REM sleep across the 24 h period.

Considered together, these results support the perspective that orexin neuron loss results in poor maintenance of wake, a common symptom of orexin deficiency in mouse models and human narcolepsy (Hara et al., 2001; Mochizuki et al., 2004; Tabuchi et al., 2014; Scammell, 2015).

Age of Orexin Neuron Loss Mildly Affected NREM Sleep

Adult-onset mice spent less time in NREM sleep during the light period than DOX+ controls, $t(42) = -2.06$, $p = 0.045$, but there was no difference in percent time in NREM sleep during the light period between young-onset mice and DOX+ controls (**Table 1**).

Orexin Neuron Numbers Declined Similarly in Young and Adult Mice

After removal of DOX, the number of orexin neurons declined rapidly over 3 weeks, $F(1) = 341.21$, $p < 2 \times 10^{16}$, but this decline was unaffected by age of DOX removal or sex (**Figure 7**). There were no significant interaction effects (group × time, sex × time, or group × sex × time), indicating that there were no group differences in orexin count across time. Thus, any differences in behavior cannot be explained by differences in the rate of orexin neuron loss.

DISCUSSION

As cataplexy and sleepiness are often severe in children with narcolepsy, we investigated whether the symptoms of narcolepsy are worse when the orexin neurons are lost in young mice (onset 4 weeks) compared to adult mice (onset 14 weeks). We found that age of orexin neuron loss did not affect cataplexy severity, but female mice had more cataplexy than male mice and cataplexy duration tended to increase over time. Young-onset mice also tended to spend more time in REM sleep during the dark period than DOX+ controls. With both young- and adult-onset orexin neuron loss, wake bouts in the dark period were only about half the duration seen in controls, but there was no reduction in the total amount of wake over 24 h. Overall, this experiment did

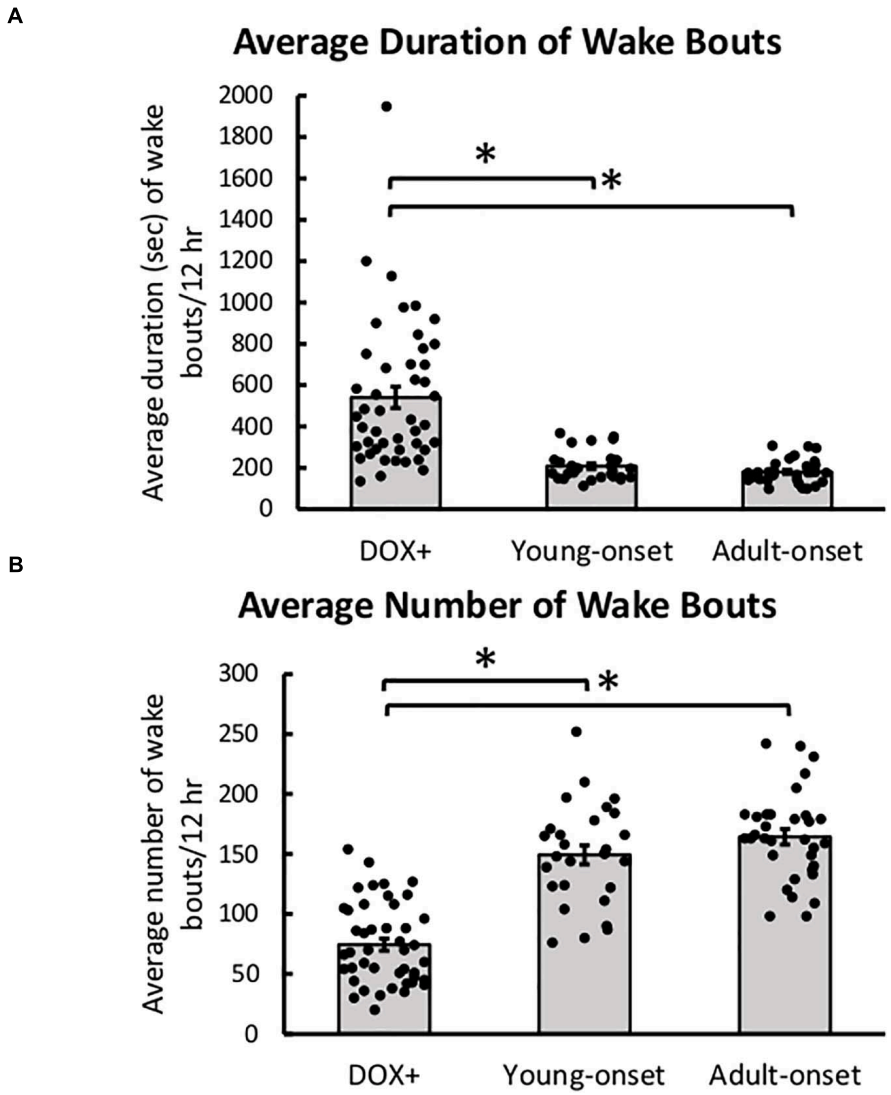

FIGURE 6 | Poor maintenance of wake with orexin neuron loss. **(A)** Average duration of wake bouts and **(B)** average number of wake bouts in DOX+ (control), young-onset, and adult-onset mice. *$p < 0.05$.

TABLE 1 | Time spent in each sleep/wake state during the dark period and the light period.

	DOX+ 7 weeks (*n* = 14)	Young-onset 7 weeks (*n* = 14)	DOX+ 17 weeks (*n* = 16)	Young-onset 17 weeks (*n* = 14)	Adult-onset 17 weeks (*n* = 14)	DOX+ 27 weeks (*n* = 15)	Adult-onset 27 weeks (*n* = 19)
Dark Period							
Wake	74.1 (1.5)	66.0 (1.6)	71.7 (2.2)	68.8 (2.5)	67.4 (2.4)	71.4 (2.7)	63.7 (2.0)
NREM sleep	22.9 (1.4)	27.7 (1.4)	25.4 (2.0)	24.7 (2.4)	26.6 (2.4)	25.8 (2.5)	29.8 (2.0)
REM sleep	3.0 (0.2)	4.3 (0.4)	2.9 (0.4)	3.0 (0.4)	4.3 (0.3)	2.8 (0.4)	4.0 (0.3)
Cataplexy	0 (0)	2.0 (0.8)	0 (0)	3.5 (0.7)	1.7 (0.5)	0 (0)	2.5 (0.4)
Light period							
Wake	37.7 (3.1)	39.4 (3.1)	37.5 (1.2)	40.4 (1.1)	42.2 (1.4)	37.1 (1.4)	38.7 (1.2)
NREM sleep	46.6 (3.8)	48.6 (0.9)	54.1 (1.0)	51.9 (1.0)	49.3 (1.2)	53.5 (1.4)	53.8 (1.2)
REM sleep	8.6 (0.8)	8.4 (0.5)	8.4 (0.3)	7.1 (0.3)	8.1 (0.4)	9.3 (0.3)	7.1 (0.3)
Cataplexy	0 (0)	0.4 (0.2)	0 (0)	0.4 (0.1)	0.2 (0.1)	0 (0)	0.4 (0.1)

Data are presented as mean percent time/12 h (SEM).

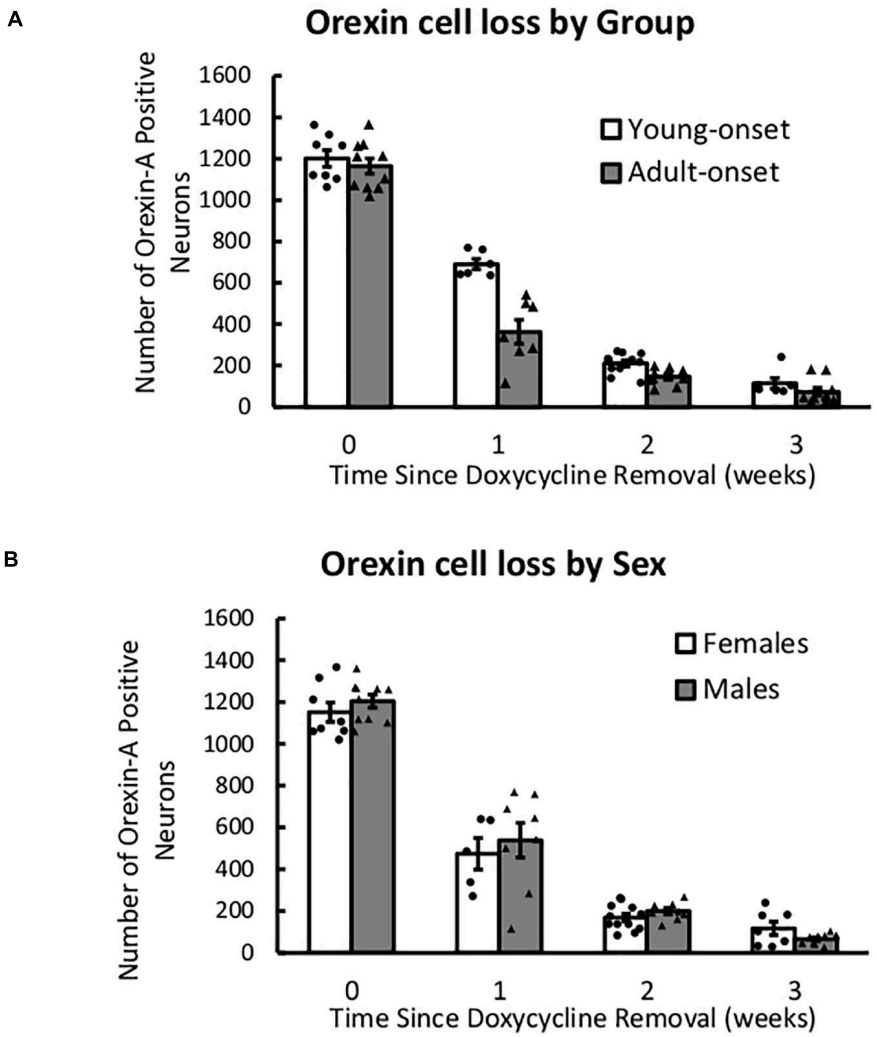

FIGURE 7 | After DOX removal, orexin neurons are lost at similar rates in young- and adult-onset mice. **(A)** Average number of orexin-A positive neurons in young-onset and adult-onset mice over time since doxycycline removal. **(B)** Average number of orexin-A positive neurons in female and male mice over time since doxycycline removal.

not support the hypothesis that young-onset orexin neuron loss would result in severe cataplexy, but it revealed an important sex difference in murine cataplexy and further characterized this important mouse model. These are the first experiments modeling young-onset narcolepsy in mice and a helpful step toward understanding the uniquely severe symptoms endured by children with narcolepsy.

Effects of Age on Cataplexy

Young-onset and adult-onset mice spent a similar amount of time in cataplexy and had a similar number of cataplexy bouts. At 3 weeks after DOX removal, the adult-onset group spent about 2% of the dark period (∼15 min) in cataplexy which is similar to prior descriptions of adult *orexin-tTA; TetO DTA* mice (Tabuchi et al., 2014; Williams et al., 2019). However, Tabuchi et al. (2014) reported that at 13 weeks after DOX removal, adult-onset mice spent about 8% (∼60 min) of the dark period in cataplexy (Tabuchi et al., 2014). This amount is much higher than our group averages, although some individual mice in our experiments showed this large amount of spontaneous cataplexy. This discrepancy could be due to differences in the recording environment and equipment, inter-individual differences in scoring cataplexy, or background mouse strain (C57/BL6 mice vary slightly between Japan and the United States).

When narcolepsy begins in childhood, symptoms are often more severe than when narcolepsy begins in adults. Children tend to be sleepier than adults (Young et al., 1988; Pizza et al., 2013), and cataplexy tends to be more severe, persistent, and frequent (Serra et al., 2008; Plazzi et al., 2011; Antelmi et al., 2017; Maski et al., 2017). Beyond typical narcolepsy symptoms, childhood narcolepsy is also associated with precocious puberty and obesity, indicating widespread disruption to multiple systems (Kotagal et al., 1990, 2004; Plazzi et al., 2006; Vendrame et al., 2008; Poli et al., 2013; Ponziani et al., 2016). Indeed, children

with narcolepsy are more likely to develop comorbidities affecting endocrine, metabolic, psychiatric, and nervous systems around diagnosis than age-matched healthy controls (Jennum et al., 2017). Our results suggest that this age-related difference may be specific to humans or may be a consequence of the autoimmune process hypothesized to kill the orexin neurons (Bonvalet et al., 2017), which is not modeled in these mice. For example, an especially aggressive autoimmune attack in children might kill the orexin neurons rapidly, leading to severe symptoms, and more disruption (Cogswell et al., 2019) whereas the neuron loss may occur more slowly in adults who develop narcolepsy. Alternatively, orexin neuron loss in adults may be less disruptive because of redundancies stabilizing different systems that have not yet developed in children.

Alternatively, some researchers maintain that narcolepsy progression is similar in children and adults but that children are typically studied closer to disease onset (Nevsimalova, 2009) or that only the most severe cases are diagnosed in children (Young et al., 1988). The first interpretation gains some preclinical support from the fact that *orexin-tTA; TetO DTA* mice removed from doxycycline at birth have far less cataplexy in adulthood than mice removed from doxycycline later (Tabuchi et al., 2014). Clinically, childhood cataplexy lessens over time and develops into a more typical, adult-like form (Plazzi et al., 2011; Pizza et al., 2013). The second hypothesis is possible as it usually takes years longer for children to be diagnosed with narcolepsy compared to adults (Guilleminault and Pelayo, 1998; Thorpy and Krieger, 2014; Maski et al., 2017), and milder cases may be overlooked. Either of these hypotheses could be supported by the lack of age effect shown here. However, because of the long diagnosis delay, most clinical studies are retrospective in nature, relying on patient and family recall of age and severity of symptoms at onset. This major limitation to much of the clinical data further emphasizes the importance of longitudinal studies in children with narcolepsy and studying age of narcolepsy onset in animal models, which provide better control than clinical research.

Sex Differences in Cataplexy

We found that female mice have more cataplexy than male mice, yet researchers debate whether narcolepsy prevalence differs between men and women. Some early epidemiological studies indicate that narcolepsy is more common in men than in women (Silber et al., 2002; Ohayon et al., 2005; Longstreth et al., 2009). However, studies that separated narcolepsy type 1 (with cataplexy) from narcolepsy type 2 (without cataplexy) found a much smaller effect (Silber et al., 2002), no sex difference in NT1 (Ohayon et al., 2002; Heier et al., 2009; Luca et al., 2013; Khatami et al., 2016), or a higher incidence in women (Longstreth et al., 2009). Given the significant diagnosis delay and frequent misdiagnoses of patients with narcolepsy (Guilleminault and Pelayo, 1998; Kryger et al., 2002; Macleod et al., 2005; Thorpy and Krieger, 2014; Maski et al., 2017), it is likely that older reports underestimated the prevalence of narcolepsy, especially in women. Importantly, considering the typical finding of an increased prevalence in men, the diagnostic delay is typically longer in women than in men (Luca et al., 2013), and women are less likely to be assessed in sleep laboratories, resulting in less access to polysomnography and likely underdiagnosis of sleep disorders (Auer et al., 2018). Overall, it seems unlikely that there is a significant sex difference in the prevalence of narcolepsy type 1 in humans.

Most studies do not assess gender differences in cataplexy severity. Cataplexy may be more common in women than in men (Ohayon et al., 2002), but there seems to be no difference in severity between men and women (Luca et al., 2013). One study found that a larger proportion of men with "high frequency" cataplexy (more than one bout a month), but the number of patients was relatively low (44) and there were few women in the study (16) (Mattarozzi et al., 2008). Future clinical studies should examine gender as a possible factor contributing to cataplexy severity.

While we suspect that the sex difference in cataplexy reported here is unlikely to parallel human narcolepsy, it may arise from thermoregulatory influences. Cataplexy is a REM sleep-like state, and REM sleep and cataplexy are likely regulated similarly. Warmer ambient temperatures increase REM sleep, and cooler temperatures decrease REM sleep in rodents (Schmidek et al., 1972; Szymusiak and Satinoff, 1981; Kumar et al., 2009; Komagata et al., 2019). Female mice prefer warmer temperatures than males, suggesting that the thermoneutral zone for females may be slightly warmer compared to males (Gaskill et al., 2009; Kaikaew et al., 2017). While cooler temperatures inhibit REM sleep, they may be more permissive to cataplexy. In the current experiment, mice were housed at 22°C, and this cool temperature may have inhibited REM sleep more in females than in males, yet persistent REM sleep pressure may have resulted in more cataplexy in females.

In addition, the estrus cycle can influence orexin levels and sleep/wake behavior. Hypothalamic orexin levels are higher during proestrus than in other stages of the estrus cycle in rats (Porkka-Heiskanen et al., 2004); however, we are not aware of any studies examining cataplexy across the estrus cycle. While the effects of the estrus cycle on sleep/wake architecture in female mice are modest and strain-specific, REM sleep is significantly reduced in the dark period during proestrus in female Sprague-Dawley rats and C57/BL6 mice (Fang and Fishbein, 1996; Koehl et al., 2003). Interesting, male rats spend more time in REM sleep than female rats overall, a finding that our results support, although a previous study in mice did not find this difference (Fang and Fishbein, 1996; Paul et al., 2006). Despite the likelihood that the sex difference shown here is a species-specific effect, this aspect of murine cataplexy has implications for future research. Cataplexy is an uncommon state, so including females may help increase the overall number of cataplexy bouts seen across groups, in addition to better modeling the patient population.

Effects of Orexin Neuron Loss on REM Sleep

Young-onset mice tend to spend more time in REM sleep during the dark period than DOX+ controls, although oddly, adult-onset mice did not show the same pattern. An increase in dark period REM sleep was expected as the orexin neurons are thought to normally suppress REM sleep during the dark period (Kantor

et al., 2009). Kantor et al. (2009) demonstrated this disinhibition of REM sleep during the subjective night in mice housed in constant darkness, and this effect has also been shown in orexin-ataxin mice and orexin knockout mice maintained on a 12:12 light:dark cycle (Hara et al., 2001; Kantor et al., 2009; Roman et al., 2018; Chowdhury et al., 2019).

Interestingly, this increase in REM sleep during the subjective dark period was originally shown in orexin-ataxin-3 mice but not orexin knockout mice, which was thought to indicate an effect of the loss of orexin neurons as opposed to the loss of the orexin peptides *per se* (Kantor et al., 2009). Our results are consistent with this hypothesis, but it is also possible that this REM sleep effect is seen soon after the loss of the orexin neurons but before some compensation occurs. This hypothesis is supported by the fact that *orexin-tTA; TetO DTA* mice that are removed from DOX in adulthood have more REM sleep in the dark period 3 weeks after DOX−, but not 4–13 weeks after DOX removal, and *orexin-tTA; TetO DTA* mice removed from DOX at birth show no increase in dark period REM sleep (Tabuchi et al., 2014). However, further research will be necessary to parse out the acute and chronic effects of orexin neuron loss vs. orexin peptide loss on REM sleep in the dark period.

Limitations

A few limitations in these experiments warrant discussion. We removed DOX from the chow of 4-week-old mice as they are roughly the same developmental stage as 11–12 years old children (Flurkey et al., 2007), but it is difficult to accurately align human ages onto mice. It is possible that orexin neuron loss prior to 4 weeks of age would produce more severe cataplexy. It is also important to consider that all cataplexy reported here is spontaneous, but the addition of palatable food (Froot Loops or chocolate) or a running wheel can dramatically increase cataplexy in orexin knockout mice (Espana et al., 2007; Clark et al., 2009; Burgess et al., 2013; Oishi et al., 2013; Mahoney et al., 2017), and young-onset mice may be more vulnerable to reward-elicited cataplexy. We did not control for estrus cycle in this experiment, which likely accounts for some of the variability in cataplexy and REM sleep in female mice; however, our data from females are only slightly more variable than males. Thus, while it is best practice to control for estrus cycle, we do not believe that this should be a barrier to including females in experiments.

CONCLUSION

In contrast to our hypothesis, we did not find more cataplexy in mice with orexin neuron loss at a young age. Instead, we found that female mice have more cataplexy overall than male mice, which is likely a species-specific effect that can aid narcolepsy research. Still, the hypothesis is open, and future research could determine whether DOX removal at birth or at weaning results in severe cataplexy in mice; examine whether age of orexin neuron loss affects reward-elicited cataplexy; characterize cataplexy in female mice as a function of estrus cycle; further parse out the acute and chronic effects of orexin neuron loss vs. orexin peptide loss on dark period REM sleep; or investigate compensatory mechanisms that develop in the weeks after orexin neuron loss. Children are uniquely burdened by narcolepsy symptoms, and research dedicated to this population is necessary to address this problem.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The animal study was reviewed and approved by the Institutional Animal Care and Use Committee of Beth Israel Deaconess Medical Center and Harvard Medical School.

AUTHOR CONTRIBUTIONS

AC and TS conceived of and designed all experiments. AY provided mice for the experiments. AC performed surgeries, recordings, perfusions, sleep stage scoring, and data analysis and drafted the manuscript. AJ performed mouse husbandry/weaning, perfusions, brain sectioning, immunostaining, imaging, image analysis, and orexin neuron counting and drafted the relevant methods section. TS, AY, and AJ made significant revisions to the manuscript. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fnins.2021.644757/full#supplementary-material

Supplementary Videos | Example videos of cataplexy bouts in SleepSign. EEG (top) and EMG (bottom) signals are presented for two representative cataplexy bouts. The three panels in the bottom left corner (from top to bottom) show: Delta Power, EMG integral, and Theta Ratio. Epochs are labeled "W" for wake or "C" for cataplexy. Asterisks indicate that the mouse transitioned between states within the 10-s epoch. In these cases, the epoch was scored as the state lasting for at least 50% of the epoch. In both mice, the EEG signal during cataplexy resembles REM sleep, the mouse is immobile for the duration of the bout, and theta activity dominates the EEG activity. Hypersynchronous theta activity is visible as brief bursts of larger amplitude EEG signal. **Video 1** Example cataplexy bout in a young-onset mouse (age 17 weeks). The cataplexy bout begins about 8 s into epoch 756 (blue numbers beneath EMG signal). The bout ends 6 s into epoch 771. **Video 2** Example cataplexy bout in an adult-onset mouse (age 17 weeks). The cataplexy bout begins 4 s into epoch 1262 (blue numbers beneath EMG signal). The bout ends 5 s into epoch 1267.

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REVIEW
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Neuropeptides as Primary Mediators of Brain Circuit Connectivity

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Across sleep and wakefulness, brain function requires inter-neuronal interactions lasting beyond seconds. Yet, most studies of neural circuit connectivity focus on millisecond-scale interactions mediated by the classic fast transmitters, GABA and glutamate. In contrast, neural circuit roles of the largest transmitter family in the brain–the slow-acting peptide transmitters–remain relatively overlooked, or described as "modulatory." Neuropeptides may efficiently implement sustained neural circuit connectivity, since they are not rapidly removed from the extracellular space, and their prolonged action does not require continuous presynaptic firing. From this perspective, we review actions of evolutionarily-conserved neuropeptides made by brain-wide-projecting hypothalamic neurons, focusing on lateral hypothalamus (LH) neuropeptides essential for stable consciousness: the orexins/hypocretins. Action potential-dependent orexin release inside and outside the hypothalamus evokes slow postsynaptic excitation. This excitation does not arise from modulation of classic neurotransmission, but involves direct action of orexins on their specific G-protein coupled receptors (GPCRs) coupled to ion channels. While millisecond-scale, GABA/glutamate connectivity within the LH may not be strong, re-assessing LH microcircuits from the peptidergic viewpoint is consistent with slow local microcircuits. The sustained actions of neuropeptides on neuronal membrane potential may enable core brain functions, such as temporal integration and the creation of lasting permissive signals that act as "eligibility traces" for context-dependent information routing and plasticity. The slowness of neuropeptides has unique advantages for efficient neuronal processing and feedback control of consciousness.

Keywords: hypothalamus, neuropeptides, orexin, hypocretin, arousal, neural circuit

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FUNCTIONAL CIRCUIT CONNECTIVITY, FAST AND SLOW

In modern neuroscience textbooks, coverage of functional interactions between neurons and their postsynaptic targets remains biased toward fast (millisecond-level on/off) interactions mediated by small-molecule neurotransmitters such as ACh, GABA, and glutamate (Bear et al., 2015). This is presumably due to the enduring influence of the insightful electrophysiological studies of fast neurotransmission by giants of 20th century neuroscience, such as Bernard Katz and John Eccles, which attracted a number of Nobel prizes.

While these fast interactions are undoubtedly one fundamental aspect of brain function, most neurotransmitters in the brain do not operate on these rapid timescales. For example, neuropeptides–which are the largest known class of neurotransmitters (>100)–alter postsynaptic

neuronal activity at timescales more similar to typical behaviors and important sensory associations, typically seconds to minutes (Hokfelt et al., 2003; Burdakov, 2004; Salio et al., 2006; Burbach, 2011; Svensson et al., 2018).

These slower actions of neuropeptide transmitters are often described as "modulatory." This term is not clearly defined, but implies a qualitative difference, or that the main role of the peptides is to change the action of fast neurotransmitters (van den Pol, 2012). However, at the level of neural circuit functional connectivity and control of neuronal spiking, the actions of at least some behaviorally-vital neuropeptides are not conceptually different from fast transmitters: they are simply slower.

The role of neuropeptides in behavioral control, and more specifically, in sleep regulation and vigilance state switching, and their impact on the sleep electroencephalogram has been long known (Steiger and Holsboer, 1997). Instead of reviewing the current knowledge of the role of those peptides in sleep and sedation, we will focus on a few proof-of-concept examples to illustrate their role in neural circuit connectivity–and therefore in brain and behavioral states–beyond the merely modulatory roles which they are often attributed.

As an illustration of this, consider the actions of the prototypical fast transmitter glutamate vs. those of the neuropeptide transmitter orexin/hypocretin (de Lecea et al., 1998; Matsuki and Sakurai, 2008; Schöne et al., 2014). Both are stored in vesicles located in presynaptic axonal terminals, and released upon electrical stimulation of the axons (de Lecea et al., 1998; Torrealba et al., 2003; Schöne and Burdakov, 2012; Schöne et al., 2014). Both bind to specific postsynaptic receptors coupled, either directly or *via* cytosolic messengers, to the opening of ion channels in the postsynaptic membrane (Sakurai, 2007; Kukkonen and Leonard, 2014; Bear et al., 2015). However, glutamate release is typically initiated very rapidly, by one or a few presynaptic action potentials; its action is also terminated similarly promptly by a combination of extracellular diffusion and glutamate reuptake by neurons and glia, resulting in a millisecond-level on/off dynamics for ionotropic glutamate signals (Bear et al., 2015). In contrast, orexin release seems to require more prolonged presynaptic firing, and orexin-evoked postsynaptic depolarization can persist for many seconds or even minutes, presumably due to slow orexin diffusion and/or breakdown, no known reuptake mechanisms, and the long half-lives of intracellular messengers generated by orexin G-protein coupled receptors (GPCRs) (Schöne et al., 2014).

Thus, functional neural circuits in the brain can be created either by slowly-acting neurotransmitters such as neuropeptides, or by fast-acting classic neurotransmitters. In the rest of this short review, specific examples of neuropeptidergic brain circuits will be presented from the above-mentioned perspective. Our focus will be narrow and somewhat subjective, concentrating on recent insights from studies of lateral hypothalamic neuropeptidergic neurons linked to control of arousal and vigilance state switching. However, some general concepts will be proposed and experiments for probing them further will be outlined.

BRAIN-WIDE PROJECTING PEPTIDERGIC NEURONS OF THE NON-NEUROENDOCRINE HYPOTHALAMUS

Functionally-speaking, the hypothalamus is usually thought of as comprising two parts: the endocrine hypothalamus consisting of neurons controlling pituitary hormone release, and the non-neuroendocrine hypothalamus, which represents most of the hypothalamus in terms of volume and contains large and heterogeneous neurons that mono-synaptically innervate much of the brain (Peyron et al., 1998; Bittencourt, 2011; Bear et al., 2015).

It is the latter, non-neuroendocrine, hypothalamus that is the focus of this review. It is a complex confederation of neuronal clusters (nuclei). Most of the non-neuroendocrine hypothalamic neurons examined so far appear to synthesize and/or use classic neurotransmitters such as GABA and glutamate (Atasoy et al., 2012; Dicken et al., 2012; Schöne and Burdakov, 2012; Jego et al., 2013; Romanov et al., 2017; Mickelsen et al., 2019). In addition, most (if not all) hypothalamic neurons express a peptide neurotransmitter. Many of these neuropeptides are generally thought to be made only in the hypothalamus, for example orexin, discussed in our earlier example, is made exclusively by neurons of the lateral hypothalamus (LH) (de Lecea et al., 1998; Sakurai et al., 1998). This makes hypothalamic neuropeptidergic-producing neurons a very attractive "model system" for studying the role of neuropeptide transmission in brain-wide neural computation, brain state control, and behavior. This is because, in contrast to brain-wide synthesized transmitters such as glutamate, the origin of hypothalamus-unique neuropeptide signals is always known. This solves a major problem of interpretation in systems neuroscience, by allowing neuropeptidergic influences to be interpreted with precise knowledge of their normal origin. For example, if changes in neuronal firing in a particular brain area are observed upon exogenous application of orexin neuropeptide in brain slices, specific hypotheses can be formulated about LH interactions with these areas (Burdakov et al., 2003; van den Top et al., 2004; Sakurai, 2007; Burdakov and Gonzalez, 2009; Belle et al., 2014; Hay et al., 2014; Burdakov, 2020).

INFORMATION REPRESENTED BY LH NEUROPEPTIDERGIC NEURONS

Here we choose once more the LH neuropeptide orexin to exemplify some emerging concepts in neuropeptide-mediated function and neuronal connectivity, given that the LH has been long-known to be crucial for normal behavioral and brain state control. Orexin neurons play a key role in sleep/wake regulation (Inutsuka and Yamanaka, 2013) and a rather large body of evidence supports their critical role in stabilizing behavioral states (Chemelli et al., 1999; Lin et al., 1999; Hara et al., 2001; Mochizuki et al., 2004; Ma et al., 2018). Importantly, the role of orexin neurons in sleep/wake and behavioral state regulation

has been shown to be primarily linked to orexin peptide release–rather than their "classic" co-transmitters (Ma et al., 2018). The peptide orexin A is itself wake promoting (Hagan et al., 1999), an effect possibly mediated *via* the histaminergic system (Huang et al., 2001).

Lateral hypothalamus lesions, either at crude anatomical level or more recently at cell-type-specific level, produce profound motor, cognitive, and sleep-wake abnormalities [reviewed in Saper et al. (2005)]. Changes in LH neuron firing are sufficient to generate diverse and profound behavioral and brain state alterations, from sleep-wake switching to specific goal-directed behaviors (Bernardis and Bellinger, 1996; Adamantidis et al., 2007; Jego et al., 2013; Mahler et al., 2014; Stuber and Wise, 2016; Blomeley et al., 2018). The firing of orexin neurons is generally higher during wake (especially active waking) and lower during sleep (Lee et al., 2005). Their stimulation increases the probability for mice to transition from sleep to wakefulness (Adamantidis et al., 2007; Carter et al., 2009), while their inhibition during the inactive (light) phase has the opposite effect in mice, promoting NREM sleep (Tsunematsu et al., 2011). Specific lesions of LH orexin neurons, such as orexin peptide knockout or orexin cell ablation, can substantially alter normal timing of vigilance state transitions in response to external context (narcolepsy) (Lin et al., 1999; Ma et al., 2018), demonstrating the critical role of orexin for moment-to-moment sensorimotor control (Chemelli et al., 1999; Hara et al., 2001; Karnani et al., 2020). This crucial role in vigilance state regulation is further supported by the fact that orexin neurons send excitatory inputs to all known wake-promoting brain regions (Scammell et al., 2017).

In vivo, the changes in LH orexin neuron firing rate can occur both rapidly and slowly, and alter behaviors either quickly (subsecond) or slowly (minutes to hours) (Adamantidis et al., 2007; Karnani et al., 2019). The slow changes in activity of orexin neurons are thought to represent changes in the internal body state. The LH is historically known as a glucose-sensing brain area (Oomura et al., 1969). LH glucose-sensing has more recently been mapped onto neurochemical cell types, such as orexin and melanin-concentrating hormone (MCH) neurons (Yamanaka et al., 2003; Burdakov et al., 2005; Gonzalez et al., 2008; Karnani et al., 2011; Venner et al., 2011). The LH also contains cellular and molecular sensing pathways for numerous other indicators of body state, including hormones such as leptin and ghrelin, as well as dietary amino acids (Yamanaka et al., 2003; Leinninger et al., 2009; Karnani et al., 2011; Lam et al., 2011; Burdakov et al., 2013). In addition to nutrients and hormones, orexin neurons also sense acid and CO_2 levels, which may assist in respiratory control (Williams et al., 2007; Williams and Burdakov, 2008; Sunanaga et al., 2009). These inputs usually change LH neural firing on slow timescales, from seconds to minutes (e.g., Yamanaka et al., 2003; Williams et al., 2008). In contrast, external sensory inputs such as sound and light can alter orexin cell firing on subsecond timescales, presumably *via* direct synaptic inputs that orexin neurons receive from the rest of the brain (Mileykovskiy et al., 2005; Gonzalez et al., 2016; Karnani et al., 2020).

Lateral hypothalamus orexin cell firing may thus communicate a combined representation of fast and slow sensory variables. The consequent control of fast and slow behaviors and brain state transitions by orexin neurons has been recently reviewed elsewhere (Kosse et al., 2015; Herrera et al., 2017; Burdakov, 2019, 2020; Adamantidis et al., 2020). Below, we focus on circuit effects of endogenous orexin peptide release that may lie between orexin cell firing and behaviors or brain state transitions.

OREXINERGIC VS. GLUTAMATERGIC REPRESENTATIONS OF OREXIN CELL FIRING IN DOWNSTREAM NEURONS

Orexin neurons co-express several neurotransmitters in addition to orexins, such as the fast transmitter glutamate and the neuropeptide dynorphin (Chou et al., 2001; Schöne and Burdakov, 2012). Upon selective optogenetic stimulation of orexin neurons, the membrane potential responses of postsynaptic neurons have been analyzed using brain slice patch-clamp recordings in several brain regions.

Inside the hypothalamus, the neural circuit between LH orexin and tuberomammillary histamine neurons has been examined. During constant-frequency optogenetic stimulation of orexin cells, histamine cell firing did not follow the temporal pattern of orexin cell stimulation (i.e., a square wave), but responded with a temporal dynamics reminiscent of the sum of a first order derivative and an integral of the presynaptic orexin cell activity (Schöne et al., 2014; **Figure 1A**). Pharmacological dissection of these responses indicated that glutamate mediated only the initial transient component of the postsynaptic response, with glutamate transmission seemingly "running out of steam" after a couple of seconds of sustained orexin cell firing. In turn, the integrative sustained component of the responses, which accounted for most downstream spikes in the orexin→histamine circuit, was mediated by orexin neuropeptide transmission, specifically by orexin type-2 GPCRs (Schöne et al., 2014). Pharmacological blockade of glutamate-driven spiking did not affect orexin-driven spiking and vice versa, suggesting that each co-transmitter acts in an isolated manner, without orexin-glutamate cross-modulation. This ability of orexin and glutamate to translate distinct features of orexin cell firing activity into sustained integrative, and transient derivative-like responses, respectively, illustrates how neuropeptides can create functional slow neural circuits that are operationally distinct from classic fast neural circuits. The integral-like nature of orexin-induced postsynaptic firing has been compared to integral feedback control signals, which are likely to be essential for stable feedback control of brain states (Kosse and Burdakov, 2014; Schone and Burdakov, 2017).

In several other hypothalamic regions, orexin-induced postsynaptic excitatory responses have also been reported, although they were created by exogenous application of orexin to brain slices rather than by endogenous orexin neurotransmission as in the above-described study (Follwell and Ferguson, 2002; Burdakov, 2004; van den Top et al., 2004; Sakurai, 2007). Interestingly, in some cases, only weak or non-existent "classic" (i.e., fast glutamatergic or GABAergic) orexin→target circuit connectivity was found in targets that display robust responses

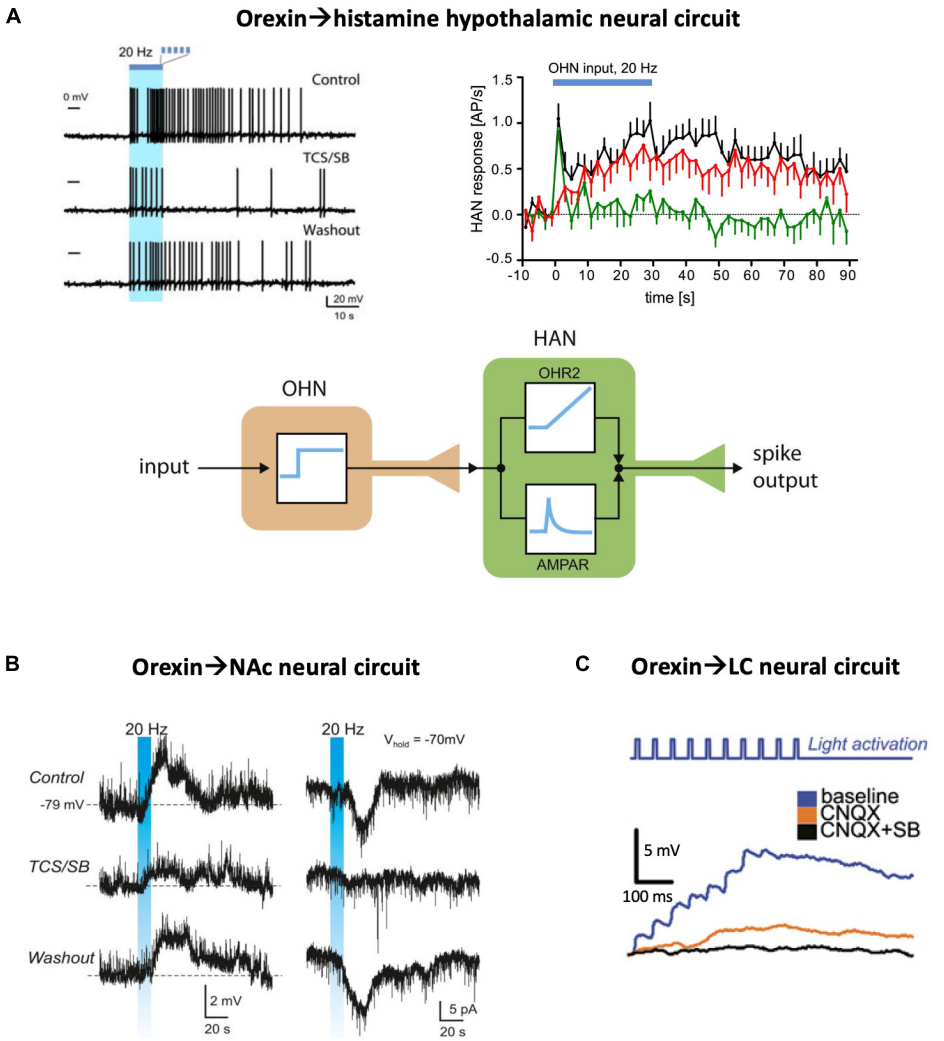

FIGURE 1 | Optogenetic evidence for neuropeptide-mediated neural circuit connectivity. **(A)** Hypothalamic orexin neuron (OHN) → histamine neuron (HAN) circuit. Top left, example of HAN firing response to optogenetic stimulation of OHNs (blue bar), in the presence and absence of orexin receptor blockers (TCS/SB = OX1 and OX2 receptor antagonists SB334867 and TCS-OX2-29). Top right, the same data plotted across many trials, illustrating glutamate (green) and orexin (red) circuit connectivity (AP/s = action potentials per second). Bottom, a conceptualization of the orexin and glutamate transmission as integral-like and derivative-like parallel signals. Source: Schöne et al., 2014. **(B)** Circuit between hypothalamic orexin neurons and accumbal D2 neurons. Stimulation of orexin cell axons (blue bar) creates orexin-receptor-blockade-sensitive excitation in the postsynaptic D2 neurons (left column, membrane potential recordings; right column, membrane current recordings). Source: Blomeley et al., 2018. **(C)** Circuit between hypothalamic orexin neurons and LC noradrenaline neurons, in the presence and absence of glutamate (CNQX = AMPA receptor blocker) and orexin (SB = SB334867 orexin receptor blocker) receptor transmission. Adapted from Sears et al., 2013.

to exogenous orexin. One of such targets is LH GAD65 neurons, which are robustly excited by orexin and are required for generating normal locomotion in mice (Kosse et al., 2017). This example is interesting, because based on traditional "gold standard" connectivity mapping–i.e., simultaneous pre- and postsynaptic patch-clamp recordings or channelrhodopsin-assisted circuit mapping which focus on fast connectivity and ignore slow connectivity–it would be concluded that the orexin→GAD65 LH microcircuit does not exist (Burdakov and Karnani, 2020). Yet, when examined from a neuropeptidergic perspective, the LH GAD65 neurons display robust machinery for strong orexin→GAD65 neuropeptidergic coupling, which appears important for activation of LH GAD65 neurons *in vivo* (Kosse et al., 2017).

Outside the hypothalamus, there is also considerable evidence that neuroexcitatory orexin transmission forms functional peptidergic circuits. One example is the recently described circuit between orexin neurons and dopamine-inhibited D2 receptor-expressing medium spiny neurons of the nucleus accumbens shell (Blomeley et al., 2018). Optogenetic excitation of orexin neurons creates depolarization waves in these D2 neurons, and these waves are blocked by orexin receptor antagonists demonstrating a functional $LH_{orexin} \rightarrow NAc_{D2}$ neuropeptidergic circuit (**Figure 1B**). This circuit is proposed to control

NAc-dependent action-selection, in particular risk taking, based on orexin cell activity (Blomeley et al., 2018). In contrast to the strong effects of endogenously-released orexin, glutamatergic transmission in the same circuit appears rather weak (Blomeley et al., 2018). Similar neuropeptide-mediated circuits, albeit with a stronger glutamatergic component, have been reported between orexin neurons and locus coeruleus neurons (Sears et al., 2013; **Figure 1C**).

From such studies, it can be concluded that orexin neuropeptide transmission is able to create functional intra- and extra-hypothalamic neural circuits in its own right, beyond a mere modulation of fast neurotransmitters.

HOW UNIQUE ARE NEUROPEPTIDES IN THE SLOWNESS OF THEIR ACTIONS?

Describing transmitters as fast and slow is a concise way to capture their important operational characteristics. Yet, the dichotomy between fast and slow transmitters can also be viewed as somewhat artificial. Canonical "fast" transmitters, such as GABA/glutamate, also have "slow" receptors (typically GPCRs) that exert well-characterized long-lasting effects on neuronal function (Bormann, 2000; Niswender and Conn, 2010). Non-neuropeptide, intermediate-sized transmitters such as amines and acetylcholine also act on a plethora of neuronal GPCRs coupled, among other effectors, to plasmalemmal ion channels and thus to membrane excitability (Bear et al., 2015). Since non-neuropeptide transmitters are capable of slow and lasting control of neuronal excitability *via* such mechanisms, one may justifiably ask: Why does the brain need neuropeptides? What is unique about neuropeptides that other transmitters cannot achieve?

The resources invested by the brain into making neuropeptides (transcription, translation, and trafficking) are presumably not trivial. Thus, it is tempting to speculate that the 100 + neuropeptides in the brain play some unique functions, and did not evolve solely to serve as a "redundant safeguard" should something go awry with the other transmitters (or as convenient molecular markers for neuroscientists). We speculate that their unique function does not arise at the receptor level, since, as alluded above, other transmitters have similar types of receptors, namely GPCRs linked to long-lasting postsynaptic effects. It could, instead, stem from what happens to neuropeptides in the extracellular space, during the time between release and receptor binding. Unlike other neurotransmitters, which are rapidly cleared from the extracellular space by uptake into neurons and glia by specialized membrane transporters (Bear et al., 2015), neuropeptides have no known specific clearance mechanisms. Neuropeptide diffusion from their release sites will also be slower than the other transmitters, due to their larger size. While there is little quantitative information yet about how far neuropeptides spread from their natural release sites in different brain areas, and how long they stay in the extracellular space following release, we speculate that the spatiotemporal scales involved are likely to be longer than for other neurotransmitters. This may contribute to the unique reasons why neuropeptides have evolved among other transmitters. Alternatively, or in addition, these unique reasons could relate to certain advantages of neuropeptides for evolution itself. The 1 gene/1 (prepro)peptide encoding relationship of peptides vs. the enzymatic multistep synthetic pathways of other neurotransmitters might offer the evolutionary advantage of single gene duplication for generation of new neuronal identities.

EMERGING CONCEPTS AND FUTURE DIRECTIONS

In order to gain a broader understanding of functional neural circuits, we propose that neuropeptide-mediated postsynaptic signals should be routinely analyzed alongside the classic small-molecule neurotransmitters in circuit connectivity screens. While such broader analysis is becoming commonplace in functional dissections of hypothalamic circuits, it is still relatively unusual in other neural circuits, e.g., in cerebral cortex where neuropeptides such as NPY and somatostatin are also abundantly expressed (Karnani et al., 2016). Thanks to the current genetic tools, studies are able to focus on neuronal populations that express a given neuropeptide. And yet, in most cases, the exact role of the neuropeptidergic release vs. that of the co-released classic neurotransmitter(s) is rarely unraveled. For example, the role of galaninergic neurons in sleep regulation has been rather extensively studied, in particular the sleep-promoting role of galanin-expressing GABAergic neurons of the ventrolateral preoptic area (Steiger and Holsboer, 1997; Sherin et al., 1998). A more recent study also showed that galaninergic neurons of the dorsomedial hypothalamus can be divided into two distinctly-projecting subsets: one suppressed during REM sleep and whose activation promotes NREM sleep and opposes REM sleep, the other with exact opposite patterns and effects (Chen et al., 2018). But these studies fail to clarify which of GABA or galanin–that these neurons co-express–mediates the reported effect on sleep regulation (Sherin et al., 1998; Chen et al., 2018). Thus, expanding circuit connectivity screens to neuropeptides and their specific actions may shed light on the mechanisms through which neural circuits solve the challenging task of exerting stable control over the brain and body in a rapidly changing world (Kosse and Burdakov, 2014).

Several emerging features of peptidergic neurotransmission should be kept in mind while probing neuropeptidergic connectivity of a neural circuit. First, there may be a presynaptic frequency threshold for neuropeptide release that is higher than that for small transmitters such as GABA or glutamate (Verhage et al., 1991; Schöne et al., 2014). It is therefore important to screen a range of presynaptic frequencies. Second, neuropeptide release and/or action seems to build-up slowly during steady presynaptic stimulation, and also decays slowly, sometimes over many seconds, rather than a few milliseconds as in the case of glutamate or GABA fast transmission (Schöne et al., 2014; Blomeley et al., 2018). It is therefore important to screen the effects of prolonged presynaptic firing trains (especially where prolonged firing is normally displayed by the presynaptic neurons *in vivo*), and to allow sufficient time for the postsynaptic response

to appear. Third, while the above-chosen example of orexin illustrates how it directly creates postsynaptic excitation, this is not the case of all neuropeptides. Even within the LH, the MCH neuropeptide, which is made by neurons intermixed with (but distinct from) orexin neurons, does not appear to have direct effects on postsynaptic membrane potential but rather seems to act by altering GABA or glutamate signaling, which could supply a lasting permissive signal [akin to an eligibility trace (Gerstner et al., 2018)] for creating or erasing certain kinds of memory (Adamantidis and de Lecea, 2009; Izawa et al., 2019; Kosse and Burdakov, 2019; Burdakov and Peleg-Raibstein, 2020; Concetti et al., 2020). Thus, "modulatory" actions of neuropeptides should continue to be examined, even though some neuropeptides such as orexin do not require them to form functional neural circuits.

In summary, the ability of neuropeptidergic postsynaptic effects to substantially outlast presynaptic firing may bind together fast and slow brain functions in a way that cannot be achieved by fast transmitters alone. Slow on/off neuropeptide signals may thus enable core brain functions, such as creating temporal eligibility traces for memory formation and information routing. Neuropeptides that directly affect the firing of postsynaptic neurons can, in addition, create functional neural circuits able to perform control-relevant computations such as signal integration. This gives peptidergic neural circuits unique advantages for efficient neuronal processing and feedback control of consciousness.

AUTHOR CONTRIBUTIONS

DB and MG wrote this review. Both authors contributed to the article and approved the submitted version.

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Medial Parabrachial Nucleus Is Essential in Controlling Wakefulness in Rats

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Activation of the parabrachial nucleus (PB) in the brainstem induced wakefulness in rats, suggesting which is an important nucleus that controls arousal. However, the sub-regions of PB in regulating sleep-wake cycle is still unclear. Here, we employ chemogenetics and optogenetics strategies and find that activation of the medial part of PB (MPB), but not the lateral part, induces continuous wakefulness for 10 h without sleep rebound in neither sleep amount nor the power spectra. Optogenetic activation of glutamatergic MPB neurons in sleeping rats immediately wake rats mediated by the basal forebrain (BF) and lateral hypothalamus (LH), but not the ventral medial thalamus. Most importantly, chemogenetic inhibition of PB neurons decreases wakefulness for 10 h. Conclusively, these findings indicate that the glutamatergic MPB neurons are essential in controlling wakefulness, and that MPB-BF and MPB-LH pathways are the major neuronal circuits.

Keywords: chemogenetics, glutamatergic neurons, optogenetics, parabrachial nucleus, rat, wakefulness

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INTRODUCTION

Wakefulness has been reported to be controlled by multiple neuronal systems, such as histamine neurons in the tuberomammillary nucleus (TMN) (Huang et al., 2006), noradrenaline (NA) neurons in the locus coeruleus (LC) (Hagan et al., 1999), orexinergic neurons in the lateral hypothalamus (LH) (Chemelli et al., 1999), and GABAergic and cholinergic neurons in the basal forebrain (BF) (Anaclet et al., 2015; Xu et al., 2015). However, lesions or inactivation of single arousal system demonstrated that none of these arousal nuclei are key players in initiating or maintaining wakefulness (Blanco-Centurion et al., 2007), which indicates that wakefulness may be regulated by arousal promoting networks, or that more essential nuclei controlling wakefulness remain unidentified.

In humans, brainstem stroke patients often experience coma symptoms when brain damage is confined in the upper pontine tegmentum (Parvizi and Damasio, 2003). In rats, bilateral chemical lesions of the parabrachial nucleus (PB) area, including the precoeruleus, induce a deep coma

state with behavioral unresponsiveness (Fuller et al., 2011). Recently, Qiu et al. (2016) showed that chemogenetic activation of the PB induced long lasting arousal via the BF and LH in rats; however, the wakefulness induced by activation of PB may be secondary to other behavior or physiologic functions (Scammell et al., 2017). The effect of reversible inhibition of the PB on wakefulness regulation is needed. In addition, specific lesions of glutamatergic neurons in the external and crescent parts of lateral PB (LPB) but not the medial part of PB (MPB) decreased hypercapnia-evoked arousal, while specific deletion of glutamatergic MPB neurons increased non-rapid eye movement (NREM) sleep in mice (Kaur et al., 2013). Moreover, many of the LPB neurons that express calcitonin gene-related peptide are responding to CO_2, and activation of these neurons in the LPB induced wakefulness, while inhibition of these neurons prevented arousal to CO_2 in mice (Kaur et al., 2017). In addition, electrical activation of the LPB can induce reanimation during continuous isoflurane anesthesia (Muindi et al., 2016), and chemogenetic activation of glutamatergic PB neurons shorten the anesthesia recovery time in mice (Wang et al., 2019; Xu et al., 2020). These results indicated that the PB may be a powerful candidate in sleep-wake regulation, and the MPB and LPB may play different roles. Moreover, the sub-regions of the PB that are involved in natural wakefulness and the cellular types of the downstream targets are still unclear.

In the current study, we employ a chemogenetic strategy to activate PB neurons, and elucidate the role of MPB and LPB in promoting wakefulness. In addition, optogenetic strategy is employed to activate the glutamatergic MPB neurons and their terminals in the BF, LH and ventral medial thalamus (VM), to reveal which cell types of BF, LH, or VM neurons, are responsible for PB in controlling arousal. Lastly, inhibition of PB by genetically engineered ivermectin (IVM)-gated human glycine receptor (IVMR) was employed to decrease wakefulness in rats. These results will clearly reveal subregions of the PB and their neural circuits in controlling wakefulness.

MATERIALS AND METHODS

Animals

Pathogen-free adult Sprague–Dawley rats (male, weighing 45–55 g, 3 weeks old, or 200–220 g, 6 weeks old) were purchased from the Sino-British SIPPR/BK Lab. Animal LTD., Shanghai, China. The rats were housed at an ambient room (temperature, 23 ± 1°C and relative humidity, 60 ± 5%) under automatically controlled 12 h/12 h light/dark cycle condition (07:00/19:00). The animals had access the water and food *ad libitum* during the study (Zhang et al., 2017; Dong et al., 2019). All experiments were carried out in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and approved by the Animal Care and Use Committee of Fudan University. No sample size calculation was performed. The sample size used in present study is depended on the expected variations between rats and is comparable to many previous reports using similar techniques. Additionally, no method of randomization or blinding of treatment was used in present study.

Generation of Adeno-Associated Viral (AAV) Vectors

The AAVs of serotype rh10 for AAV-hSyn- hM3Dq-mCherry, AAV-hSyn-mCherry were generated by tripartite transfection into 293A cells, separately, as we described previously (Lazarus et al., 2011; Oishi et al., 2017). The AAV-hSyn-IVMR-eGFP and AAV-hSyn-eGFP were purchased from Shanghai Taiting biological Co. Ltd. (Shanghai, China). The AAV-CaMKIIα-ChR2-mCherry and AAV-CaMKIIα-mCherry were purchased from Obio Technology Co. Ltd. (Shanghai, China).

Stereotaxic AAV Injection and Electrode Implants

The animals were anesthetized with chloral hydrate (10% in saline, 350 mg/kg), using aseptic techniques, hM3Dq or mCherry (200 nL/injection) was injected stereotaxically into the MPB (AP = −8.0 mm, ML = ± 1.5 mm, DV = −5.8 mm), or LPB (AP = −8.0 mm, ML = ± 2.2 mm, DV = −5.0 mm) according to the rat brain atlas of Paxinos and Watson (2007) in 6-week-old rats. Another batch of rats were microinjected with IVMR or eGFP (200 nL/injection) into the PB (AP = −8.0 mm, ML = ± 1.7 mm, DV = −5.8 mm) bilaterally. Then, the rats were implanted the EEG and EMG electrodes as described before (Zhang et al., 2013; Chen et al., 2019; Li et al., 2020; Shen et al., 2020). For optogenetics, ChR2 or mCherry (200 nL/injection) was stereotaxically injected into the MPB region, and 2 weeks later, the EEG/EMG electrodes and guide cannula for optic fibers were implanted. Following surgery, rats were housed individually for 2 weeks (Wu et al., 2015; Oishi et al., 2017; Luo et al., 2018; An et al., 2020).

EEG/EMG Recording and Sleep-Wake Scoring

Rats were allowed 14 days recovery from surgery before the EEG/EMG recording. Each rat was connected to an cable for EEG/EMG recording in a chamber and habituated for 3 days before the recording. The EEG/EMG signals (EEG: 0.5–30 Hz, EMG: 20–200 Hz, sampling rate: 128 Hz) were recorded at baseline and under chemogenetic or optogenetic manipulation conditions using Vitalrecorder software (Kissei Comtec, Nagano, Japan). Then the vigilance states were automatically scored offline by 10 s epochs into three stages, including wake, REM or NREM sleep, using Sleepsign (Kissei Comtec, Nagano, Japan), according to previously established criteria (Xu et al., 2014; Li et al., 2020; Shi et al., 2020). As a final step, defined sleep-wake stages were checked visually, and corrected if necessary. The amount of time spent in each vigilance stage was determined from the scored data. The EEG power spectral density was converted into a dataset in 10-s epochs for 0–25 Hz, 24 h in length of sleep-wake behavior in the chemogenetics data or 3 h in length in the optogenetics data. The bit map represents the EEG power spectra generated by MATLAB (The MathWorks, Inc., Massachusetts) (Litvak et al., 2011).

Drug Treatments

Clozapine-*N*-Oxide (CNO) was purchased from LKT Laboratories, Inc. (Saint Paul, MN, United States). The CNO (0.03, 0.1, or 0.3 mg/kg) was dissolved in saline and intraperitoneally injected in rats at 09:00 for hM3Dq rats. The IVM were purchased from Sigma-Aldrich (Missouri), and dissolved in isopropanol at the dosage of 10 mg/kg, The IVM and isopropanol were administrated at 20:00 on a 2-day schedule.

Behavioral Analysis

The behaviors of the rats after saline or CNO injection were analyzed using video recordings as we described previously (Oishi et al., 2017). Briefly, behaviors during the first hour and the third hour after saline or CNO treatment were scored in 4 s epochs as attentive wake, characterized by non-specific motor activity (for example, head bobbing and low neck muscle activity.) or quiet wake, during which animals were quiet without walking (Lepski et al., 2012), and grooming (including head washing, body grooming, and paw or leg licking), exploring, eating, drinking and sleep when the behavior accounted for more than 50% of the epoch.

In vivo Optogenetics

Fourteen days after the surgery for implanting the EEG/EMG electrodes and optic guide cannula, the EEG/EMG recording cables were connected to the amplifier and fiber optic cables (1-m long, 200-μm diameter, Newdoon Inc., Hangzhou, China) were placed inside the implanted cannula simultaneously, and fiber-optic rotary joints (Doric Lenses, Québec, Canada) were used for unrestricted *in vivo* illumination. Rats were acclimatized for 2 days before the photostimulation sessions. Light pulse trains were programmed using a pulse generator (Nihon Kohden, Tokyo, Japan) that provided simultaneous input into 2 blue light lasers (473 nm, 100 mW intensity; SLOC, Shanghai, China). For acute optogenetic procedure, each stimulation train was applied 60 s after a stable NREM or REM sleep event as detected by real-time online polysomnographic recording. For the chronic photostimulation experiments, blue light stimulation (5-ms pulses, 50 trains of 20 Hz for MPB, 25 trains of 40 Hz for BF, LH and VM, main interval 30 s) was applied for 1 h during 9:00-10:00.

In vitro Electrophysiology

For *in vitro* electrophysiologic recording, 3-week-old rats were injected with recombinant AAVs carrying ChR2 or mCherry (200 nL/injection) for the optogenetics experiment into the MPB (AP = −7.0 mm, ML = ± 1.6 mm, DV = −5.8 mm). After 3–4 weeks of postoperative recovery, rats were anesthetized and perfused transcardially with ice-cold modified artificial cerebrospinal fluid (aCSF) containing (in mM) 0.4 Vitamin C, 0.5 $CaCl_2$, 1.2 $NaH2PO_4$, 2 Na-pyruvate, 2.5 KCl, 3 $MgSO_4$, 10 glucose, 23 $NaHCO_3$, 252 sucrose, and saturated with 95% O_2 and 5% CO_2 (pH 7.2–7.4, 301–305 Osm). Coronal slices (250 mm thick) containing the PB were cut using a vibratome (VT1200S, Leica, Germany) and incubated for 1 h at 32°C in a holding chamber in oxygenated aCSF containing (in mM) 1.25 $NaH2PO_4$,1.3 $MgSO_4$, 2 $CaCl_2$, 3 KCl, 10 glucose, 26 $NaHCO_3$, and 124 NaCl.

Whole-cell recordings were performed using patch electrodes (4–6 MΩ) containing (in mM) 0.3 EGTA, 0.3 Na-GTP, 4 Mg-ATP, 10 KCl, 10 Na-phosphocreatine, 10 HEPES, 125 potassium gluconate, and 0.2% biocytin (PH 7.3; Osmolarity, 290 ∼ 300 mOsm). The slice was transferred to a recording chamber which was continuously perfused with oxygenated aCSF at a flow rate of 2–3 ml/min (32°C). The PB was identified by its localization relative to the superior cerebellar peduncle and fourth ventricle under visual guidance using a fluorescence microscope (Olympus, Tokyo, Japan). Recorded PB neurons were further distinguished from other cells by positive fluorescence. Coronal sections of the PB, BF, LH, or VM (300 μm) were collected. ChR2 was stimulated by a 473 nm blue light laser (SLOC, Shanghai, China). The tip of the optical fiber was placed 500 μm above the recording cell. Cells with series resistance changed by > 20% were discarded.

Single-Cell Reverse Transcription (RT-PCR)

After each recording, cytoplasm was aspirated into the patch pipette by applying negative pressure, and expelled into a PCR tube (Axygen, Massachusetts) as previously described (Xu et al., 2015). The presence of mRNAs coding for ChAT, VGluT2, and VGAT was detected by single cell RT-PCR, according to the manufacturer's instructions (**Supplementary Table 1**). Then, PCR products were visualized by Safe Gel-stained 1.5% agarose gel electrophoresis.

Immunohistochemistry

Animals were deeply anesthetized with chloral hydrate (400 mg/kg, i.p.) and perfused transcardially with saline followed by 4% paraformaldehyde. Brain samples were removed and postfixed in 4% paraformaldehyde overnight at 4°C, and cryoprotected in 20% sucrose-phosphate buffer (4°C) until sunk to the bottom. The brain samples were then frozen and sectioned in the coronal plane at 30 μm using a Leica freezing cryotome (CM1520, Leica, Germany). The staining was performed on free-floating sections as previously described (Zhang et al., 2013; Xu et al., 2019). In brief, sections were incubated with the primary antisera (rabbit anti c-Fos, 1:10000, Millipore, Massachusetts). at room temperature overnight. Then the sections were rinsed by PBS and incubated for 1.5 h in biotinylated anti-rabbit secondary antiserum (1:1000, Jackson Immunoresearch Laboratories, Pennsylvania). All tissue sections were manipulated with avidin-biotin complex (1:1000, Vector Laboratories, California) for 1 h, and immunopositive cells were visualized black by reaction with 3,3-diaminobenzidine (DAB) with nickel (DAB Substrate Kit, Vector Laboratories, California). After rinsing with PBS, the sections were once more incubated with the primary antibody for anti-Dsred (1:5000, Takara Bio Inc., Shiga, Japan). Following this incubation with secondary antibody and avidin-biotin complex, the sections were visualized brown by reaction with DAB without nickel. Following additional washes in PBS, sections were then mounted, dried, dehydrated, and

cover slipped. The hM3Dq or ChR2 expression was identified by the expression of mCherry positive neurons by either only staining the DsRed/mCherry, or viewing the native fluorescence of PB by microscope (Olympus, BX51, Tokyo, Japan) with a boundary that encompassed >90% of all mCherry-containing neurons. IVMR expression was identified by staining against eGFP (1:1000, life technologies, California). The c-Fos positive neurons were counted to evaluate the potential neuronal circuits mediating the wake-promoting effect of MPB, including MPB, CG, M1, S1, LS, BNST, CeA, BF, MDM, LPMR, PV, VM, LH, PSTN, TMN, MGV, PAG, VTA, and LC. Cell counting was performed on three adjacent sections (separated by 90 μm) from four rats, the average counting per section per side was used to represent the data.

According to the rat brain atlas in stereotaxic coordinates by Paxinos and Watson (2007), the boundaries of PB was defined along the superior cerebellar peduncle. For all the rats were included in the analysis only if above 80% of the labeled neurons were central in the target region (MPB or LPB, respectively) bilaterally with a small transfection of adjacent area. Of the rats for analysis from the optogenetic study, 7 cases of mCherry expression missed the MPB bilaterally were excluded, and 3 missed the BF bilaterally and 2 missed the LH bilaterally were excluded. In total, 12 cases of rats were excluded from the optogenetic study *in vivo*.

Statistical Analysis

All results were expressed as the mean ± SEM. Statistical analysis between 2 groups was performed using the paired or unpaired two-sided Student's *t*-test. In all cases, $P < 0.05$ were taken as the level of significance.

RESULTS

MPB but Not LPB Neurons Were Involved in Controlling Wakefulness in Rats

To determine the sub-regions of the PB neurons in controlling wakefulness, the AAV vector containing the excitatory mutant human M3 muscarinic receptors (hSyn-hM3Dq-mCherry-AAV, hM3Dq) were expressed in bilateral parts of either the MPB or LPB. As shown in **Figures 1A,B**, the hM3Dq/mCherry fusion protein was successfully expressed in the MPB (**Figure 1A**) or LPB (**Figure 1B**), as indicated by superimposed mCherry expression areas in 8 AAV-injected rats, respectively. Administration of CNO (0.3 mg/kg) promoted long-lasting wakefulness in rats expressing hM3Dq in the MPB (**Figures 1C,E**) without rebound in sleep or changes in the power spectrum for NREM and REM sleep after long-lasting wakefulness induced by CNO (**Figure 2**), similar to activation of the entire PB (Qiu et al., 2016). In contrast, there was no significant change in the sleep-wake profiles of rats after activation of only the LPB (**Figures 1D,F**). In addition, the CNO treated MPB-hM3Dq rats showed an increased theta EEG power spectra of wakefulness compared with the saline-treatment (**Figure 1G**), while the CNO-treatment did not change the EEG power spectrum of wakefulness in the LPB-hM3Dq rats (**Figure 1H**). These data clearly indicate that MPB neurons play a crucial role in controlling wakefulness.

We analyzed the behaviors of rats during long-lasting arousal induced by CNO. During 10 h after CNO administration (light period), rats spent most of their time in "attentive wake" characterized by head bobbing without moving around, while these rats after saline treatment spent more time in "quiet wake" and sleep during the first hour, followed by more sleep during the subsequent periods (**Figure 3** and **Supplementary Movies 1**, **2**). After saline treatment, the rats showed exploratory behaviors, such as exploring, sniffing and rearing during the first hour (**Figure 3B** and **Supplementary Movie 2**). The mCherry-expressing control rats treated with CNO at 9:00 also exhibited a similar behavior as the hM3Dq rats treated with saline. They spent more time in sleep after an initial hour of "quiet wake" after injection (**Figure 3C**).

To test whether CNO activates hM3Dq-expressing neurons *in vivo*, colocalization of c-Fos, a marker for neuron activity, with mCherry was examined after CNO or saline treatment. c-Fos was robustly expressed in MPB neurons after CNO administration, as compared to the saline control, indicating that hM3Dq effectively activated MPB neurons *in vivo* (**Supplementary Figure 1**). Moreover, c-Fos was highly expressed in many other nuclei such as the cerebral cortex, BF, thalamus, LH, TMN, ventral tegmental area (VTA), periaqueductal gray (PAG) and LC. This observation suggests that activation of MPB neurons strongly increased the activity of wake-promoting neurons.

Optogenetic Activation of Glutamatergic MPB Neurons Immediately Initiated Wakefulness

To clarify the role of the MPB neurons in initiating arousal, we employed the optogenetic strategy to activate glutamatergic MPB neurons using an AAV vector carrying an excitatory channelrhodopsin-2 (ChR2) with a CaMKIIα promoter [CaMKIIα-ChR2 (H134R)-mCherry-AAV, ChR2]. ChR2 was bilaterally expressed in MPB neurons (**Figure 4A**) and the optical fibers were placed within the MPB boundaries (**Figure 4B**). For the whole-cell current clamp recording conditions, short pulses of blue light (5–10 ms) elicited single spikes in MPB neurons (red curve, **Figures 4C,D**), whereas pulses longer than 10 ms induced two action potentials (light gray curve, **Figure 4C**). The trains of short blue light pulses entrained the firing of ChR2-expressing MPB neurons up to 50 Hz with high fidelity ($n = 13$, **Figure 4E**). We subsequently used a pulse duration of 5 ms and frequency of 20 Hz to stimulate the MPB glutamatergic neurons in the following optogenetic study *in vivo*. After patch clamp recordings, the cell type of the ChR2-expressing neurons, which can be elicited firing by light, was identified using single cell reverse-transcription PCR. The presence of a 315 bp-band specific for the vesicular glutamate transporter 2 (VGluT2, **Figure 4F**) suggests that firing was evoked in ChR2-expressing glutamatergic MPB neurons *in vitro* by blue-light stimulation.

Next, we implanted optic fibers and EEG electrodes on the rat skull with dental cement and placed EMG wire electrodes into the nuchal muscles. We then stimulated the glutamatergic

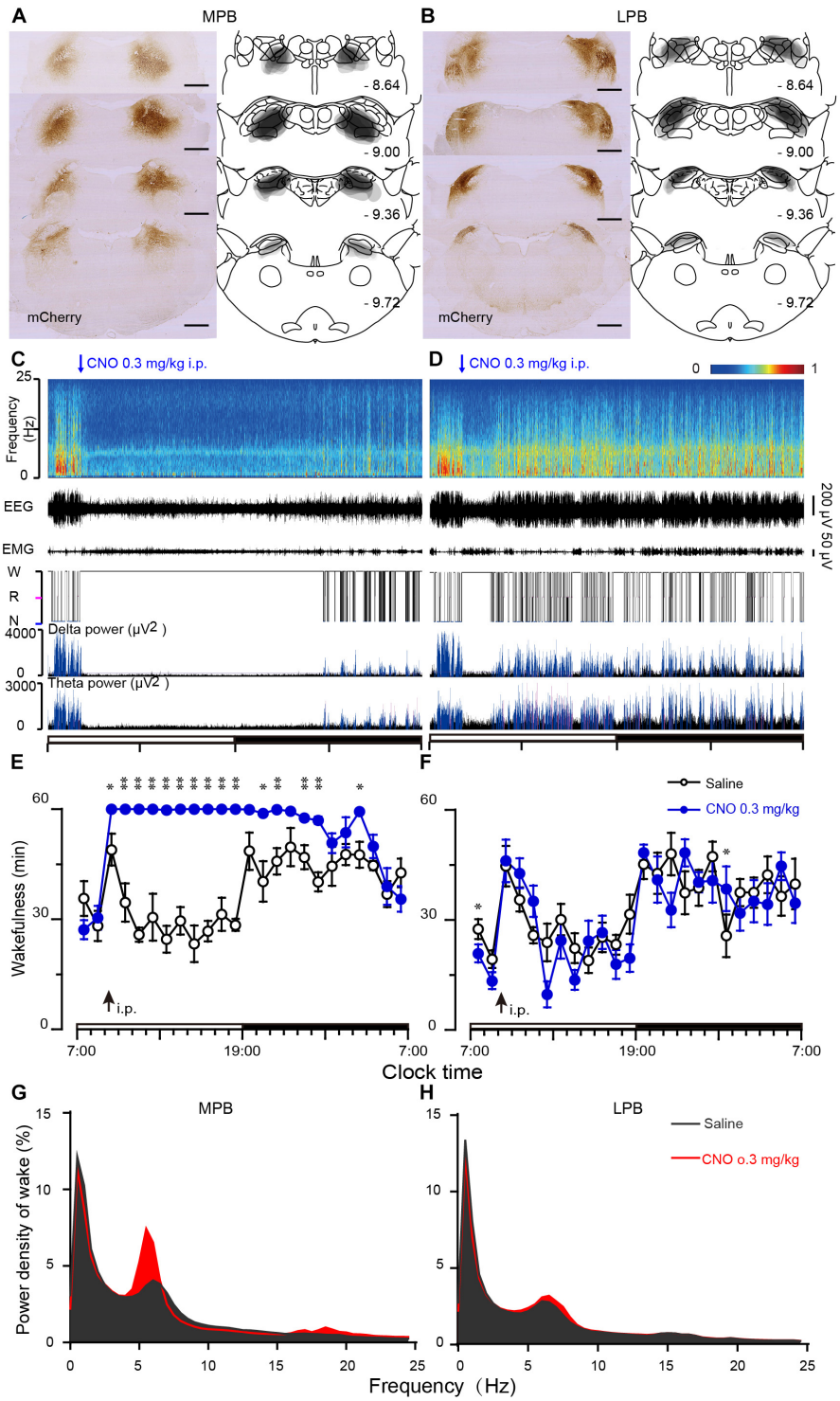

FIGURE 1 | Chemogenetic activation of MPB (but not LPB) neurons induces wakefulness. **(A,B)** Schematic diagram indicating the position of hM3Dq-mCherry protein was confined to the MPB **(A)** or LPB **(B)**. Superimposed mCherry expression areas in the MPB **(A)** and LPB **(B)** of 8 AAV-injected rats in each group, are shown in the right panels (Paxinos and Watson, 2007). Scale bars: 1 mm. **(C,D)** Typical examples of EEG power spectra, EEG, EMG, hypnogram, and relative magnitude changes in delta and theta power over 24 h for a rat expressing hM3Dq at MPB **(C)** or LPB neurons **(D)** when given CNO (0.3 mg/kg). **(E,F)** Time courses of wakefulness after administration of CNO (0.3 mg/kg) or saline in rats expressing hM3Dq in MPB **(E)** or LPB neurons **(F)**. The arrow indicates the time point of CNO or saline administration, and open and closed bars above the *x*-axis indicate light and dark periods, respectively. **(G,H)** EEG Power spectrum of wakefulness during 10 h (9:00–19:00) after the CNO injection in MPB-hM3Dq **(G)** and LPB-hM3Dq rats **(H)**. $^{*}P < 0.05$, $^{**}P < 0.01$, compared with saline ($n = 8$).

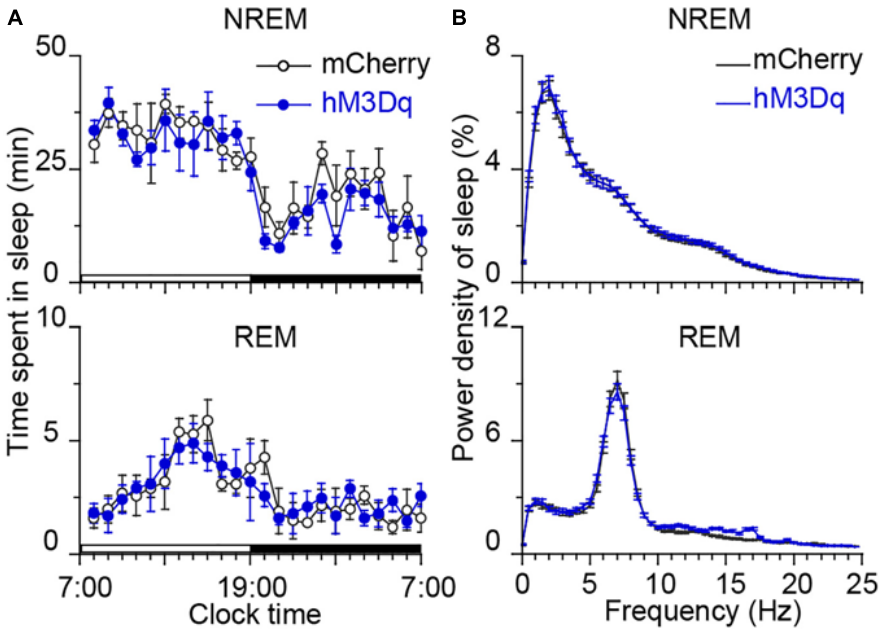

FIGURE 2 | Time course of sleep and power spectra starting 22 h after CNO treatment. **(A)** No bouts of NREM and REM sleep after the CNO treatment. Each circle represents the hourly mean amount of sleep. The horizontal open and closed bars on the *x*-axes indicate the 12-h light and 12-h dark periods, respectively. **(B)** EEG power spectra of NREM and REM sleep during the following day in rats injected with AAV-hSyn-hM3Dq-mCherry in MPB (n = 8).

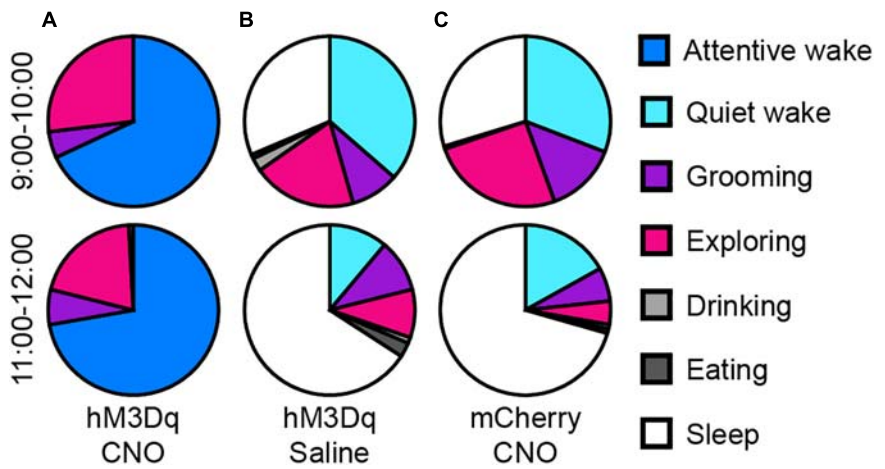

FIGURE 3 | Activation of glutamatergic PB neurons produces attentive wake in rats. After injection of CNO at 9:00, rats are mainly in attentive wake (do not move but with head bobbing) with modest amounts of time spent exploring, grooming, or rearing for more than 10 h **(A)**. After injection of saline, hM3Dq rats are mainly asleep with a small amount of time in quiet wake and exploring behavior **(B)**. Injections of CNO in mCherry control rats at 9:00 produced similar behavior to the saline treated hM3Dq rats, mainly asleep with a small amount of time in quiet wake and exploring, grooming, and feeding **(C)** (n = 4).

MPB neurons *in vivo* with brief pulses (5 ms) of blue light in the frequency range of 1–20 Hz when the rats were sleep. Acute bilateral light stimulation produced immediate transitions from sleep to wakefulness with an average latency of 2.75 s in ChR2 rats at 20 Hz (**Supplementary Movie 3**). There was no striking change of the waveforms in mCherry-expressing control rats (n = 6, **Figure 4G** and **Supplementary Movie 4**). The probability of a sleep-to-wake transition increased with the stimulation frequency or bilateral stimulation in ChR2 rats (**Figure 4J**). Unilateral activation of MPB neurons also promoted transition from sleep to wakefulness with a lower probability than bilateral stimulation (**Figure 4I**), while there was no change in mCherry-expressing control rats (**Figure 4H**). Then chronic bilateral stimulation protocol (5 ms, 20 Hz, 50 trains, main interval 30 s) was applied to the MPB neurons *in vivo* for 1 h. As a result, activation of MPB neurons induced immediate transition from NREM sleep to wakefulness and the wakefulness was maintained for 1 h. After stopping stimulation,

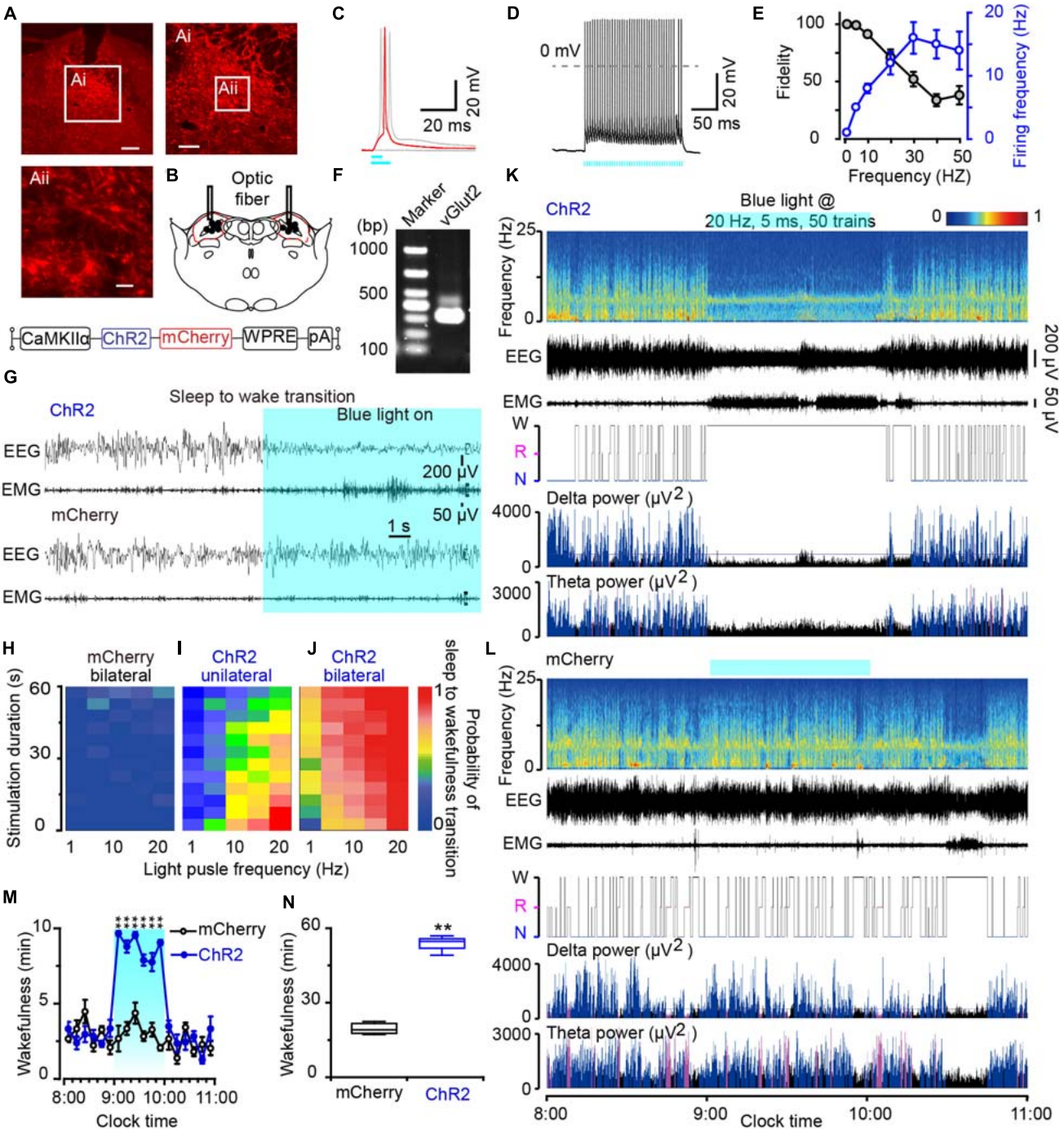

FIGURE 4 | Photostimulation of MPB glutamatergic neurons induced a rapid wakefulness response. **(A)** Coronal brain section with native mCherry fluorescence confirmed ChR2-mCherry was confined in the MPB. Scale bars: A = 200 μm, Ai = 100 μm, Aii = 20 μm. **(B)** The schematic diagram showing the cannula trace and the optic fibers targeting the MPB. **(C)** A single action potential (red trace) evoked by 5 ms blue light pulses, and double action potentials (gray trace) evoked by >10 ms blue light pulses from a ChR2-expressing neuron. **(D)** Firing traces from a ChR2-expressing MPB neuron evoked by 10 Hz (5 ms pulses) photostimulation. **(E)** Neuronal firing showing an effective entrainment up to 20 Hz optogenetic stimulation of MPB neurons. **(F)** Single-cell RT-PCR analysis of ChR2-expressing neurons in MPB indicates the band of 315 bp represents VGluT2. **(G)** EEG/EMG examples of the sleep to wakefulness transition following optogenetic stimulation in a rat with bilateral ChR2 (top) or mCherry (bottom) expression in MPB glutamatergic neurons. **(H–J)** The probability of sleep to wakefulness transition in the frequency range of 1–20 Hz in mCherry controls **(H)**, stimulating the MPB unilaterally **(I)**, and bilaterally **(J)**. **(K,L)** Typical examples of EEG power spectra, EEG, EMG, hypnogram, and relative magnitude changes in delta and theta power in a rat injected with AAV-CaMKIIα-ChR2-mCherry **(K)** or AAV-CaMKIIα-mCherry **(L)** bilaterally. **(M,N)** The time course **(M)** and total amount **(N)** of wakefulness during optogenetic stimulation. $^{**}P < 0.01$ compared with the mCherry groups ($n = 6$).

the wakefulness level of animals quickly returned to the baseline level (**Figures 4K,M,N**), while such effects were not observed in the MPB mCherry-expressing control rats (**Figures 4L,M,N**). These data show that the activation of glutamatergic MPB neurons resulted in immediate transitions from stable sleep to a continuous wakefulness.

Activation of Glutamatergic MPB Neurons Controlled Wakefulness Through BF or LH Connections

Although mapping of the c-Fos/mCherry expression may indicate a possible neuronal pathway for the promotion of wakefulness by the MPB, it is still unclear whether the robust expression of c-Fos is a direct effect of MPB activation or a secondary effect caused by consolidated arousal. Due to the low temporal resolution of c-Fos mapping, it is impossible to determine a causal link between nuclei activation and wakefulness. To demonstrate the neuronal circuits mediating the arousal promoting effect of the MPB, we expressed ChR2 in MPB (**Supplementary Figure 2**) and stimulated the ChR2-expressing glutamatergic MPB axons in the BF (**Figures 5A–C**), LH (**Figures 5J–L**), or VM (**Figures 6A–C**).

An increased firing frequency and excitatory post-synaptic current (EPSC) were elicited by blue light pulses in BF (**Figure 5D**), LH (**Figure 5M**), and VM neurons (**Figure 6D**). The signals evoked by stimulation of MPB neuron terminals were blocked by CNQX (6-cyano-7-nitroquinoxaline-2,3-dione, a competitive AMPA/kainate receptor antagonist) and AP5 [(2R)-amino-5-phosphonovaleric acid, a selective NMDA receptor antagonist], demonstrating that MPB-evoked signals are glutamatergic (**Figures 5D,M**), which is consistent with the single cell PCR results (**Figure 4F**). After patch clamp recordings, single-cell RT-PCR analysis revealed that all BF neurons that received excitatory afferent signals from MPB neurons were positive for the vesicular GABA transporter (VGAT, 250 bp) (6/6), but negative for ChAT (7/7) or VGluT2 (9/9) (**Figure 5E**). By contrast, glutamatergic signals from the MPB excited VGAT (8/8) or VGluT2 (7/7) positive neurons in the LH (**Figure 5N**). These results indicate that glutamatergic MPB neurons projected onto BF GABAergic neurons and LH GABAergic or glutamatergic neurons.

Next, EEG/EMG recordings together with optogenetic stimulation MPB terminals in the BF, LH, or VM were performed *in vivo* to reveal the neuronal circuits of the MPB in controlling wakefulness. Sleep-to-wake transition were decreased after blue light pulses at 5 ms in the frequency range of 20–40 Hz were bilaterally applied in the BF (**Figures 5F,G**) and LH (**Figures 5O,P**), but not in the VM (**Figure 6E**). To determine the effects of chronic stimulation on the wake-promoting effect of MPB axons, a long-term photostimulation protocol (5 ms, 40 Hz, 25 trains, main interval 30 s) for 1 h with sleep-wake recording were performed from 9:00 to 10:00 (when the sleep pressure is high in rats). Compared to control rats, bilateral optogenetic stimulation of MPB axons in the BF (**Figures 5H,I**) or LH (**Figures 5Q,R**) increased waking time by 2.59- and 2.87-fold, respectively, whereas wakefulness was not affected by stimulation of MPB axons in the VM (**Figures 6F,G**). These results provide fundamental evidence that glutamatergic MPB neurons excited BF GABAergic neurons or LH GABAergic and glutamatergic neurons, but not the VM neurons to initiate wakefulness.

Chemogenetic Inhibition of PB Neurons in Rats Decreased Arousal

To explore the effect of inhibition of PB neurons in sleep-wake behavior, we employed an inhibitory chemogenetic tool, known as IVMR. IVMR are based on inhibitory human α1 glycine receptor with the mutations F207A and A288G to remove glycine sensitivity while producing IVM sensitivity (Lynagh and Lynch, 2010; Hu et al., 2014), and able to reduce neuronal excitability by mediating the influx of Cl^- (Hu et al., 2014; Obenhaus et al., 2016). AAV carrying IVMR (hSyn-IVMR-eGFP-AAV, IVMR) or eGFP (hSyn-eGFP-AAV, eGFP) was bilaterally injected into the PB. Immunohistochemistry against GFP revealed that IVMR fused to eGFP was successfully expressed in PB neurons, as indicated by superimposed eGFP expression areas in the PB of 6 AAV-injected rats (**Figure 7A**). During the dark period (when the rats are usually very active), i.p. administration of IVM (10 mg/kg) into rats expressing inhibitory IVMR in the PB neurons decreased the amount of wakefulness, as EEG delta amplitude was increased while the EMG amplitude decreased (**Figure 7B**). The vehicle treatment (isopropanol, IPA) did not significantly alter the sleep-wake profiles (**Figure 7C**). The inactivation of PB neurons bilaterally by IVM strongly decreased wakefulness for 10 h during the active period, as compared to the IPA control (**Figure 7D**). The total amount of wakefulness during 10 h decreased to 58% of the wake amount after IPA administration. By contrast, IVM did not change sleep-wake behavior in the eGFP control rats (**Figures 7D,E**). These data clearly indicated that PB neurons are essential for the maintenance of wakefulness under baseline conditions in rats.

DISCUSSION

In the current study, we employed chemogenetics to manipulate the activity of PB neurons, and found that activation of the MPB, but not the LPB by hM3Dq promotes wakefulness for 10 h, whereas inhibition of PB by IVMR resulted in a decrease in wakefulness for 10 h, indicating that MPB neurons are essential in controlling wakefulness in rats.

A pioneer clinical study used MRI scanning and postmortem histological analysis showed that brainstem stroke patients with coma have lesions or damage in the bilateral pontine tegmentum including PB, DR, and LC (Parvizi and Damasio, 2003). Later, chemical lesion of the entire PB and adjacent nuclei by orexin-saporin caused a behavioral unresponsiveness state with low-frequency cortical EEG in rats (Fuller et al., 2011), indicating the potential role of the pons in regulating wakeful consciousness. Moreover, chemogenetic activation of the PB induced long-lasting wakefulness during the light period when rats are normally mostly asleep (Qiu et al., 2016). Here we confirm and extend the findings that activation of the MPB is sufficient to induce robust continuous wakefulness for up to 10 h

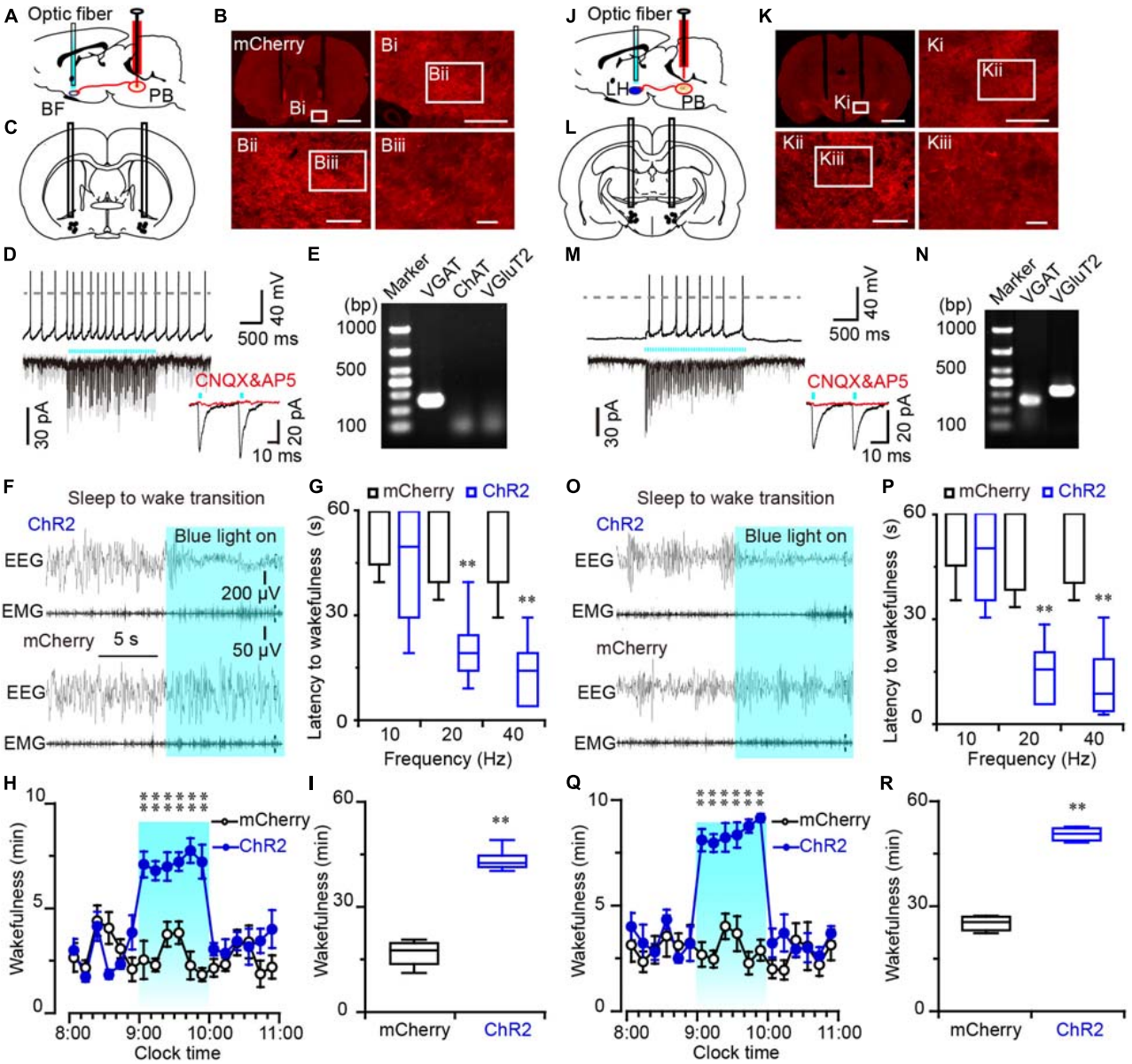

FIGURE 5 | Photostimulation of ChR2-expressing MPB axonal terminals in the BF or LH evoked waking response in rats. **(A,J)** Diagram depicting the location of the optic fiber in the BF **(A)** or LH **(J)** of a rat injected with AAV-CaMKIIα-ChR2-mCherry or AAV-CaMKIIα-mCherry in the MPB. **(B,K)** Coronal sections with native mCherry fluorescence confirmed the expression of ChR2 protein in the BF **(B)** or LH region **(K)**. Scale bars: B, K = 2 mm; Bi, Ki = 500 μm; Bii, Kii = 200 μm; Biii, Kiii = 50 μm. **(C,L)** The schematic diagram showing the cannula traces and the optic fibers targeting the BF **(C)** or LH **(L)**. **(D,M)** Photostimulation of ChR2-expressing MPB axons increased firing frequency and evoked synchronized EPSCs in a BF **(D)** or LH neuron **(M)**; the evoked EPSCs were blocked by CNQX and AP5 (red trains). **(E,N)** Single-cell RT-PCR analysis of ChR2-expressing neurons in BF **(E)** and LH **(N)** indicates the bands represent VGluT2 or VGAT, respectively. **(F,O)** EEG/EMG examples of sleep to wakefulness transition following optogenetic stimulation in the BF **(F)** or LH **(O)** of a rat with bilateral ChR2 (top) or mCherry (bottom) expression in the MPB, respectively. **(G,P)** Latency of sleep to wakefulness after optogenetic stimulation in the frequency range of 10–40 Hz in BF **(G)** or LH **(P)**. **(H,I,Q,R)** The time course **(H,Q)** and total amount **(I,R)** of wakefulness during optogenetic stimulation in BF and LH, respectively. $^{**}P < 0.01$ compared with mCherry groups ($n = 6$).

(**Figure 1E**). In addition, reversible chemogenetic inhibition of PB neurons by IVMR decreases wakefulness for 10 h during the dark period (**Figure 7D**), when rats are normally very active showing foraging, feeding, or exploring behaviors.

PB can be divided along the superior cerebellar peduncle into three main parts: MPB, LPB, and Kolliker-Fuse nucleus (KF) (Fulwiler and Saper, 1984; Singh et al., 2019). Several pioneer studies have revealed that LPB plays an important role in transmitting viscero- and somatosensory information to the forebrain, including pain, feeding and thermoregulation (Morrison and Nakamura, 2011; Le May et al., 2021), and the KF mainly involved in the respiration regulation (Chamberlin, 2004;

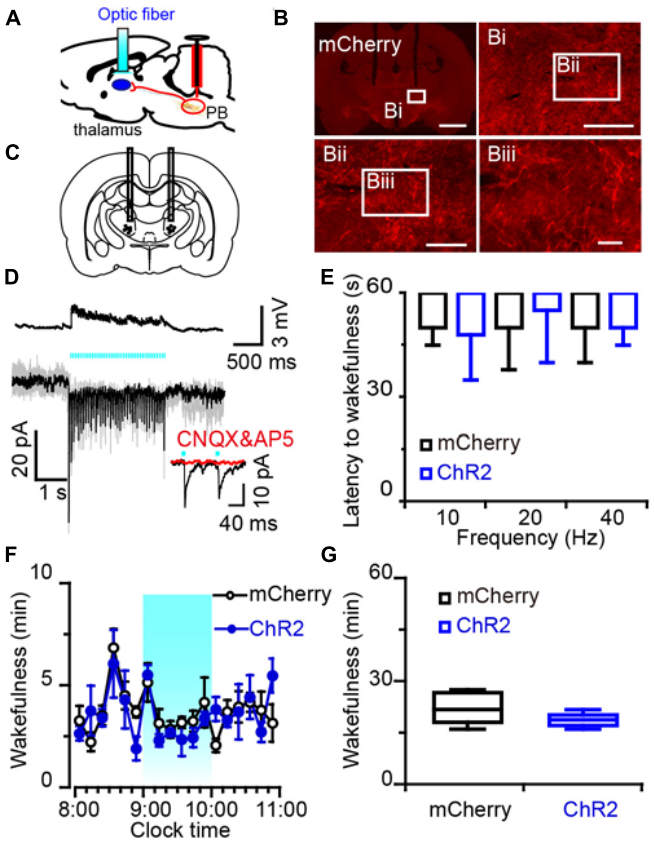

FIGURE 6 | Photostimulation of MPB axonal terminals in the VM had no effect on wake response in rats. **(A)** Diagram depicting the location of the optic fiber in the VM of a rat injected with AAV-CaMKIIα-ChR2-mCherry or AAV-CaMKIIα-mCherry in the MPB. **(B)** Coronal sections with native mCherry fluorescence confirmed the expression of ChR2 protein in the VM. Scale bars: b = 2 mm; bi = 500 μm; bii = 200 μm; biii = 50 μm. **(C)** The schematic diagram showing the cannula traces and the optic fibers targeting the VM. **(D)** Optogenetic stimulation of MPB axons increased firing frequency and evoked synchronized EPSCs in VM neurons. Evoked EPSCs were blocked by CNQX and AP5 (red trains). **(E)** Latency of sleep to wakefulness after photostimulation in the frequency range of 10–40 Hz. **(F,G)** The time course **(F)** and total amount **(G)** of wakefulness during optogenetic stimulation in the LH, compared with mCherry groups (n = 6).

Yang et al., 2020). To investigate the effect of MPB and LPB in sleep-wake regulation, hM3Dq were delivered into the MPB and LPB by stereotactic injection. We found that chemogenetic activation of MPB neurons induced continuous wakefulness, whereas activation of the LPB did not promote wakefulness in rats under basal conditions. To avoid non-specific expression of hM3Dq in the adjacent area of the targeted nucleus, the injection volume of the AAV was adjusted and confirmed by the immunostaining against the mCherry. The injection sites were centered in the MPB or LPB. In some rats, a small number of mCherry-containing neurons were observed in adjacent regions, such as the precoeruleus area (**Figures 1A,B**), raising the question of whether activation of the adjacent regions of MPB may induce wakefulness? However, from the previous lesion studies, lesions of the LC (Lu et al., 2006; Blanco-Centurion et al., 2007), PPT and LDT (Lu et al., 2006) have had no significant effect on wakefulness in rats. Only the lesion involved in the MPB region resulted in the dramatic decrease of wakefulness in rats (Fuller et al., 2011). Together with these pioneer studies, our results indicated the MPB is an essential nucleus in controlling wakefulness.

Kaur et al. (2013, 2017) reported that the specific lesion of glutamatergic LPB neurons did not alter the normal amounts of wakefulness or the EEG power spectrum. While blocking LPB signals decreased EEG arousal in response to hypercapnia, indicating that LPB neurons are important in hypercapnia-induced wakefulness, but not in controlling natural arousal (Kaur and Saper, 2019). Electrical activation of the glutamatergic LPB also induced reanimation (active emergence) during continuous isoflurane anesthesia, with a behavioral arousal and a significant decrease in EEG delta power in mice (Muindi et al., 2016). These results indicated that the LPB may regulate wakefulness secondary to the processing of viscero- and somatosensory information.

In contrast, specific deletions of glutamatergic MPB neurons decreased spontaneous wakefulness accompanied by an increase in both amount and EEG delta power of NREM sleep in mice (Kaur et al., 2013), and the population firing of MPB neurons was inhibited during sevoflurane-induced loss of consciousness (Xu et al., 2020), indicating the glutamatergic MPB neurons play an important role in controlling wakefulness. Moreover, chemogenetic inactivation of PB decreased wakefulness during

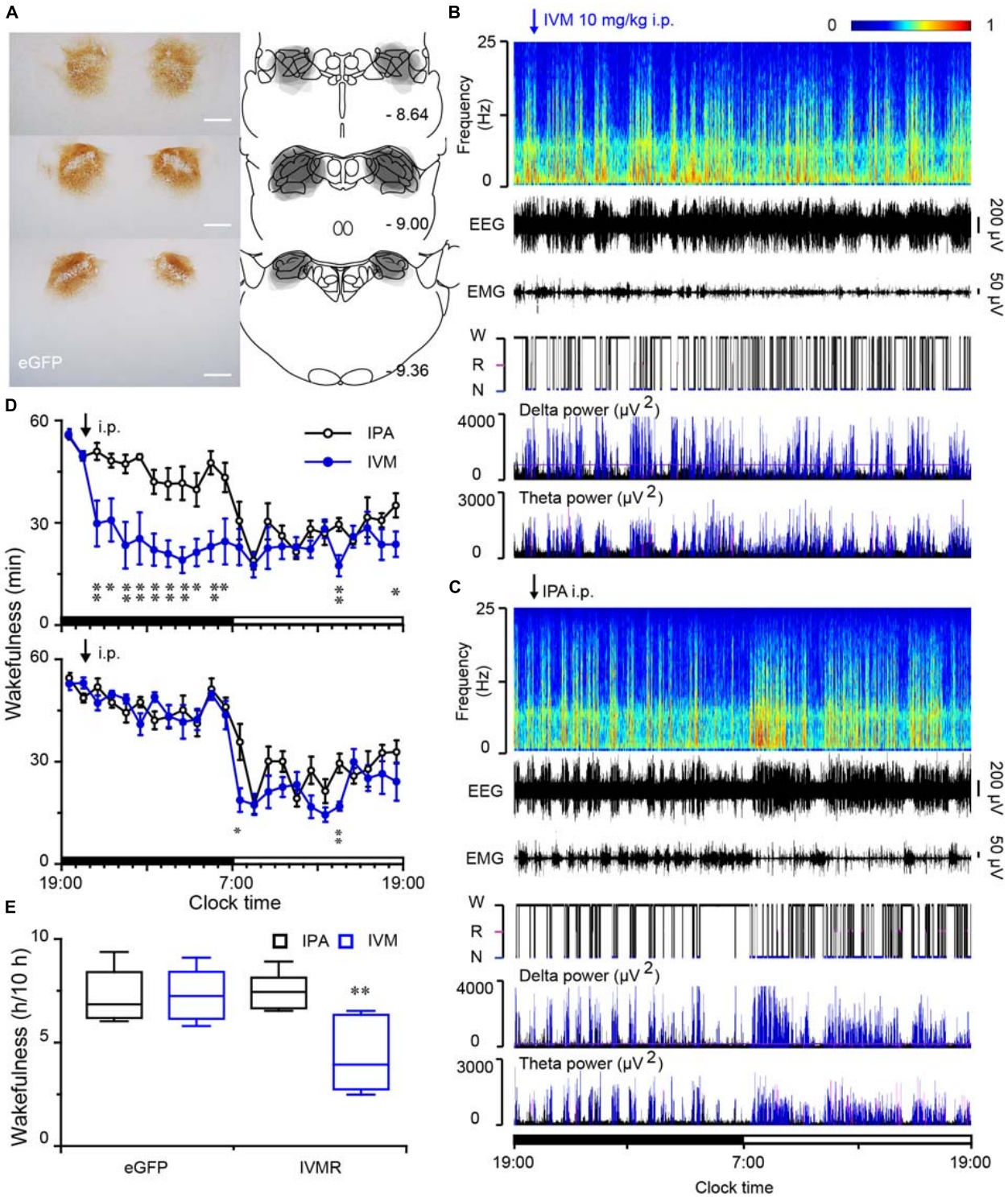

FIGURE 7 | Chemogenetic inhibition of PB neurons decreased wakefulness in rats during the active period. **(A)** Brain section stained against eGFP to confirm IVMR expression in the PB. Injection sites were mapped on coronal atlas drawings at three different levels containing the PB. Superimposed eGFP expression areas in the PB of 6 AAV-injected rats are shown in the right panels. Scale bars: 1 mm. **(B,C)** Typical examples of EEG power spectra, EEG, EMG, hypnogram, and relative magnitude changes in delta and theta power over 24 h for the same rat given IVM **(B)** or IPA **(C)**. W, wakefulness; R, REM sleep; N, NREM sleep. **(D)** Time courses of wakefulness in rats after the administration of IVM (10 mg/kg) or IPA in IVMR rats and eGFP controls, respectively. **(E)** Amount of wakefulness during 10 h after IVM or IPA injection in IVMR and eGFP rats. $^{*}P < 0.05$, $^{**}P < 0.01$, compared with the IPA treatment ($n = 6$).

the dark period (**Figure 7**), further indicating that the MPB is essential for controlling natural wakefulness. As previous reports have demonstrated that PB neurons are almost exclusively glutamatergic neurons (Liu and Jones, 1996; Yokota et al., 2007; Niu et al., 2010), some of which also express the calcitonin gene-related peptide, mu opioid receptors, or the corticotropin-releasing factor (Palmiter, 2018). The diversity of cell types in the MPB and LPB may lead to varying physiological functions. Although previous studies showed the projections of the MPB in rats are similar to those of the LPB (Saper and Loewy, 1980), how PB neurons innervate particular cell types in the targeted nuclei is still unclear. Further experiments are needed to identify the specific neural circuits of MPB or LPB for a specific behavior. Therefore, we consider that the MPB neurons are important for spontaneous wakefulness, while a subpopulation of LPB neurons may regulate arousal caused by visceral sensory distress, such as pain, extreme temperatures or respiratory insufficiency in rats.

Video analysis showed that the hM3Dq rats that received CNO injections are alert but are minimally moving around. The typical observed behavior, termed as attentive wake, is the movement of the head up and down and sideways without moving, accompanied by an increased theta EEG in the frequency range of 5–6 Hz (Valle et al., 1992; Lepski et al., 2012). During long-lasting wakefulness induced by MPB activation, rats may wish to gain a sense of visual depth, because PB is involved in visceral sensory regulation, such as taste, body temperature or anxiety (Baird et al., 2001; Bourgeais et al., 2001). By contrast, saline-treated hM3Dq rats or CNO-treated mCherry-expressing control rats spent more time in quiet wake (without head bobbing) with minimal grooming, exploring or feeding behavior during the first hour after the i.p. injection. In conclusion, our data provides fundamental evidence for the essential role of MPB in controlling wakefulness.

Previous studies proposed that possible neuronal PB circuits for promoting wakefulness are PB-BF/POA and the PB-LH (Anaclet et al., 2014; Qiu et al., 2016). Double staining against mCherry/c-Fos after activation of MPB neurons revealed that the expression of c-Fos increased remarkably in many regions including wake promoting nuclei such as the BF, thalamus, LH, VTA, and LC. The c-Fos expression pattern may indicated possible neuronal pathways mediating the wake promoting effect of MPB; however, c-Fos expression does not provide evidence for a causal relation between the observed neural activity and the ability to induce wakefulness.

Qiu et al. (2016) combined a retrograde strategy by injection of AAV6-Cre into the POA-BF, LH, or thalamic nucleus and the injection of AAV-DIO-hM3Dq into the PB area to activate specific neuronal circuits for sleep wake regulation. They found that activation of PB-BF or PB-LH pathway increased wakefulness for 4–5 h, which is much less than that induced by direct activation of PB somata. The reason for the short wakefulness duration may be due to the hM3Dq dense expressed in the LPB and spare in the MPB, which is in line with our finding that the LPB did not regulate the spontaneous wakefulness (**Figure 1F**). In addition, due to the injection of AAV6-Cre into huge areas of BF or PH, it is difficult to retrogradely label the exact target and the cell types in the MPB areas. Moreover, retrograde activation of the PB-LH/PH pathway may activate the PB-POA-BF pathway, or vice versa. Optogenetic methods used here to target the MPB terminals are much more accurate since they target the fibers issued from MPB neurons and are therefore more specific than retrograde-cre recombined floxed hM3Dq in which they target neurons projecting to the structures which could have collaterals to other structures.

To specific target the glutamatergic neurons in MPB, the excitatory ChR2 with a CaMKIIα promoter was employed and later confirmed by patch clamp recording and single cell PCR for the mCherry positive neurons (**Figures 4**, **5**). Here, manipulation of the glutamatergic MPB neurons using optogenetics with high timing precision enabled us to analyze causality between neural activity and initiation and maintenance of wakefulness (Zhang et al., 2007). We found that acute photostimulation of glutamatergic MPB neurons expressing ChR2 immediately initiated and maintained wakefulness for 1 h during stimulation. Then optogenetic activation of MPB terminals revealed the neuronal circuits for controlling wakefulness mediated by GABAergic neurons in the BF and GABAergic and glutamatergic neurons in the LH.

The BF has been reported to contain glutamatergic, GABAergic and cholinergic neurons which regulate sleep-wake behaviors (Anaclet et al., 2015; Xu et al., 2015; Chen et al., 2016). The GABAergic neurons in the BF showed fast firing during wakefulness and REM sleep, tested by *in vivo* juxtacellular recordings (Hassani et al., 2009b). Furthermore, a pioneer electron microscopy results revealed that the GABAergic neurons in BF preferentially target cortical interneurons (Freund and Meskenaite, 1992). Later, a study using transgenic mice demonstrated that BF GABAergic projection neurons share many similarities with cortical interneurons, such as the fast firing, brief spikes and electrical coupling (McKenna et al., 2013). In addition, the cholinergic and glutamatergic neurons in the BF are also more active during wakefulness and REM sleep than during NREM sleep. Furthermore, activation of cholinergic, glutamatergic or parvalbumin-positive GABAergic neurons rapidly induces wakefulness (Xu et al., 2015), while inhibition of BF cholinergic neurons increased EEG delta power spectrum and decreased wakefulness (Anaclet et al., 2015; Chen et al., 2016). Here, we found that the glutamatergic MPB neurons excited the GABAergic BF neurons to initiate and maintain wakefulness, agreed with previous literature.

We demonstrated that the glutamatergic MPB neurons excite the GABAergic and/or glutamatergic neurons in the LH to control wakefulness. The LH contains several types of neurons critically implicated in the wake-sleep regulation, including glutamatergic, GABAergic, orexinergic and melanin-concentrating hormone containing neurons (Lin, 2000; Hara et al., 2001; Gerashchenko and Shiromani, 2004; Hassani et al., 2009a). Alam and Mallick (2008) reported that activation of glutamatergic neurons in and around the LH promoted arousal and suppressed both NREM and REM sleep, indicating that glutamatergic neurons of the LH play an important role in maintaining wakefulness. Additionally, optogenetic activation of LH GABAergic neurons exerts a strong wake-promoting effect

in mice (Herrera et al., 2016), and similar effect was observed with chemogenetic activation of GABAergic LH neurons (Venner et al., 2016). In line with these findings, we postulate that glutamatergic MPB neurons innervate GABAergic and/or glutamatergic LH neurons to control wakefulness (**Figure 5**). However, whether there are other cell types in the LH mediated the wake promoting effect of glutamatergic MPB neurons still remained to be answered.

The thalamus is a large mass of gray matter and can be divided into many distinct portions, including the mediodorsal, paracentral, ventral lateral, submedius, VM, and so on. The thalamus participates in regulating many physiologic functions, such as relay of sensory and motor signals to the cerebral cortex, regulation of consciousness, and mediation of sleep and alertness (Kinomura et al., 1996; Maquet et al., 1996). PB neurons projected densely to midline and intralaminar thalamic nuclei of the rat (Herkenham, 1979; Krout and Loewy, 2000). We found that the increased c-Fos expression in the intralaminar, mediodorsal, lateral posterior and VM thalamic nuclei, after activation of glutamatergic MPB. However, a previous study showed lesion of the thalamic nuclei (Fuller et al., 2011), or activation of the PB-midline and intralaminar thalamus pathway did not alter sleep-wake behaviors significantly (Qiu et al., 2016). While the activities of matrix cells in the VM is high during wakefulness and low in NREM sleep, optogenetic activation of VM cells induced rapidly transitions from NREM sleep to arousal and chemogenetic inhibition of VM matrix cells decreased wakefulness (Honjoh et al., 2018), suggesting the VM plays a role in promoting arousal. Here, we clarified the functional connections between glutamatergic MPB and the VM by patch clamp recording, and demonstrated that this neural circuit is unexpected to be responsible for the wake-promoting effects of the MPB (**Figure 6**), although there are studies showing the VM participates in the catalepsy (Ossowska et al., 1986; Wullner et al., 1987).

In conclusion, we demonstrated the glutamatergic MPB neurons are essential in controlling wakefulness, and the wake-promoting effect of MPB mediated by BF GABAergic neurons and LH GABAergic or glutamatergic neurons in rats.

DATA AVAILABILITY STATEMENT

The original contributions generated for this study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding authors.

ETHICS STATEMENT

The animal study was reviewed and approved by Animal Care and Use Committee of Fudan University.

AUTHOR CONTRIBUTIONS

Z-LH, W-MQ, JL, and QX conceived and designed the study. QX, D-RW, HD, and LC carried out the experiments. QX, D-RW, Z-LH, and W-MQ analyzed the data. YC and ML provided the AAVs. QX, G-HC, W-MQ, and Z-LH wrote the manuscript. All authors contributed to the study and approved the final version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fnins.2021.645877/full#supplementary-material

Supplementary Figure 1 | Mapping of c-Fos expression in the rat brain after chemogenetic activation of MPB neurons. **(A–I)** Representative photomicrographs of c-Fos (black color) and mCherry (brown color) immunostaining in the rat brain. **(J)** The number of c-Fos-immunoreactive neurons in the rat brain after CNO or saline treatment. $^{**}P < 0.01$ compared to Saline, assessed by student's *t*-test ($n = 4$). MPB, medial parabrachial nucleus; CG, cingulate cortex; M1, primary motor cortex; S1, primary somatosensory cortex; LS, lateral septal nucleus; BNST, bed nucleus of stria terminalis; CeA, central amygdaloid nucleus; BF, basal forebrain; MDM, mediodorsal thalamic nucleus; LPMR, lateral posterior thalamic nucleus; PV, paraventricular thalamic nucleus; VM, ventromedial thalamic nucleus; LH, lateral hypothalamus; PSTN, parasubthalamic nucleus; TMN, tuberomammillary nucleus; MGV, medial geniculate nucleus; PAG, periaqueductal gray; VTA, ventral tegmental area; LC, locus coeruleus. Scale bars: CNO left panel, Saline left panel = 500 μm; CNO right panel, Saline right panel = 100 μm.

Supplementary Figure 2 | Bilateral AAV injection sites in the rats MPB region. **(A)** Typical coronal brain sections of native mCherry fluorescence confirmed that the ChR2 protein was expressed in the MPB area at three brainstem levels. **(B–D)** Superimposed mCherry expression areas in the MPB of 6 AAV-injected rats are shown in each panel for the optogenetic stimulation of MPB axons in the BF **(B)**, LH **(C)**, and VM **(D)**. Scale bars: 1 mm.

Supplementary Table 1 | The primers for single-cell RT-PCR.

Supplementary Movie 1 | A MPB-hM3Dq rat whose behaviors were captured after the CNO injection. EEG and EMG recordings, and a video show that the rat spent most of its time in "attentive wake" characterized by head bobbing without moving around during the activation of MPB neurons by hM3Dq.

Supplementary Movie 2 | A MPB-hM3Dq rat whose behaviors were captured after the saline injection. EEG and EMG recordings, and a video show that the rat spent more time in quiet wake, and sleep, and little time in grooming, exploring, eating and drinking during the first hour, followed by more sleep during the subsequent hours after saline treatment.

Supplementary Movie 3 | A rat whose wakefulness was induced by blue light illumination of glutamatergic MPB neurons expressing ChR2. EEG and EMG recordings, and a video show that the rat was sleeping for at least 1 min before 1-min light illumination, which quickly produced wakefulness. The duration of the behavioral states of wakefulness and sleep are indicated above the EEG trace.

Supplementary Movie 4 | A rat whose wakefulness was not induced by blue light illumination of glutamatergic MPB neurons expressing only mCherry. EEG and EMG recordings, and a video show that the rat was sleeping during the 4.5-min recording period, including the 1-min illumination period.

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BRIEF RESEARCH REPORT
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Cholinergic-Induced Specific Oscillations in the Medial Prefrontal Cortex to Reverse Propofol Anesthesia

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General anesthesia is a drug-induced reversible state comprised of altered states of consciousness, amnesia, analgesia, and immobility. The medial frontal cortex (mPFC) has been discovered to modulate the level of consciousness through cholinergic and glutamatergic pathways. The optogenetic tools combined with *in vivo* electrophysiological recording were used to study the neural oscillatory modulation mechanisms in mPFC underlying the loss of consciousness (LOC) and emergence. We found that optogenetic activation of both cholinergic and glutamatergic neurons in the basal forebrain (BF) reversed the hypnotic effect of propofol and accelerated the emergence from propofol-induced unconsciousness. The cholinergic light-activation during propofol anesthesia increased the power in the β (12–20 Hz) and low γ (20–30 Hz) bands. Conversely, glutamatergic activation increased the power at less specific broad (1–150 Hz) bands. The cholinergic-induced alteration to specific power bands after LOC had opposite effects to that of propofol. These results suggested that the cholinergic system might act on more specific cortical neural circuits related to propofol anesthesia.

Keywords: optogenetic, basal forebrain, cholinergic, glutamatergic, general anesthesia, emergence

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INTRODUCTION

General anesthesia is a reversible, anesthetic drug-induced state in which patients undergo an amalgamation of altered states of consciousness, analgesia, amnesia, and immobility (Brown et al., 2011). One of the biggest mysteries of modern medicine is how anesthetic drugs induce unconsciousness and how patients subsequently recover from general anesthesia (Kennedy and Norman, 2005). Neural oscillatory dynamics that are readily visible in the physiological measurements (electroencephalogram, EEG, and local field potential, LFP) are used to empirically characterize the anesthesia state (Ching and Brown, 2014). Different anesthetics induced altered specific oscillation band changes during the loss of consciousness (LOC) because of the discrepant molecular targets in the brain (Blain-Moraes et al., 2014, 2015). Propofol, one of the $GABA_A$ receptor agonist anesthetics, exhibited specific activation of the delta band and theta-gamma coupling during the LOC and emergence, and is commonly used in clinical trials (Breshears et al., 2010). However, due to the lack of current literature on the formation of propofol-induced

oscillation change in microcircuits, a detailed understanding of the neural mechanism is warranted.

Recent studies suggest that both LOC and emergence from general anesthesia is brought about by the modulation of ascending arousal systems, such as glutamatergic and cholinergic systems, in the central nervous system (Ren et al., 2018; Wang et al., 2019). It is widely accepted that acetylcholine (Ach) in the cortex is predominately derived from cells located in the basal forebrain (BF) (Zant et al., 2016). Moreover, BF cholinergic neurons have been shown to play an imperative role in the sleep-wake cycle transition (Han et al., 2014). Selective modulation of the BF cholinergic neurons has been found to change the sedative potency of general anesthetics and the duration of loss of the righting reflex (LORR) during anesthesia (Laalou et al., 2008; Leung et al., 2011; Luo et al., 2020). Intriguingly, systematic administration of physostigmine promoted arousal in human patients during propofol anesthesia (Xie et al., 2011). BF cholinergic neurons strongly innervate the medial prefrontal cortex (mPFC) to partly exert the wake-promoting effect (Bloem et al., 2014; Ahrlund-Richter et al., 2019). The BF cholinergic activation in the prefrontal cortex has been also demonstrated to contribute with paramount value in the depth of consciousness (Pal et al., 2018). However, the characteristics and functions of the pathways projecting from the BF to mPFC on the altered states of consciousness induced by propofol remain to be elaborated.

The BF innervating the frontal cortex contains glutamatergic neurons (∼55%), as well as cholinergic neurons (∼10%) (Gritti et al., 2006). Previous studies have shown that glutamatergic neurons in the BF regulate the sleep-wake cycle (Xu et al., 2015; Peng et al., 2020). However, there is no evidence showing the role of glutamatergic neurons in the BF on the altered states of consciousness induced by general anesthesia.

To investigate these questions, we used optogenetic activation of two different neurotransmitters, cholinergic and glutamatergic in the BF, to underlie the mPFC oscillatory mechanisms of altered states of consciousness induced by propofol general anesthesia.

MATERIALS AND METHODS

Animals

Adult wild-type (6–8 weeks old) C57BL/6 mice and ChAT-ChR2-EYFP transgenic mice (ChAT-ChR2-EYFP mice as a generous gift from Prof. Duan Shuming, Institute of Neuroscience, School of Medicine, Zhejiang University) were used. During the experiment procedures, all animals were given water and regular mice chow *ad libitum* and housed individually under climate-controlled conditions with a 12-h light/dark cycle, with lights on at 7:00 AM. The temperature in the room was maintained at 21–23°C. All the procedures were conducted according to guidelines approved by the Animal Care Committee of the Zhejiang University (Hang Zhou, Zhejiang, China).

Virus Injection

Wild-type C57BL/6 mice were anesthetized with sodium pentobarbital (1% wt/vol) and AAV-CaMKIIα-hChR2 (H134R)-mCherry virus (Shumi Technology, Wuhan, China) was bilaterally injected into the BF (AP = −0.6; ML = 0.8; DV = −4.8). We injected 0.1–0.3 μl of the virus into each location at 0.01–0.03 μl/min. The syringe was not removed until 15–20 min after the end of infusion to allow the diffusion of the virus. After injection, mice were allowed 2–3 weeks for recovery and virus expression.

LFP Recording

Mice were deeply anesthetized with sodium pentobarbital (induction 1% wt/vol) and fixed in a stereotaxic device (RWD, China). After exposing, cleaning, and disinfecting the skull bone, four electrodes made by nickel chromium(California Fine Wire,United States)were implanted. Targeting the mPFC (AP = 1.5; ML = 1; DV = −1.5, Atlas of Paxinos and Watson), two screw electrodes were fixed into the frontal (AP = 2; ML = 1) and parietal (AP = −3; ML = 1.5) cranium for grounding. Mice were allowed to recover for at least 7 days. Continuous LFP was recorded at 1 KHz using Central Acquisition system (Cerebus system, Blackrock Technology, United States). The LFP signals were amplified and filtered (0.5–500 Hz) for further analysis. The LFP recording was sustained for 15 min before and after propofol delivery.

Light Stimulation

Optical fibers were implanted into the BF (AP = −0.6; ML = 0.8; DV = −4.8) according to the Atlas of Paxinos and Watson of previous work in the ChAT-ChR2-EYFP mice (Han et al., 2014). For light stimulation, the optical fibers were bilaterally implanted in BF. Laser light was generated using a fiber-coupled 473 nm solid-state laser diode (473 nm, Lasercentury, Shanghai, China) and was delivered *via* the ceramic ferrule. Laser light stimulation was driven by software-generated TTL pulses (10 ms@20 Hz for 5/30 s for 30 min post propofol) (Anilab, Ningbo, China) (Han et al., 2014; Peng et al., 2020). Wild-type mice with optical fibers implanted served as control mice. For electrophysiology data analysis, the TTL pulse was also recorded by the Cerebus recording system simultaneously.

Anesthetic Performance During Propofol

LORR was used as the behavioral time-point to investigate the hypnotic properties of propofol (AstraZeneca, United Kingdom), following previously described methods with slight modifications (Leung et al., 2013). To determine the propofol dosage required to induce LORR, an initial bolus of 50 mg/kg was given to the mouse intraperitoneally. 15 min absorption time was set after injection. LORR was considered if there were two failed attempts to right itself (four paws grounded) within 30 s after being placed supinely. Subsequently, recurring 25 mg/kg was administered and retested until LORR was achieved. The percentage of mice showing LORR at each dose of propofol was established in the control and optical groups, and the ED_{50} (50% effective dose) and ED95 (95% effective dose) values of propofol were estimated from the dose-response equation described in **Statistical Analysis**. The time to LORR and time for recovery of righting were investigated with 200 mg/kg propofol (ED_{95}), in order to assess propofol induction and emergence time.

Histology

To verify the validity of the fibers' placement and virus expression, the mice were perfused with saline and a 4% paraformaldehyde in 0.1 M PBS. After perfusing, the brains were removed, post-fixed overnight in 4% paraformaldehyde, and then immersed in a 30% sucrose solution at 4°C for cryoprotection. 30 μm-thick coronal slices were collected and stored in PBS at 4°C. Finally, the sections were rinsed in 90% glycerol, cover slipped, and imaged by the fluorescence microscope (Olympus VS 120, Japan).

Statistical Analysis

All values are shown as mean ± SEM. Statistical analyses were performed using Graphpad Prism (version 5.01, Graphpad Prism, Inc., San Diego, CA, United States). LORR dose-response data were curve-fitted by non-linear regression with Prism to give the half-maximal effective concentration–dose values (ED_{50} ± SEM) with the equation $Y = Y_{min} + (Y_{max} - Y_{min})/[1 + 10^{log(ED50 - X) \times m}]$, where Y is the percentage of the population showing LORR, Y_{min} and Y_{max} are the minimal and maximal values of Y, respectively, ED_{50} is the drug dose for a half ($Y_{max} - Y_{min}$), X is the logarithmic drug dose, and m is the Hill slope constant. The F-test for non-linear regressions was then used to determine whether the calculated ED_{50} was significantly different between groups. At the time of the onset of LORR, the recovery of righting reflex (RORR) was compared using an unpaired Student *t*-test.

For LFP data analyses, data were obtained from the mPFC at 200 mg/kg propofol. The recorded signals were pre-filtered with Matlab 2010 (Mathworks, United States) to exclude artifacts. All the 15 min data were used to plot spectrogram using FFT multi-taper function of the MATLAB chronux toolbox[1]. For spectrogram analysis, the spectrogram data at the initial 100 s when the mice were awake were used as the baseline reference to calculate the mean and standard deviation for normalizing [Normalized Z = (the point value -mean)/Standard deviation]. After spectrogram normalization, the laser-stimuli-triggered spectrogram changes were averaged from all the stimuli in 15 min recording period ($n = 36$). For comparisons of spectrogram power 5 s before and after light stimulation, we used one-way repeated-measures ANOVA, followed by Bonferroni *post hoc* tests. *P* value less than 0.05 (two-tailed) is considered to be statistically significant.

RESULTS

The Activation of $BF^{Ach} \rightarrow$ mPFC on Propofol Anesthesia

To investigate the cholinergic function during the propofol-induced anesthesia, we firstly performed an optogenetic activation of the BF cholinergic neurons during propofol-induced anesthesia in mice with graded propofol (**Figure 1A**). The behavior response of the proportion of mice LORR,

[1]http://chronux.org/

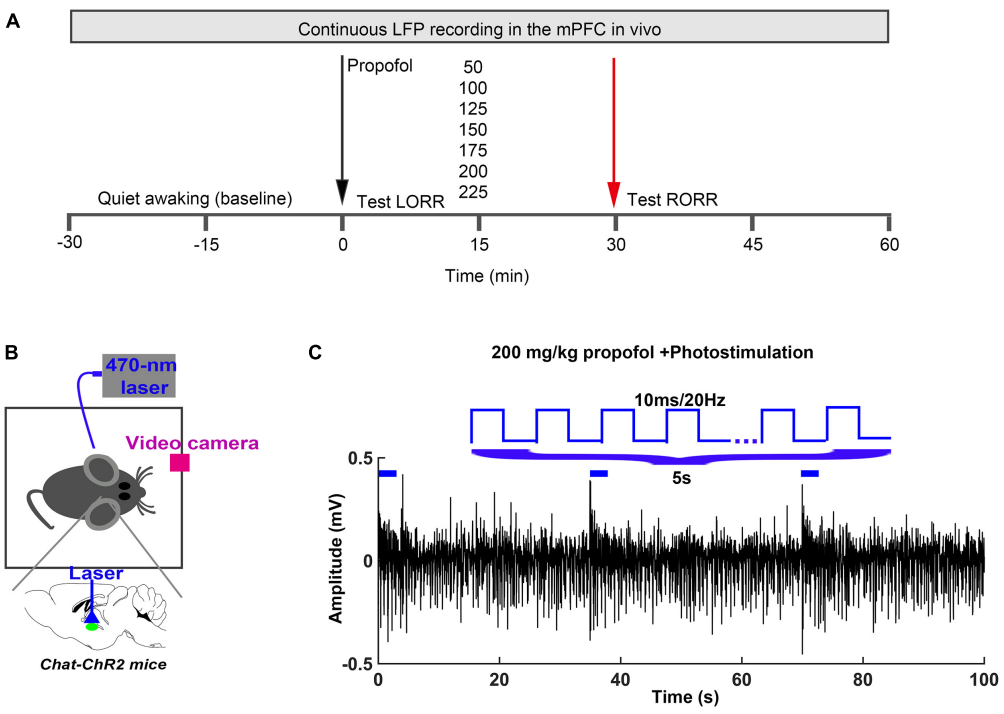

FIGURE 1 | Experimental procedures **(A)** *In vivo* LFP recording and behavioral test timeline. **(B)** Schematic of optogenetic stimulation of ChR2-expressing BF cholinergic neurons. **(C)** The parameter of light stimulation during the *in vivo* recording.

induction time, and emergence time (LORR to RORR), were recorded by synchronous video recording (**Figure 1B**). After intraperitoneal injection (i.p.) of propofol, a 473 nm blue light (10 ms@20 Hz, for 5/30 s) was continuously delivered to the BF of a ChAT-ChR2-EGFP(ChAT) transgenic mouse for 15 min (**Figure 1C**). The typical placement of the electrodes, fibers, and the expression of ChAT were shown in the **Figures 2A,B**. The sensitivity of ChAT mice to propofol was significantly reduced by the stimulation of light, characterized by a rightward shift to the dose-response curve (**Figure 2C**). The effective dose instigating 50% (i.e., ED_{50}) of the LORR in the wild-type mice was 157.7 mg/kg (95% CI, 138.3–179.8 mg/kg, and $n = 12$), and was significantly different ($P = 0.0008$) to ChAT mice with 197.4 mg/kg (95% CI, 178.4–206.9 mg/kg, and $n = 12$) (**Figure 2C**). Based on the ED_{95} value, we chose the 200 mg/kg propofol dose to test LORR and the emergence time. The LORR was significantly prolonged following the light activation of BF cholinergic neurons in ChAT mice, compared with wild-type mice ($P < 0.05$, $n = 6$ for WT, and ChAT, respectively) (**Figure 2D**). The emergence time was significantly reduced by light stimulation ($P < 0.05$, $n = 6$ for WT, and ChAT, respectively) (**Figure 2E**). These results showed that selective activation of cholinergic neurons in the BF not only delays the time to unconsciousness but also promotes emergence from propofol anesthesia.

Brain network oscillations are ubiquitous in mammals. They are assumed to be an imperative signature for various cognitive abilities, such as learning (Brickwedde et al., 2019), memory (Liu et al., 2013), attention (ElShafei et al., 2019), and consciousness (Mukamel et al., 2014). Understanding the mechanisms and functions of these oscillations is necessary to understand how the brain carries out complex functions. During general anesthesia, the most practical technique for tracking the various states of the brain is the EEG/LFP, which measures scalp/local electrical potentials generated by cortical oscillations. The effects of propofol anesthesia on macroscopic dynamics are noticeable in EEG readings, which display several stereotypical oscillation patterns, including increased delta (0.5–4 Hz) power, decreased gamma (25–40 Hz) power, and an alpha (∼10 Hz) rhythm that is coherent across the frontal cortex (Lewis et al., 2012). Here, we placed the electrodes into the mPFC to acquire the LFP oscillations during the awake-immobility state and 200 mg/kg propofol administration (**Figures 2F,G**). During the immobility state, low-voltage, high-frequency activity was recorded in the mPFC, with high power over 30 Hz (**Figure 2F**). After exposure to the propofol at a dosage of 200 mg/kg i.p., an apparent increase in slow activity in the mPFC was observed, with comparatively lower power over 30 Hz (**Figure 2G**). After an average of 300 s LORR time, there was an increase in the delta (0–4 Hz) and theta (5–12 Hz) range and a decrease in power at higher frequencies (12–20, 20–30, 30–80, and 80–150 Hz) ($n = 7$, **Figure 2H**, left).

To understand the cortical neural oscillation dynamic changes during the BF cholinergic stimuli, we also recorded the LFP of the mPFC during light stimulation in the same region. The power changes occurred in frequencies between 12 and 30 Hz in one typical mouse during the light stimuli within propofol administration (**Figure 2I**). The averaged normalized spectrograms during 5 s light stimulation under propofol-induced anesthesia showed a 20–30 Hz power increase (**Figure 2J**). The light stimulation effectively decreased power in the delta (0–4 Hz) band and increased the power in the beta (12–20 Hz) and low gamma (20–30 Hz) bands ($P < 0.001$, $P < 0.001$, and $P < 0.001$, respectively) (**Figure 2K**). These alterations to specific bands were inversely identical to propofol-induced power changes after LOC in **Figure 2H**. The neural mechanism of propofol-induced LOC is associated to the BF-mPFC cholinergic ascending projection system.

The Activation of $BF^{Glu} \rightarrow$ mPFC on Propofol Anesthesia

To investigate the glutamate function in BF, we bilaterally injected the virus AAV-CamKIIα-ChR2-mCherry into the BF of the C57BL/6 mice (CamKIIα) 2–3 weeks before behavioral testing (**Figure 3A**). The optical fibers were bilaterally implanted over the injection sites (**Figure 3B**). A blue light was delivered with the same parameters as previously used at a physiologically relevant frequency of 20 Hz (**Supplementary Figure 1**) (Xu et al., 2015; Peng et al., 2020). Compared with the control group, light stimulation of CamKIIα mice reduced their sensitivity to propofol anesthesia, characterized by a significant right shift of the dose-response curve (**Figure 3C**). ED_{50} in the control group was 148.3 mg/kg (95% CI, 138.9–158.3 mg/kg, and $n = 10$). In contrast, in the CamKIIα group, the ED_{50} (182.8 mg/kg, 95% CI, 147.2–227.2 mg/kg, and $n = 10$) was significantly increased (**Figure 3C**). The ED_{95} of 200 mg/kg propofol i.p. was selected to test LORR and the emergence time. The induction time showed a significant increase between the CamKIIα and wild-type groups ($P < 0.05$, $n = 6$ for WT and CamKIIα groups, respectively) (**Figure 3D**). The emergence time of the CamKIIα group in response showed a decrease, but this was not significant compared with the control group ($P > 0.05$, $n = 6$ for WT and CamKIIα, respectively) (**Figure 3E**). Taken together, these results indicate that modulation of glutamatergic neurons in the BF can also reverse propofol-induced unconsciousness.

We also recorded the LFP in the mPFC during light stimulation of BF glutamatergic neurons. The oscillation characteristics in the awake immobility were found to be identical to that in the ChAT mice (**Figure 3F**). Obvious power changes could be seen during all the light stimulation across 15 min recording under propofol anesthesia (**Figure 3G**, left). Selective light activation of glutamatergic neurons in the BF induced a shift from a low-frequency, high-amplitude slow oscillatory pattern to an active high-frequency, low-amplitude pattern (**Figure 3G**, right). The normalized averaged power showed a transient increase in the 20–80 Hz bands during the light stimulation (**Figure 3H**). A further *post hoc* Bonferroni test indicated that the 5 s light stimulation induced a significant increase in all bands during propofol anesthesia compared with that before the light stimulation (**Figure 3I**). Compared with the previous cholinergic activation-induced oscillation bands change, the glutamate activation showed more broad band changes during the propofol-induced general anesthesia, which suggested that the underlying neural mechanism may be different.

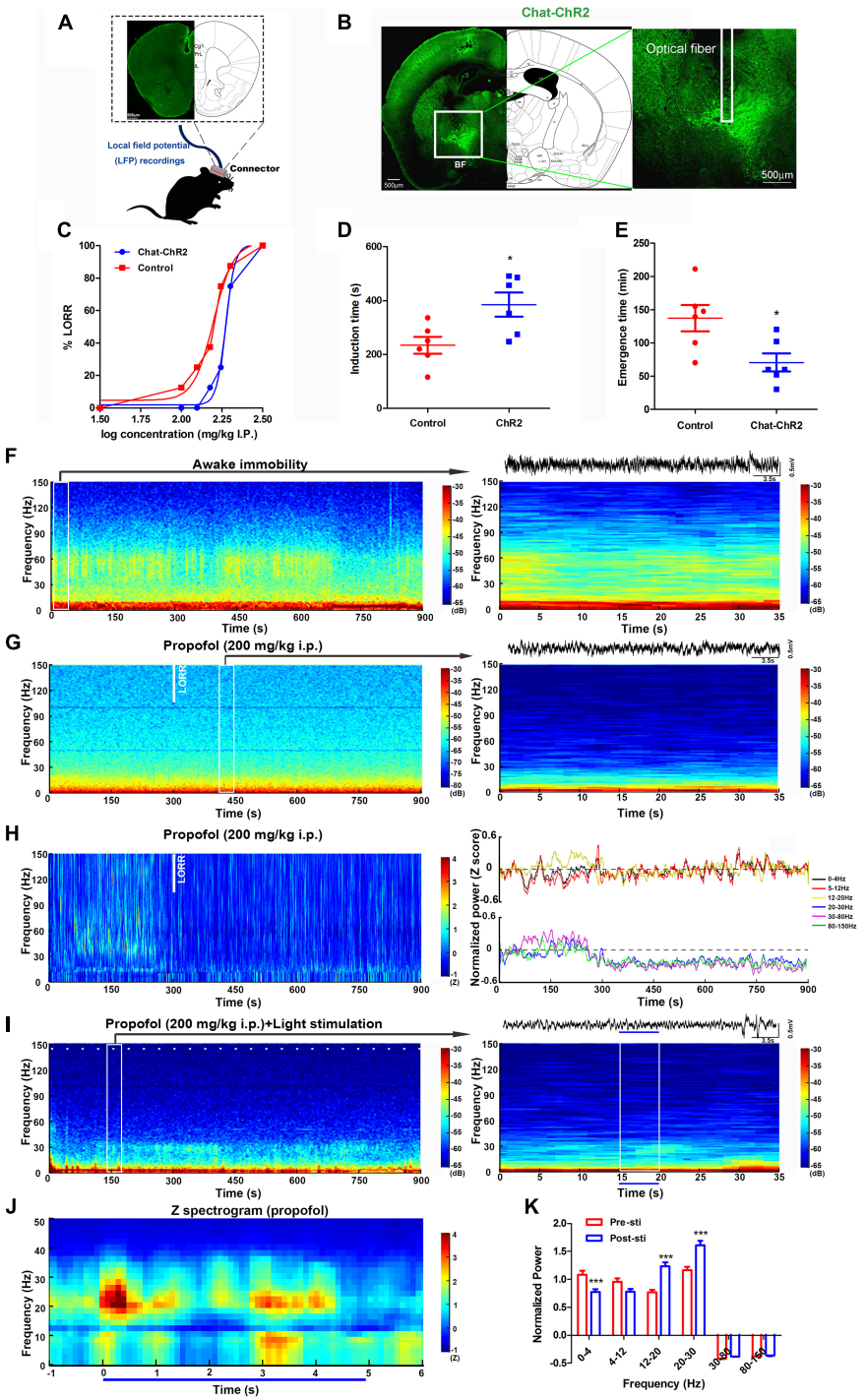

FIGURE 2 | Optogenetic activation of cholinergic neurons in the BF reversed propofol anesthesia with mPFC LFP change. **(A)** The location of electrodes embedded in the mPFC. **(B)** Typical placement of optical fibers over the injection site and ChR2-expressing cholinergic neurons. **(C)** The dose-response curve plotted from the proportion of mice losing the righting reflex under graded propofol ($n = 12$ in both groups). Induction **(D)** and emergence **(E)** time after stimulation of BF cholinergic neurons when exposed to propofol at the dosage of 200 mg/kg ($n = 6$ both). $^*P < 0.05$, compared with the control group. **(F)** Typical mouse spectrogram during the awaking state (left), and an enlarged, 35 s spectrogram with raw LFP data (right). **(G)** 15-min spectrogram (left) and a 35 s enlarged time window with raw LFP data (right) from a typical mouse during propofol anesthesia. **(H)** Normalized spectrogram averaged from six mice (left) and power differential at 0–4, 5–12, 12–20, 20–30, 30–80, and 80–150 Hz (right). **(I)** 15-min spectrogram (left) and an enlarged 35-s time window with raw LFP data from a typical mouse during propofol anesthesia with light stimulation (right). White dashes (left) and blue line (right) represent light stimulation. **(J)** Normalized spectrogram computed from nine mice under light stimulation during propofol at 200 mg/kg, trial = 52). **(K)** *Post hoc* analysis of the power at 0–4, 5–12, 12–20, 20–30, 30–80, and 80–150 Hz for 5 s before and after light stimulation during propofol at 200 mg/kg. "0" means the onset of propofol administration in the Figures **(G,H,I)**. $^{***}P < 0.001$, compared with the pre-sti group at every frequency range.

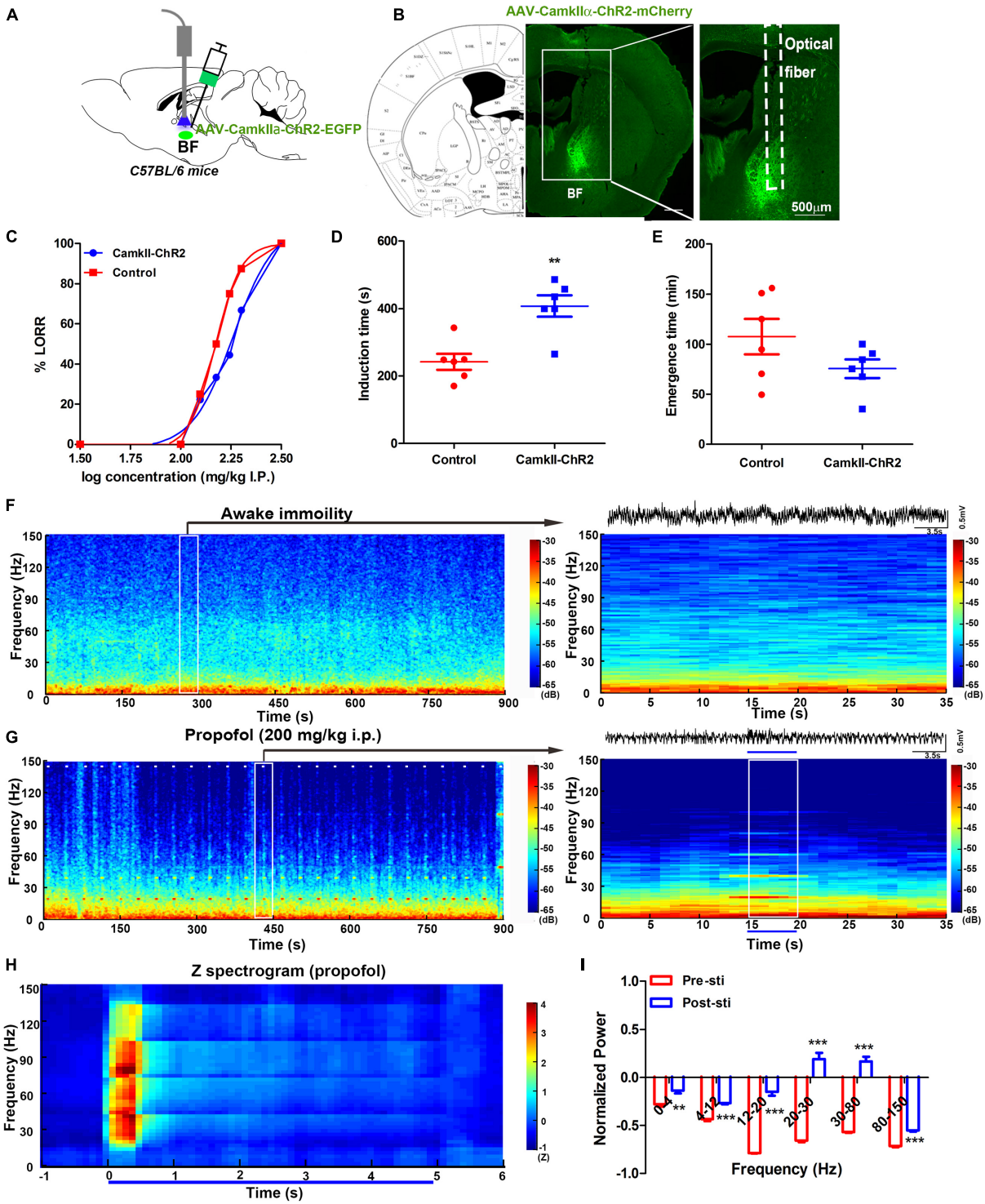

FIGURE 3 | Selective activation of glutamatergic neurons in the BF reversed propofol anesthesia with mPFC LFP change. **(A)** Schematic of optogenetic activation of BF glutamatergic neurons. **(B)** Selective expression of the AAV-CamKII-ChR2 virus in the BF glutamatergic neurons. **(C)** Dose-response curve from graded propofol after light stimulation (10 mice in both groups). Induction **(D)** and emergence **(E)** time after stimulation of BF glutamatergic neurons when exposed to propofol at a dose of 200 mg/kg (six mice in both). $^{**}P < 0.01$, compared with the control group. **(F)** Typical mouse spectrogram during the quiet awaking state for 15 min (left) and an enlarged 35 s time window with raw LFP data(right). **(G)** Typical mouse spectrogram during propofol anesthesia, accompanied by light stimulation for 15 min (left) and 35 s time window with raw LFP data (right), white dashes (left) and the blue line (right) represent light stimulation. **(H)** Normalized spectrogram computed from six mice under light stimulation during propofol at 200 mg/kg trial = 51). **(I)** *Post hoc* analyses at 0–4, 5–12, 12–20, 20–30, 30–80, and 80–150 Hz for 5 s before and after light stimulation during propofol at 200 mg/kg. "0" means the onset of propofol administration in the Figures **(F,G)**. $^{**}P < 0.001$ and $^{***}P < 0.001$, compared with the pre-sti group at every frequency range.

DISCUSSION

In summary, we demonstrated that the selective activation of both cholinergic and glutamatergic neurons in the BF could reverse the hypnotic effect of propofol. Additionally, they promote emergence with activation of alternative mPFC oscillation bands. Propofol induced specific power increases at 12–20 Hz during the wake-LOC state transition. This pattern appears similar to that observed in the human scalp EEG, characterized by broad-band β oscillations that coalesce into α oscillations after losing consciousness (Vertes, 2002; Flores et al., 2017). Selective activation of BF cholinergic neurons significantly decreased the delta power, which is an index to the unconsciousness state (Flores et al., 2017), but increased the power at 12–20 and 20–30 Hz, which was thought to antagonize the anesthesia-promoting effect of propofol in the mPFC. However, promoting the glutamate system could induce a systematic non-targeted change throughout all frequencies, suggesting the complex brain networks involving in the activation of the pathway projection may not have specificity as a cholinergic system. Moreover, previous work demonstrated that distinct consciousness patterns could be induced by different neuropharmacological agents (Kenny et al., 2016). The level of consciousness could be dissociated from cholinergic, behavioral levels, and neurophysiologic oscillations (Pal et al., 2020). These findings might explain the difference in cortical activation between cholinergic and glutamatergic neurons induced in our study. Indeed, the glutamatergic and cholinergic neurons in the BF project across the cortex, including the mPFC, but there is no comparison with any other cortical node (e.g., posterior parietal cortex) to understand if there is anything unique about the oscillations, which is a limitation of our study. Nevertheless, our findings suggest that cholinergic and glutamatergic arousal projections from the BF are sufficient to induce emergence in the mPFC from general anesthesia. Activating the cholinergic systems may modulate specific conscious related circuits, can provide a novel approach to accelerating recovery from general anesthesia, and treat or eliminate consciousness-related disorders such as hypoxia, postoperative delirium, and cognitive dysfunction.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The animal study was reviewed and approved by Animal Care Committee of the Zhejiang University.

AUTHOR CONTRIBUTIONS

LeW, MY, and WX conceived the project and wrote the manuscript with input from all co-authors. WZ, YW, YG, and NS provided computational support. HD, JR, LY, LaW, and FY supported mouse work. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fnins.2021.664410/full#supplementary-material

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Altered Functional Connectivity in the Resting State Neostriatum After Complete Sleep Deprivation: Impairment of Motor Control and Regulatory Network

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Wang H, Yu K, Yang T, Zeng L, Li J, Dai C, Peng Z, Shao Y, Fu W and Qi J (2021) Altered Functional Connectivity in the Resting State Neostriatum After Complete Sleep Deprivation: Impairment of Motor Control and Regulatory Network. Front. Neurosci. 15:665687. doi: 10.3389/fnins.2021.665687

Sleep loss not only compromises individual physiological functions but also induces a psychocognitive decline and even impairs the motor control and regulatory network. In this study, we analyzed whole-brain functional connectivity changes in the putamen and caudate nucleus as seed points in the neostriatum after 36 h of complete sleep deprivation in 30 healthy adult men by resting state functional magnetic resonance imaging to investigate the physiological mechanisms involved in impaired motor control and regulatory network in individuals in the sleep-deprived state. The functional connectivity between the putamen and the bilateral precentral, postcentral, superior temporal, and middle temporal gyrus, and the left caudate nucleus and the postcentral and inferior temporal gyrus were significantly reduced after 36 h of total sleep deprivation. This may contribute to impaired motor perception, fine motor control, and speech motor control in individuals. It may also provide some evidence for neurophysiological changes in the brain in the sleep-deprived state and shed new light on the study of the neostriatum in the basal ganglia.

Keywords: sleep deprivation, putamen, caudate, resting state-fMRI, functional connectivity, motor control network

INTRODUCTION

Total sleep deprivation (TSD) refers to a physiological state of less than 4 h of continuous sleep for at least 24 h. TSD has been shown to not only harm individual physiological functions and increase the risk of developing cardiovascular disease and obesity (St-Onge and Zuraikat, 2019; Yu et al., 2020) but also cause a psychocognitive decline including loss of mood, learning, and memory, which in turn triggers individual behavioral disorders and can even cause operational accidents (Killgore, 2010; Tantawy et al., 2013; Goldstein and Walker, 2014; Xie et al., 2015; Lo et al., 2016; Krause et al., 2017; Cunningham et al., 2018; Peng et al., 2020).

Accidents are associated not only with psychocognitive decline but also with impaired fine motor control. Fine motor control is a high-level cognitive function of humans, which belongs to the

category of voluntary movement, and occurs throughout the whole process of human daily life and social activities, such as reading, writing, speech, working, and some sports (Stippich et al., 2007; Bracci et al., 2012; Hao et al., 2016). Because fine movement is very important in daily life, much research has been conducted in the field of cognitive neuroscience, applying anatomical, physiological, and molecular biological methods to study voluntary movement and the mechanisms that govern it. The basic anatomical structures, physiological functions, and interconnections among various structures involved in voluntary motor control in animals and humans have now been established. In recent years, with the rapid development of medical imaging, functional magnetic resonance imaging (fMRI) techniques have provided further insights into the *in vivo* study of neural mechanisms, as well as into the imaging mechanism of fine motor control (Lee et al., 2010; Plow et al., 2010). Resting-state functional MRI (rs-fMRI) reflects spontaneous activity *via* the blood oxygenation level dependent (BOLD) signal in the brain, which is closer to the physiological state. Relative to the task state, rs-fMRI is convenient to operate, provides repetitive, stable, and reliable information, and can be analyzed in many ways. Therefore, rs-fMRI has obvious advantages for studying brain spontaneous activity, functional connectivity among various brain regions, development and plasticity of brain function, and neuropsychiatric disorders, and is a recent focus of research on brain function (Friston, 2004; Zhu et al., 2020).

In general, voluntary motor signals emanate from the area of the precentral gyrus of the cerebral cortex through descending pyramidal system conduction pathways to the anterior horn of the spinal cord and then through spinal nerves to the corresponding motor neurons, causing skeletal muscle contraction. The precentral gyrus plays an important role in voluntary movement of the soma and is a somatomotor high-level control center. Multiple precentral gyrus regions are involved in composing the somatomotor cortex, including the primary motor cortex (M1), premotor area (PM), and supplementary motor area (SMA), which are located within and lateral to the brain in the precentral gyrus. These different regions of the precentral gyrus play different roles in the achievement of motor function, acting in the form of network connections (Mayka et al., 2006). However, the brain regions involved in motor control function are extensive, and voluntary motor information undergoes regulatory control by the extrapyramidal system in addition to the pyramidal system. These include the spinal cord, thalamus, secondary somatosensory cortex (**Supplementary Table 2**), medial insular cortex (IC), anterior cingulate cortex (ACC), PM, SMA, and M1. Furthermore, the limbic system, basal ganglia (BG), thalamus, orbitofrontal cortex (OFC), prefrontal cortex (PFC), ACC, PM, SMA, and M1 regions make up the locomotor modulatory excitatory pathway (Tanaka and Watanabe, 2012).

However, because of limitations in research conditions, the mechanisms of motor control involved in the basal ganglia have not been thoroughly elucidated regarding the initiation, programming of voluntary movement, and the execution of movement in humans. The BG are important nuclei in extrapyramidal transmission pathways that receive afferent signals from the cortex to feed back to the cerebral cortex after integration. From a functional point of view, the BG can be divided into six functional nuclei, including the striatum (STR), external globus pallidus (GPe), internal segment of the globus pallidus, substantia nigra pars reticulata (GPi-SNr), substantia nigra pars compacta (SNc), and subthalamic nucleus (STN) (Rodriguez-Sabate et al., 2016).

In recent years, functional connectivity analyses have provided invaluable approaches for studying the human brain on the brain-network level. Using fMRI approaches, investigators have found specific patterns of functional connectivity in the sensorimotor network between the BG and the primary motor cortex and cerebellum (Carlson, 2009; Sokolov et al., 2012), in which the STR, together with the STN and substantia nigra, mainly constitute the subcortical circuits regulating locomotion, in concert with the cerebral cortex and cerebellum regulating voluntary movements, muscle tone, and postural reflexes (Wall et al., 2013). Therefore, the BG play a very important role in the sensorimotor network and mainly participate in the motion control and regulation network (Bostan et al., 2013; Zhang et al., 2018) responsible for physiological functions such as motor control, motor learning, functioning, and behavior (Lanciego et al., 2012).

Among the anatomical structures of the BG, the STR includes the caudate and lentiform nucleus, which are connected anteriorly ventrally; the lentiform nucleus is further divided into the putamen and the pallidus; the caudate nucleus and putamen are phylogenetically more recent structures of the STR, together called the neostriatum, and the pallidus is the oldest part of the STR, called the old STR. All three are structurally and functionally closely linked (Alexander et al., 1986), and because of the unique anatomical properties of the STR, many studies have combined the three nuclei as STR in the past to analyze motor control and regulation functions. Few studies have separated the neostriatal caudate nucleus and putamen for functional analysis.

Neuroanatomical studies have shown that the putamen has direct anatomical connections with the M1 and SMA (Viñas-Guasch and Wu, 2017). In addition, the putamen has been shown to play an important role in motor control, with the putamen receiving voluntary motor information from corticothalamic projections for integrative processing and descending projections through ganglion brainstem networks for their characteristic motor control functions (Moustafa et al., 2018).

Moreover, fine motor control is critically involved in the transmission integration of multisensory information and is coupled with higher cognitive functions, such as learning and memory (Grosbras et al., 2011; Wacker et al., 2011; Lechak and Leber, 2012). Therefore, a thorough exploration of the neurophysiological basis underlying individual changes in motor control function after TSD is an important approach for understanding the impact of human sleep and physiological rhythms on cognitive behavior. We hypothesized that impaired fine motor control after TSD is associated with altered patterns of functional connectivity in the neostriatum, and thereby designed a 36 h TSD experiment with functional imaging data acquisition before and after sleep deprivation. The aim of this study was to identify the neurofunctional mechanisms by which sleep

deprivation affects fine motor control by analyzing changes in the pattern of functional connectivity between the putamen and caudate nucleus bilaterally after TSD using resting-state fMRI.

MATERIALS AND METHODS

Subjects

Thirty healthy adult males at university, aged 18–24 years (21.94 ± 1.73), were enrolled according to the following criteria: right handedness, normal uncorrected or corrected visual acuity, no history of alcohol and drug abuse, and no history of mental or neurological diseases. The Pittsburgh sleep quality index test scores of all subjects were less than five points, which indicated that all subjects had good sleep habits. Subjects had no history of severe physical disease or traumatic brain injury. The subjects were required not to consume alcohol, coffee, or other irritant food and drink the week before and during the experiment. The trial was approved by the Research Ethics Committee of Beihang University. The trial process and precautions were explained to the subjects before the trial. All participants voluntarily participated in the trial and provided informed consent.

Research Methods

At the beginning of the experiment, the subjects registered their information at 16:00 on the first day and were ready to rest at 20:00. All participants underwent sleep monitoring from the first night onward, and relevant index tests was performed at 06:00 on the second day before rs-fMRI scanning including a series of emotional state scales and working memory tests. The second relevant index tests and rs-fMRI scanning were conducted at 20:00 on the third day after a 36-h sleep deprivation (SD). The participants were only allowed to perform non-strenuous activities during the 36-h period, such as conversing, reading, gaming, and working on a computer. Moreover, participants were not permitted to smoke, drink, or consume any stimulants including coffee, chocolate, soft drinks, or alcohol. Our SD laboratory used the medical sleep monitoring room in the PLA Air Force General Hospital, with complete supporting medical facilities. During the whole process of sleep deprivation, medical staff took turns on duty to ensure the health status of the subjects, and the researchers also took turns on duty to monitor the status of the subjects.

MRI Data Acquisition

All MRI scans were conducted at the MRI Department of the PLA Air Force General Hospital. Before the scanning, the subjects were asked to take preparations (remove the magnetic items they carried, wear shoe covers, wear earplugs, etc.). The subjects lay flat on the MRI table, and their heads were fixed with sponges and bandages. Ge 3.0T MR750 equipment and a special 8-channel head coil were used to collect the MRI signals. During the scan, the subjects were asked to close their eyes and keep their head still and not think about anything and the whole procedure of the subjects in the scanner was about 40 min.

The rs-fMRI images were collected using a plane-echo imaging sequence. There were 190 images. The specific scanning parameters were as follows: repetition time, 2,000 ms; echo time, 30 ms; scanning field, 240 mm × 240 mm; layer thickness, 3 mm; layer spacing, 1 mm; turning angle, 90°; and acquisition matrix, 64 × 64. The number of layers was 35 (the scan positioning line was parallel to the anterior posterior commissural line). High-resolution T1 images were acquired using the FSPGR-BRAVO sequence. The parameters were as follows: repetition time, 8.208 s; echo time, 3.22 ms; turning angle, 12°; scanning field, 240 mm × 240 mm; inversion time, 450 ms. It is important to ensure that the subjects do not fall asleep during rs-fMRI scanning. Therefore, before each scan, they communicated with the subjects through a microphone to remind the subjects to keep awake. After each scan, subjects were asked whether they remained awake during the scan. In addition, the MRI equipment we used had a camera inside, and throughout each scan, the operator monitored the subject's body movements and other states through the camera. Combined with their subjective reports, we can confirm that no subjects fell asleep during the scan.

MRI Data Preprocessing

The raw MR data were analyzed using MATLAB 2015b and the statistical parametric mapping (SPM12; Welcome Department of imaging neuroscience, London, United Kingdom,[1]) software package for processing. Before resting state data preprocessing, the first 10 frames of each subject were manually removed to eliminate the effect of magnetic saturation at the initial scan stage. fMRI data preprocessing was performed next, and the specific steps mainly included slice timing, alignment, co-registration between functional and structural images, spatial normalization to MNI space (3 mm × 3 mm resolution), filtering of the waveforms of each brain voxel by band-pass filters ($0.008\ \text{Hz} < f < 0.09\ \text{Hz}$) to accommodate low-frequency drift and high-frequency noise effects, and Gaussian filter (FWHM = 6 mm) to spatially smooth the filtered data. Subjects with a head motion correction displacement of more than 2 mm in the X-, Y-, and Z-axis directions and rotation of more than 1° were removed.

rs-fMRI Data Functional Connectivity Analysis

rs-fMRI data functional connectivity (FC) analysis was performed after preprocessing and was completed using the CONN toolbox[2]; FC analysis was performed on 30 subjects. To define the 116 regions of interest (ROIs) considered in this study, the CONN toolbox automated anatomical labeling (AAL) was used (Tzourio-Mazoyer et al., 2002), including 90 cerebral ROIs and 26 cerebellar ROIs. Linear regression analysis was performed to remove white matter, CSF, and six motor signals. The CONN toolbox extracts various subsites, that is, the average BOLD time series signals from all voxels included in the ROI region, and then summing the time series of signals from that subsite region and the correlation coefficient of the time series from each voxel of the remaining whole brain (ROI-Voxel). To

[1] http://www.fil.ion.ucl.ac.uk/spm/software/spm12/

[2] http://web.mit.edu/swg/software.htm

estimate the intensity of FC, the correlation coefficients were converted to Z values using Fisher r-to-z transformation after obtaining the correlation coefficient map, resulting in an FC value for each ROI region. Paired sample t-tests were used to compare the differences in voxel-wise FC values between the putamen and caudate seed points to the whole brain before and after SD, with statistical significance defined as uncorrected $p < 0.001$ and cluster > 40 (Yu et al., 2020).

RESULTS

We performed the procedure described in sections "MRI Data Preprocessing" and "rs-fMRI Data Functional Connectivity Analysis" for each subject's data, performing correlation analysis between the BOLD signal from each ROI and whole brain voxels with paired samples t-tests. Results of whole-brain FC patterns in the bilateral putamen and caudate nucleus before and after sleep deprivation are detailed in **Figures 1–4** and Tables in **Supplementary Material**.

FC Pattern Differences in Bilateral Putamen Before and After 36 h TSD

It was found that, compared to subjects after TSD in the normal wakefulness state, the left putamen had decreased FC within the left precentral gyrus [t-value = −5.76, $p < 0.001$, t-test (GLM)], decreased FC within the right precentral gyrus [t-value = −5.96, $p < 0.001$, t-test (GLM)], and decreased FC within the left postcentral gyrus [t-value = −5.56, $p < 0.001$, T-test (GLM)], with decreased FC to the right postcentral gyrus [t-value = −5.50, $p < 0.001$, t-test (GLM)], with decreased FC to the left superior temporal gyrus [t-value = −6.81, $p < 0.001$, t-test (GLM)], and with decreased FC to the left middle temporal gyrus [t-value = −6.30, $p < 0.001$, t-test (GLM)]. However, a significant enhancement was found in the FC in the right supramarginal gyrus [t-value = 5.41, $p < 0.001$, t-test (GLM)].

The results found that the right putamen showed similar results to the left putamen, with decreased FC between the right putamen and the left precentral gyrus after TSD [t-value = −5.20, $p < 0.001$, t-test (GLM)], decreased FC within the right precentral gyrus [t-value = −7.47, $p < 0.001$, t-test (GLM)], decreased FC within the left precentral gyrus [t-value = −5.73, $p < 0.001$, T-test (GLM)], and decreased FC within the right postcentral gyrus [t-value = −6.45, $p < 0.001$, t-test (GLM)]. However, no significant enhancement of FC was found in the right putamen (**Table 1** and **Figure 5**).

FC Pattern Differences in the Bilateral Caudate Before and After 36 h TSD

An analysis of the results only found changes in FC patterns before and after TSD in the left caudate nucleus; no significant results were found in the right caudate nucleus. Compared to subjects after TSD in the normal wakefulness state, the left caudate nucleus showed decreased FC within the left postcentral gyrus [t-value = −4.56, $p < 0.001$, t-test (GLM)], decreased FC within the right postcentral gyrus [t-value = −5.72, $p < 0.001$, t-test (GLM)], and decreased FC within the left inferior temporal gyrus [t-value = −6.03, $p < 0.001$, t-test (GLM)]. A significant enhancement was also found for FC within the right supramarginal gyrus [t-value = 4.82, $p < 0.001$, t-test (GLM)] (**Table 2** and **Figure 6**).

DISCUSSION

In this study, we investigated the effects of 36 h TSD on whole-brain FC in the putamen and caudate nucleus of the neostriatum at resting state using fMRI. We observed a significant decrease in connectivity between the putamen and the precentral gyrus, postcentral gyrus, superior temporal gyrus, and middle temporal gyrus in subjects after TSD, as well as a significant decrease in connectivity between the left caudate and the postcentral gyrus and inferior temporal gyrus of the cortex and significantly enhanced connectivity between the left putamen and caudate with the right supramarginal gyrus. Such results could represent impaired somatic fine motor control and speech motor function due to TSD caused by disrupted communication in the motor control and regulatory network jointly involving the putamen and caudate nucleus, which is an innovative addition to the current association study of sleep loss.

By comparing putamen and caudate connectivity changes induced by loss of sleep, we found significant reductions in FC between the putamen and parts of the precentral gyrus, caudate, and parts of the postcentral gyrus. According to the cortical BG motor control loop proposed by Wall et al., the putamen and caudate nucleus receive excitatory motor information from corticocortical projections and send post-processing feedback projections back to the cortex *via* the BG (Wall et al., 2013). Thus, the reduced FC between the putamen and caudate with parietal regions after TSD compared to normal wakefulness in subjects may have contributed to the disrupted transmission of information between the neostriatum and cortical motor and sensory areas after TSD. The current study found that there is functional consolidation of motor memory by the cerebral cortical spindles during sleep, with areas of action including the hippocampus, putamen, thalamus, and somatomotor cortex (Boutin et al., 2018). TSD not only impaired this functional consolidation but also functionally separated putamen from motor control and regulatory network, which possibly causing somatic dystonia from impaired voluntary muscle control with direct consequences for individual motor control functions.

Meanwhile, the results also revealed decreased FC between the bilateral putamen and left caudate nucleus with some areas of the postcentral gyrus. This indicated that not only disturbed motor information transmission but also somatosensory information communication processes between the cerebral cortex and the neostriatum are affected by TSD in the somatomotor functions in which the BG participate. Somatosensory signals generated by the skin and proprioceptive receptors play a crucial role in the fine control of dexterous motor movements, and individuals adjust motor commands using the acquired sensory information to enable the motor system to correct sensory errors in a timely manner; the anatomical basis of this

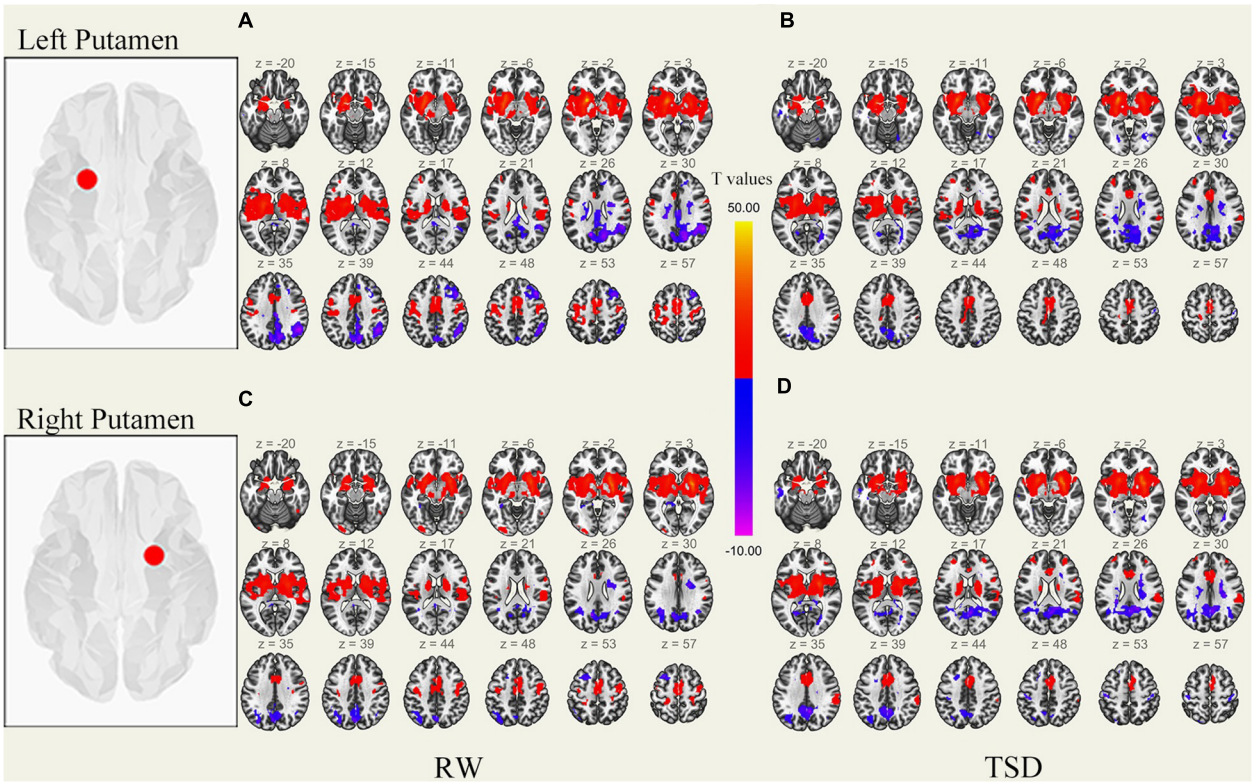

FIGURE 1 | Whole-brain functional connectivity patterns of the putamen before 36 h TSD shown at panels **(A,C)**, and after 36 h TSD shown at panels **(B,D)** (*n* = 30) (Transverse view). Warm colors indicate positive correlations, and cold colors indicate negative correlations.

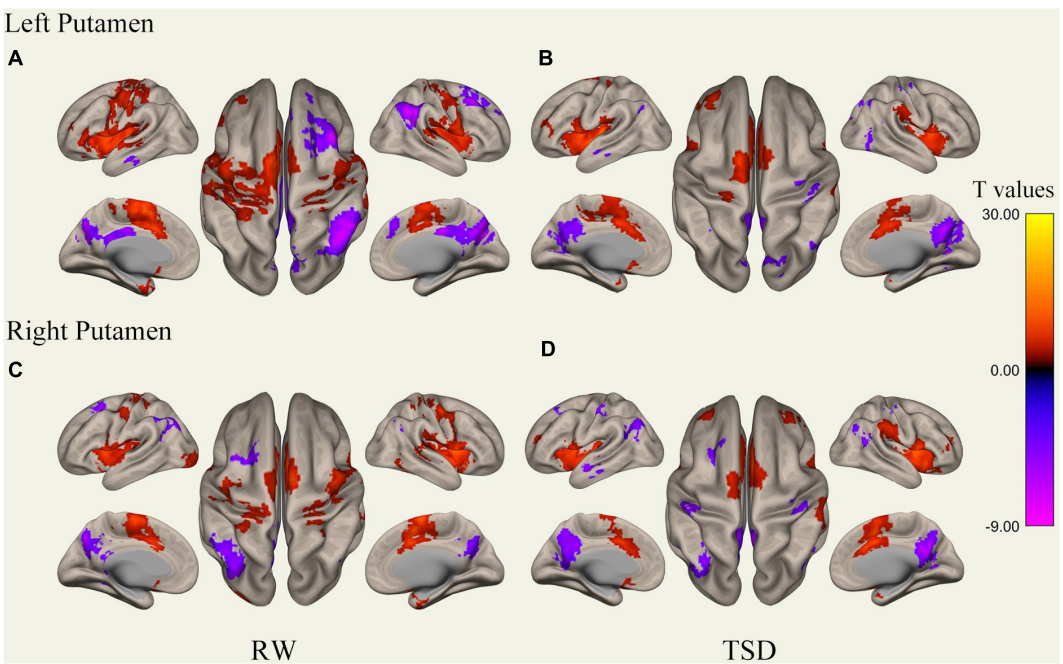

FIGURE 2 | Whole-brain functional connectivity patterns of the putamen before 36 h TSD shown at panels **(A,C)**, and after 36 h TSD shown at panels **(B,D)** (*n* = 30) (Surface view). Warm colors indicate positive correlations, and cold colors indicate negative correlations.

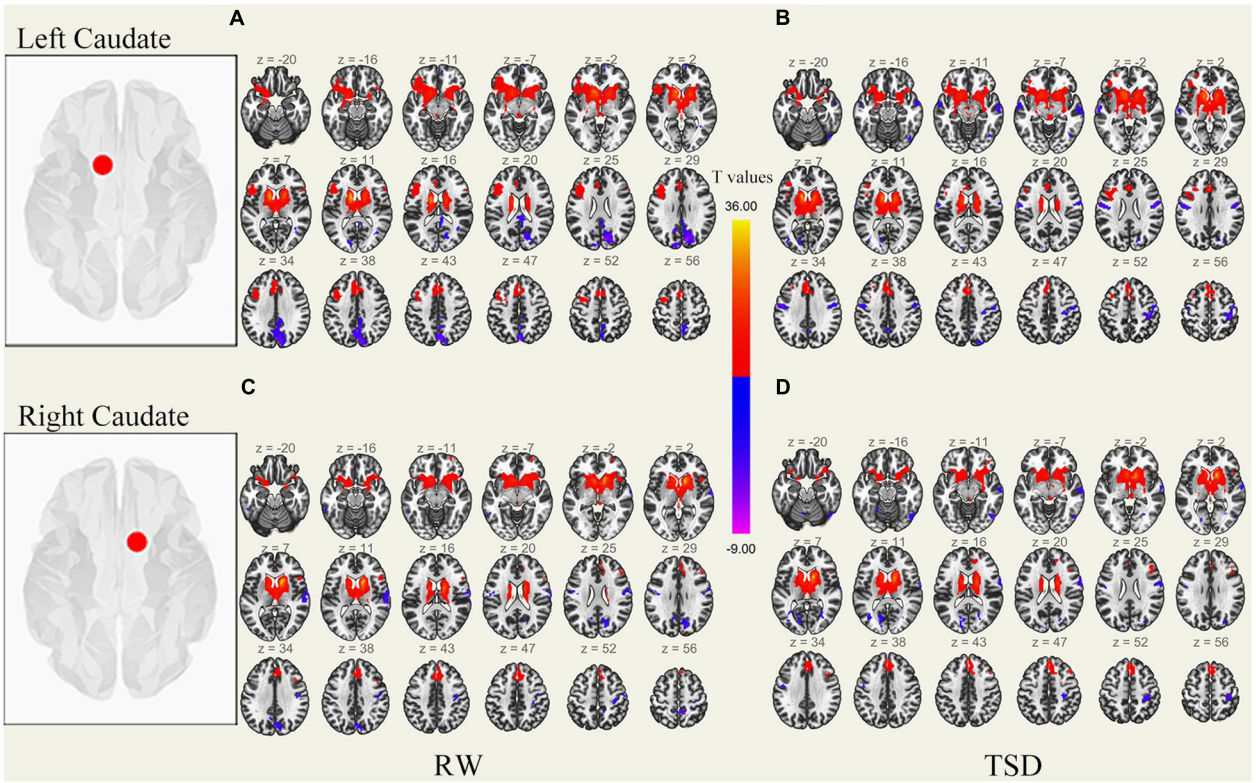

FIGURE 3 | Whole-brain functional connectivity patterns of the caudate before 36 h TSD shown at panels **(A,C)**, and after 36 h TSD shown at panels **(B,D)** (n = 30) (Transverse view). Warm colors indicate positive correlations, and cold colors indicate negative correlations.

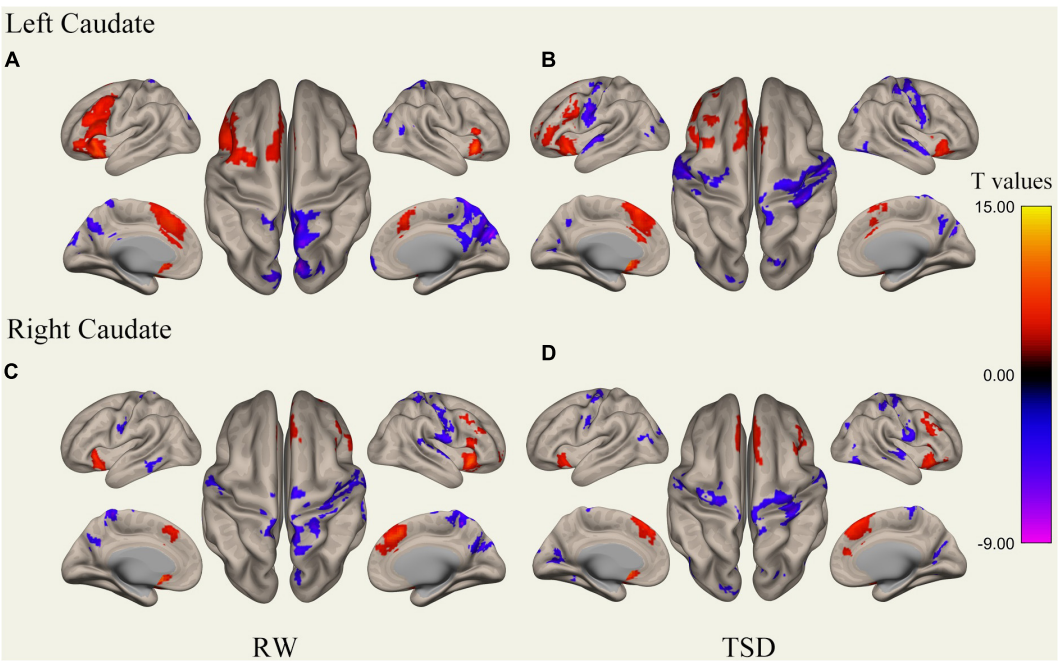

FIGURE 4 | Whole-brain functional connectivity patterns of the caudate before 36 h TSD shown at panels **(A,C)**, and after 36 h TSD shown at panels **(B,D)** (n = 30) (Surface view). Warm colors indicate positive correlations, and cold colors indicate negative correlations.

mechanism is the direct anatomical connection between the primary somatosensory cortex (**Supplementary Table 1**) and M1 (Mao et al., 2011; Feldmeyer, 2012). In addition, it is also a complex somatosensory motor integration function that allows quick and accurate movement (Johansson and Flanagan, 2009; Masato et al., 2019). Therefore, the reduced connectivity between the putamen, caudate, and postcentral gyrus may reflect a decline in individual fine motor control after TSD, which is associated with altered functional patterns in the sensorimotor network including the neostriatum.

However, increased FC was found between the putamen and caudate with the right supramarginal gyrus, which is considered a key region of the higher-order sensorimotor cortex and plays an important role in spatial processing and motor control. Furthermore, an fMRI study found that the right supramarginal gyrus is important for proprioception in patients with stroke (Ben-Shabat et al., 2015). The increased FC between the left putamen and left caudate with the right supramarginal gyrus in the sleep-deprived state may reflect a type of proprioceptive compensation, which, to some extent, compensates for the impaired motor control and regulatory network due to reduced connectivity in **Supplementary Table 1**.

It has also been found that 24-h SD causes a reduction in the density of short-distance FC in the posterior cerebellar lobes, suggesting that SD maintains cognitive performance by reducing higher-order cognition-, arousal-, and sensorimotor-related regions (Kong et al., 2018). The

TABLE 1 | Changes in whole brain FC in the bilaterally Putamen before and after 36 h TSD, size of relevant regions, coordinates of MNI and maximum statistical *t* value (n = 30).

Brain regions	Size	Talairach coordinates			*T* score
		x	y	z	
Seed: Left Putamen (after TSD > before TSD)					
Left precentral gyrus	180	−51	−3	+ 36	−5.76
Left postcentral gyrus	109	−51	−15	+ 42	−5.56
Right precentral gyrus	213	+45	−15	+ 54	−5.96
Right postcentral gyrus	120	+45	−18	+ 51	−5.50
Left superior temporal gyrus	48	−57	−12	−3	−6.81
Left middle temporal gyrus	40	−48	−36	−3	−6.30
Right supramarginal gyrus	86	+51	−42	+57	5.41
Seed: Right Putamen (after TSD > before TSD)					
Left precentral gyrus	108	−45	−15	+45	−5.20
Left postcentral gyrus	186	−42	−24	+57	−5.73
Right precentral gyrus	150	+45	−12	+54	−7.47
Right postcentral gyrus	106	+45	−18	+54	−6.45

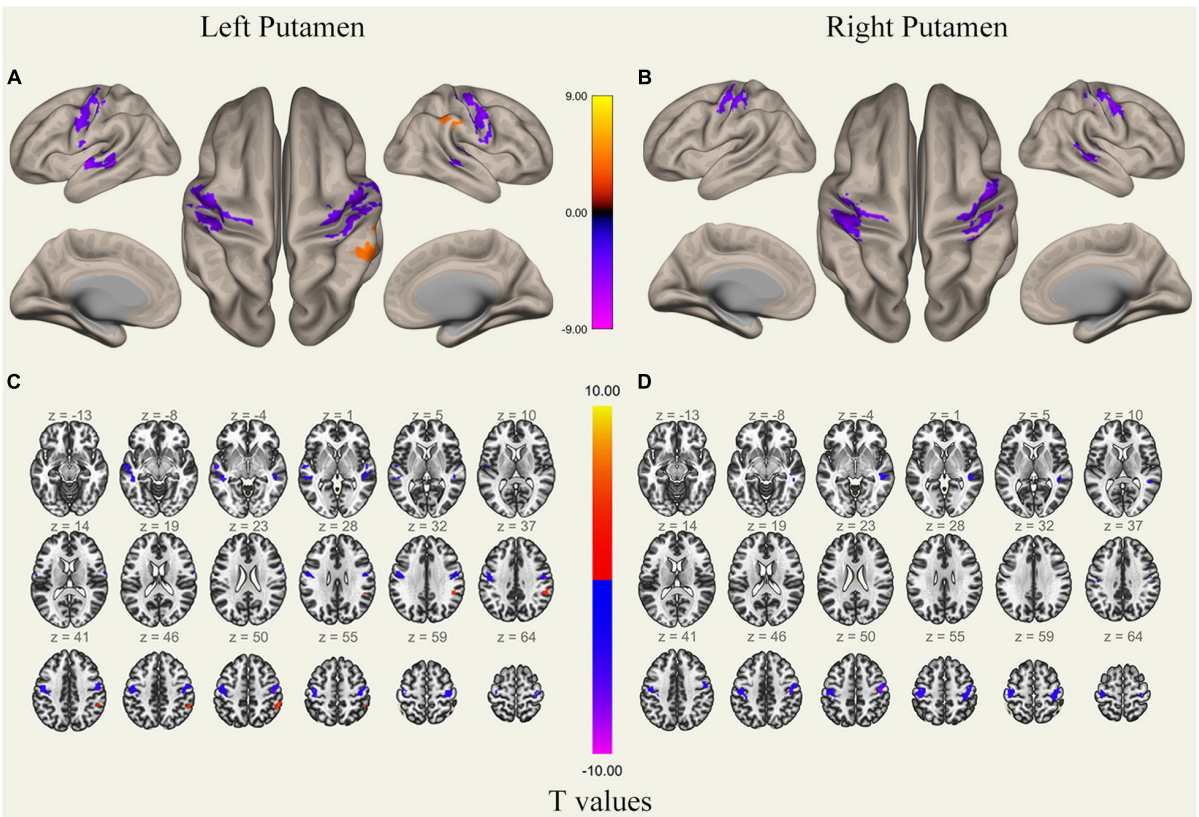

FIGURE 5 | Surface views shown at panels **(A,B)** and transverse views shown at panels **(C,D)** of brain areas that exhibited altered functional connectivity with bilaterally putamen after 36 h TSD (n = 30). Warm colors indicate increased FC, and cold colors indicate decreased FC.

TABLE 2 | Changes in whole brain FC in the left Caudate before and after 36 h TSD, size of relevant regions, coordinates of MNI and maximum statistical *t* value (n = 30).

Brain regions	Cluster size	MNI coordinates			*T* score
		x	y	z	
Seed: Left Caudate (after TSD > before TSD)					
Left postcentral gyrus	40	−45	−24	+54	−4.56
Right postcentral gyrus	53	+42	−30	+57	−5.72
Left inferior temporal gyrus	38	−60	−12	+03	−6.03
Right supramarginal gyrus	32	+63	−36	+33	4.82

conclusion of this study can be similarly explained by the findings of Kong et al. that the post-TSD brain has reduced fine motor control function to maintain cognitive functional integrity by altering the neostriatal potency in motor control and regulatory network. However, the 36-h TSD paradigm used in this study, in which neuroimaging data acquisition was not performed during the procedure, only accounts for the impairment motor control and regulatory network during prolonged sleep loss and does not specifically confirm the corresponding time point of connectivity decline.

Interestingly, the putamen was found to have reduced FC in some areas of the superior and middle temporal gyrus, which we speculate is related to motor control processes related to individual speech production, simply to the movements involved in speaking. Speaking is one of the most complex and precise motor behaviors in humans; it coordinates the movements of breathing, larynx, articulation, and facial muscles to produce speech while speaking. The underlying neural mechanisms of speech involve sensory-motor interactions that incorporate feedback information for online monitoring and control of produced speech sounds. The motor behavior of speech is regulated by speech processing in areas of the auditory cortex, as demonstrated by neurophysiological studies of this sensorimotor mechanism (Flinker et al., 2010; Behroozmand and Larson, 2011; Chang et al., 2013; Greenlee et al., 2013; Sitek et al., 2013; Behroozmand et al., 2015).

An fMRI study identifying brain regions involved in the motor control of speech showed that speech feedback processing involves complex sensorimotor networks, including the superior temporal gyrus (STG), precentral gyrus, postcentral gyrus, SMA, inferior frontal gyrus (IFG), inferior parietal lobule (IPL), and insula. It has also been shown that a more complex sensory motor network involving the bilateral STG, MTG, precentral gyrus, SMA, IFG, postcentral gyrus, IPL, insula, and putamen is involved when humans use auditory feedback for speech production and motor control (Parkinson et al., 2012), which is highly consistent with our findings. From this, we speculate that speech production motor function is impaired after TSD and that this functional impairment may result from diminished connectivity of the temporal cortex speech area with the neostriatum (Ludman et al., 2000; Xu et al., 2009; Bernstein et al., 2011; Wu et al., 2017).

Our study has some limitations. First, we only assessed male volunteers, so we cannot make generalizations to females. Only male volunteers were recruited due to the experimental conditions and the long time course of the study. This will limit the clinical utility of the findings. In the future, it would be interesting to investigate sex differences in functional connectivity changes following TSD.

Second, circadian biorhythms affect behavioral performance, and these effects differ across individuals (Montplaisir, 1981; Lavie, 2001). However, considering that 48 h of sleep deprivation may uncontrollably damage the health of the subjects and 24 h of sleep deprivation has relatively small effects, 36 h of sleep deprivation is inevitably affected by circadian rhythm. In previous similar studies, the data collected at 20:00 on the day before sleep deprivation and 08:00 on the first day were used as two baselines, but the results showed that there was no significant difference between the two baselines (Shao et al., 2014; Lei et al., 2015; Zhang et al., 2019; Peng et al., 2020; Li et al., 2021);

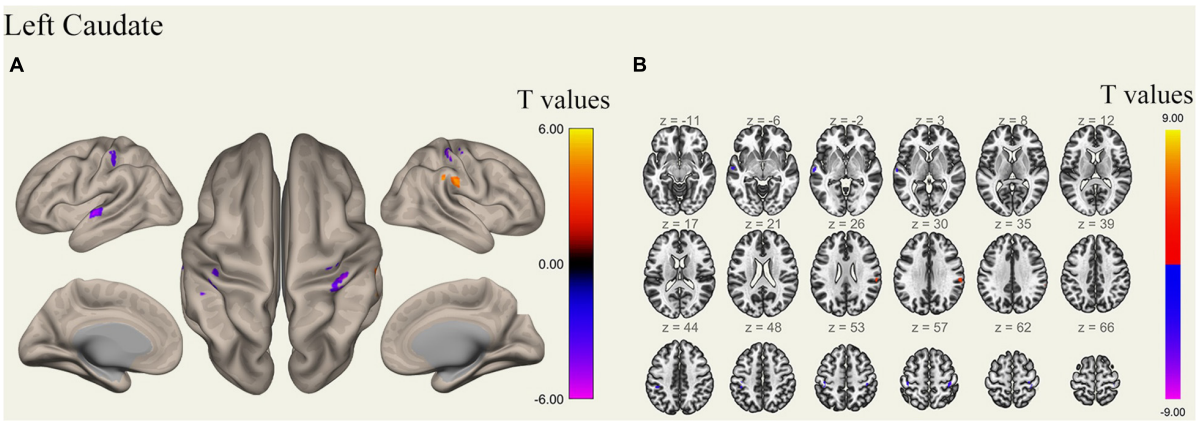

FIGURE 6 | Surface views shown at panel **(A)** and transverse views shown at panel **(B)** of brain areas that exhibited altered functional connectivity with left caudate after 36 h TSD (n = 30). Warm colors indicate increased FC, and cold colors indicate decreased FC.

therefore, only one baseline was collected in this study. However, no circadian/time of day differences in other brain functions does not mean that neostriatum connectivity is unaffected by circadian/time of day effects. Thus, we could not rule out effects of circadian rhythms on our results, and in future experiments, we will consider measuring two baseline data and using the Horne and Ostberg questionnaire to determine the chronotype of the subject to exclude the influence of circadian rhythm.

Third, although behavioral assessment was used to evaluate sleep during MRI scanning, this method may not be completely sufficient. Fluctuation in states of sleepiness, drowsiness, and sudden (even if very short) sleep episodes of a few seconds, cannot be 100% excluded, which is a major limitation of the study. Polysomnography, which is the gold standard for sleep evaluation, and other physiological monitoring methods lacking here, such as long-term EEG or ECG, should be considered in future research.

CONCLUSION

Overall, reduced connectivity between the putamen and the precentral gyrus leads to a blockade of the cortico BG motor control circuit and impaired motor control and regulatory network in individuals. In addition, connectivity between the putamen and caudate nucleus with the postcentral gyrus leads to disrupted sensory information feedback in the somatosensory motor integration system, which in turn affects individual fine motor control function. Decreased connectivity of the putamen with the STG and MTG and the caudate with the ITG may lead to impaired speech production and speech motor control in individuals due to the separation of speech and sensorimotor information in the speech sensory motor network.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Research Ethics Committee of Beihang University. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

YS and WF designed the study. HW and KY conceptualized, investigated, and visualized the data, carried out the formal analysis, and wrote the manuscript. TY, LZ, JL, CD, and ZP contributed to the data collection. YS and JQ were the guarantors of this study. All authors contributed to the article and approved the submitted version.

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Nitric Oxide Synthase Neurons in the Preoptic Hypothalamus Are NREM and REM Sleep-Active and Lower Body Temperature

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When mice are exposed to external warmth, nitric oxide synthase (NOS1) neurons in the median and medial preoptic (MnPO/MPO) hypothalamus induce sleep and concomitant body cooling. However, how these neurons regulate baseline sleep and body temperature is unknown. Using calcium photometry, we show that NOS1 neurons in MnPO/MPO are predominantly NREM and REM active, especially at the boundary of wake to NREM transitions, and in the later parts of REM bouts, with lower activity during wakefulness. In addition to releasing nitric oxide, NOS1 neurons in MnPO/MPO can release GABA, glutamate and peptides. We expressed tetanus-toxin light-chain in MnPO/MPO NOS1 cells to reduce vesicular release of transmitters. This induced changes in sleep structure: over 24 h, mice had less NREM sleep in their dark (active) phase, and more NREM sleep in their light (sleep) phase. REM sleep episodes in the dark phase were longer, and there were fewer REM transitions between other vigilance states. REM sleep had less theta power. Mice with synaptically blocked MnPO/MPO NOS1 neurons were also warmer than control mice at the dark-light transition (ZT0), as well as during the dark phase siesta (ZT16-20), where there is usually a body temperature dip. Also, at this siesta point of cooled body temperature, mice usually have more NREM, but mice with synaptically blocked MnPO/MPO NOS1 cells showed reduced NREM sleep at this time. Overall, MnPO/MPO NOS1 neurons promote both NREM and REM sleep and contribute to chronically lowering body temperature, particularly at transitions where the mice normally enter NREM sleep.

Keywords: preoptic hypothalamus, nitric oxide, sleep, calcium photometry, body temperature, tetanus-toxin light-chain

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INTRODUCTION

Numerous circuits dispersed throughout the brain induce NREM sleep, but the preoptic (PO) hypothalamus, one of the first sleep-promoting centers to be identified (Nauta, 1946), has a major role (Sherin et al., 1996; Zhang et al., 2015; Weber and Dan, 2016; Chung et al., 2017; Kroeger et al., 2018; Ma et al., 2019; Reichert et al., 2019; Reitz and Kelz, 2021). The PO area also contains neurons that are required for REM sleep (Lu et al., 2000), including REM-promoting cells in MPO (Suntsova and Dergacheva, 2004; Gvilia et al., 2006). The PO area, which contains a huge diversity of cells (Moffitt et al., 2018; Tsuneoka and Funato, 2021), also contributes to regulating many other functions, including nesting, thermoregulation, parenting, sexual behavior, water consumption, blood osmolarity, and daily torpor (Nakamura and Morrison, 2008, 2010; Morrison and Nakamura, 2011; Saper and Lowell, 2014; Abbott and Saper, 2017; Hrvatin et al., 2020; Takahashi et al., 2020; Tsuneoka and Funato, 2021).

The medial (M) and median (Mn) PO hypothalamic areas are enriched for neuronal nitric oxide (*nos1*) gene expression, as seen by *in situ* hybridization in the Allen Brain Atlas (Lein et al., 2007), and from our previous studies (Harding et al., 2018). Previously we found that NOS1 neurons in the MnPO and MPO area link NREM onset and the decrease of body temperature that accompanies sleep (Harding et al., 2018). We hypothesized that external warm sensing and NREM sleep induction through these neurons may be part of an energy conservation mechanism that optimizes sleep toward thermoneutral temperatures (Harding et al., 2020).

In addition to presumably synthesizing NO in response to excitation and calcium, MnPO/MPO NOS1 cells, depending on subtype, likely release both GABA and glutamate and/or various peptides (Moffitt et al., 2018). Here we show by calcium photometry that NOS1 neurons in MnPO/MPO have their highest activity during NREM sleep, becoming particularly active at the boundary of wake to NREM transitions, and they are also active during the latter parts of REM sleep episodes. Synaptic silencing of MnPO/MPO NOS1 cells with tetanus toxin light-chain (TeLC) expression induced bidirectional changes to NREM sleep structure: over the 24-h cycle, mice had less NREM sleep in the dark phase, and more in the light phase. Dark phase REM sleep also consolidated to longer episodes, with a reduction in REM transitions; however, both light- and dark-phase REM sleep had more delta and less theta power than in controls, possibly suggesting disrupted REM function. In addition, a shift in the core body-temperature profile to warmer temperatures and a disrupted siesta (ZT16-20) period were observed. Thus, vesicular release of transmitters from MnPO/MPO hypothalamic NOS1 neurons is needed for maintaining normal sleep and temperature profiles.

MATERIALS AND METHODS

Mice

Experiments were performed under the Animals (Scientific Procedures) Act (1986) and approved by the local ethics committee. The mice used were *Nos1-ires-Cre*$^{TM1(crE)Mgmj}$*/J* (JAX labs stock 017526), referred to here as *Nos1-Cre* mice, donated by Martin G Myers (Leshan et al., 2012), and C57BL/6J mice (supplied by Charles River United Kingdom). All mice used in the experiments were male and congenic on a C57BL/6J background. Mice were maintained on a reversed 12 h:12 h light:dark cycle at constant temperature (22 ± 1°C) and humidity with *ad libitum* food and water.

AAV Transgenes and AAV Production

We used the following *pAAV* transgene plasmids: *pAAV-FLEX-GFP-TeLC* (Murray et al., 2011), and *pAAV-FLEX-GFP* (Addgene #28304, a gift from Edward Boyden). Plasmid *pAAV-FLEX-GCaMP6s* was created by inserting the *GCaMP6s* open reading frame from *pCMV-GCaMP6s* (Addgene plasmid 40753, gift of Douglas Kim) (Chen et al., 2013), into the backbone of *pAAV-flex-hM3Dq-mCHERRY* (Krashes et al., 2011) in place of the *hM3Dq* sequence, but retaining the loxP sites. AAV transgenes were packaged in-house into capsids with a 1:1 ratio of AAV1 and AAV2 capsid proteins. The adenovirus helper plasmid *pFΔ6*, the AAV helper plasmids *pH21* (AAV1) and *pRVI* (AAV2), and the *pAAV* transgene plasmids were co-transfected into HEK293 cells and AAVs harvested on heparin columns, as described previously (Klugmann et al., 2005; Yu et al., 2015). AAVs titers were determined with an AAVpro Titration Kit (for real-time PCR) Ver. 2 (TakaRa Bio). The virus titers were as follows: *AAV-FLEX-GCaMP6s,* 1.6×10^6 viral genomes/μl; *AAV-FLEX-GFP-TeLC* 5.1×10^5 viral genomes/μl; *AAV-FLEX-GFP*, 6.1×10^6 viral genomes/μl.

Surgeries and Stereotaxic Injections of AAV

Mice underwent their first surgery at 10-weeks old. The mice required two rounds of surgery including implantation of an abdominal temperature logger, followed one week later by stereotaxic injections of AAV virus and electrode placement for electrocorticography (ECoG). For surgery, mice were anesthetized with 2% isoflurane and given appropriate analgesia. Viral infusions were performed using a steel injector (10 μl-Hamilton #701) and the aid of an electronic pump. Injections were optimized for the target with injection volumes of between 0.05 and 0.2 μl at 0.1 μl min^{-1}. The injection coordinates relative to Bregma were AP +0.34 mm, ML 0 mm, DV −4.8 and 5.2. A minimum of one week recovery was allowed before recording the EEG.

EEG and EMG Recordings, Scoring of Vigilance States and Power Spectrum Analysis

EEG and EMG were recorded from non-tethered animals using Neurologger 2A devices as described previously and electrodes placed at the same positions as our previous work in mice (Anisimov et al., 2014; Zhang et al., 2015). These positions were: AP +1.5 mm, ML −1.5 mm relative to Bregma, 1st − AP −1.5 mm, ML +1.5 relative to Bregma, 2nd Lambda −1.0 mm,

ML 0.0 mm. EMG wires were also implanted in the neck muscles. Data were recorded at a sampling rate of 200 Hz with four times oversampling. The EEG data analyzed using Spike2 software 7.18 (Cambridge Electronic Design, Cambridge, United Kingdom) or MATLAB (MathWorks, Cambridge, United Kingdom). Prior to sleep scoring the ECoG was digitally filtered (high-pass, 0.5 Hz, −3dB) and the EMG was band-pass filtered (5–45 Hz, −3dB). Power in the delta (1–4 Hz) and theta (6–9 Hz) bands was calculated, together with the RMS value of the EMG signal (averaged over 5 s), and these were used to define the vigilance states of wake, NREM and REM with an automatic script OSD7 v7.2 (in Spike2). Each vigilance state was then rechecked manually. We analyzed the sleep-state specific power spectrums following normalization to wake power within each mouse, as described previously (Ma et al., 2019).

Photometry Recordings

Photometry was performed using a 473-nm diode-pumped solid state (DPSS) laser with fiber coupler (Shanghai Laser and Optics century Co.) and adjustable power supply (Shanghai Laser and Optics century Co.), controlled by a Grass SD9 stimulator. A lock-in amplifier (SR810, Stanford Research Systems, CA, United States) drove the laser using a TTL signal at 125 Hz with an average power of 80 μW at the tip of the fiber. Using an optical fiber patch cord (Ø 200 μm, 0.22 NA, Doric Lenses) the light source passed through a fluorescence cube (FMC_GFP_FC, Doric Lenses) and then *via* a second optical patch cord (Ø 200 μm, 0.37 NA, Doric Lenses), was connected to the brain-implanted fiber *via* a ceramic sleeves (Thorlabs). The GCaMP6s output was then filtered at 500–550 nm (using a fluorescence cube) and passed to a photodiode (APD-FC, Doric Lenses) and amplified by the lock-in amplifier (time constant, 30 ms). The signal was recorded on a CED 1401 Micro box (Cambridge Electronic Design, Cambridge, United Kingdom) at 200 Hz using Spike2 software (Cambridge Electronic Design, Cambridge, United Kingdom). The maximum continuous recording length was 6 h. Photometry, EEG and EMG data were aligned offline using Spike2 and analyzed using this software or custom scripts in either MATLAB (MathWorks) or R scripts (R Core Team, 2020). Peak counting was performed using Spike2 (using peak mode), "Peaks" were counted when immediately followed by a decrease of at least the threshold amplitude (100 μV) and were outside the minimal interval between detections of 10 ms. For each transition, the photometry signal F was normalized to baseline using the function $\Delta F/F = (F-F_0)/F_0$, where F_0 is the baseline fluorescence prior to the transition. Data are presented as a percentage. Heatmaps are shown as Z-scores. Transitions coinciding with recording artifacts or large shifts in baseline (DC offset) were excluded.

Temperature Recordings

Core body temperatures were measured using an abdominally implanted temperature loggers (DSTnano, Star-Oddi, Herfølge, Denmark), sampling every 2 min, as described previously (Harding et al., 2018).

Immunohistochemistry

Mice were given pentobarbital (100 mg/kg body weight; *i.p.*), and transcardially perfused with 4% paraformaldehyde in phosphate-buffered saline (PBS), pH 7.4. Brains were removed and 40-μm-thick coronal sections cut using a Leica SM 2010R microtome. Staining was performed on free-floating sections, washed in PBS three times and permeabilized in PBS plus 0.4% Triton X-100 for 30 min, blocked by incubation in PBS plus 10% normal goat serum (NGS), 0.2% Triton X-100 for 1 h (all at room temperature) and subsequently incubated overnight with a 1:1000 dilution of anti-GFP polyclonal antibody (A-6455, ThermoFisher). Sections were washed three times in PBS before incubating with goat anti-Rabbit IgG (H + L) Secondary, Alexa Fluor® 488 conjugate (A-11034, ThermoFisher) for 2 h. Samples were then washed six times before mounting on Vectashield Antifade Mounting Medium with DAPI (H-1200, Vector Laboratories).

Statistics

Data collection were either randomized or performed in a counter-balanced manner. Data are represented as the mean ± SEM, unless otherwise stated. OriginPro 2017 was used for statistical analyses. For data that were not independent (where ANOVA was not appropriate) we employed either two-tailed or paired t-tests and then accounted for multiple comparisons using the Benjamini-Hochberg procedure at a false discovery rate of 5%. Mice were excluded from the analysis if the histology did not confirm AAV transgene expression in the MnPO/MPO area, or if the expression had spread beyond the target region. Investigators were not blinded to behavioral treatment groups.

RESULTS

Medial Preoptic Nitric Oxide Synthase 1 Neurons Are Most Active During NREM Sleep

We used calcium photometry to assess the sleep-wake activity of NOS1 neurons in the MPO area. *AAV-FLEX-GCaMP6s* was injected into the MnPO/MPO area of *Nos1-Cre* mice to generate *Nos1-MnPO/MPO-GCaMP6s* mice (**Figures 1A,B**). We then recorded calcium photometry signals from mice over 6 h while the mice behaved freely in their home cages. Many NOS1 neurons in the MnPO/MPO region were NREM sleep-active, having their highest calcium activity in NREM sleep with only sporadic activity during wakefulness. An example over a 6-min period of a transition to NREM sleep is shown in **Figure 1C**, alongside the raw photometry signal, delta power (1–4 Hz), spectrogram from 0 to 20 Hz, EEG, EMG and scored sleep state. During wakefulness only low-level calcium-induced fluorescence signal was seen (labeled "F" on the axis of **Figure 1C**), and peaks in the signal were rare. While occasional small peaks in the calcium signal occurred during wake, a specific increase in peak frequency in the calcium signal was associated with NREM sleep. This is shown as a raster plot for ten transitions in **Figure 1D** over a 6-min period and quantified in **Figure 1E**. Higher GCaMP6s signal levels and

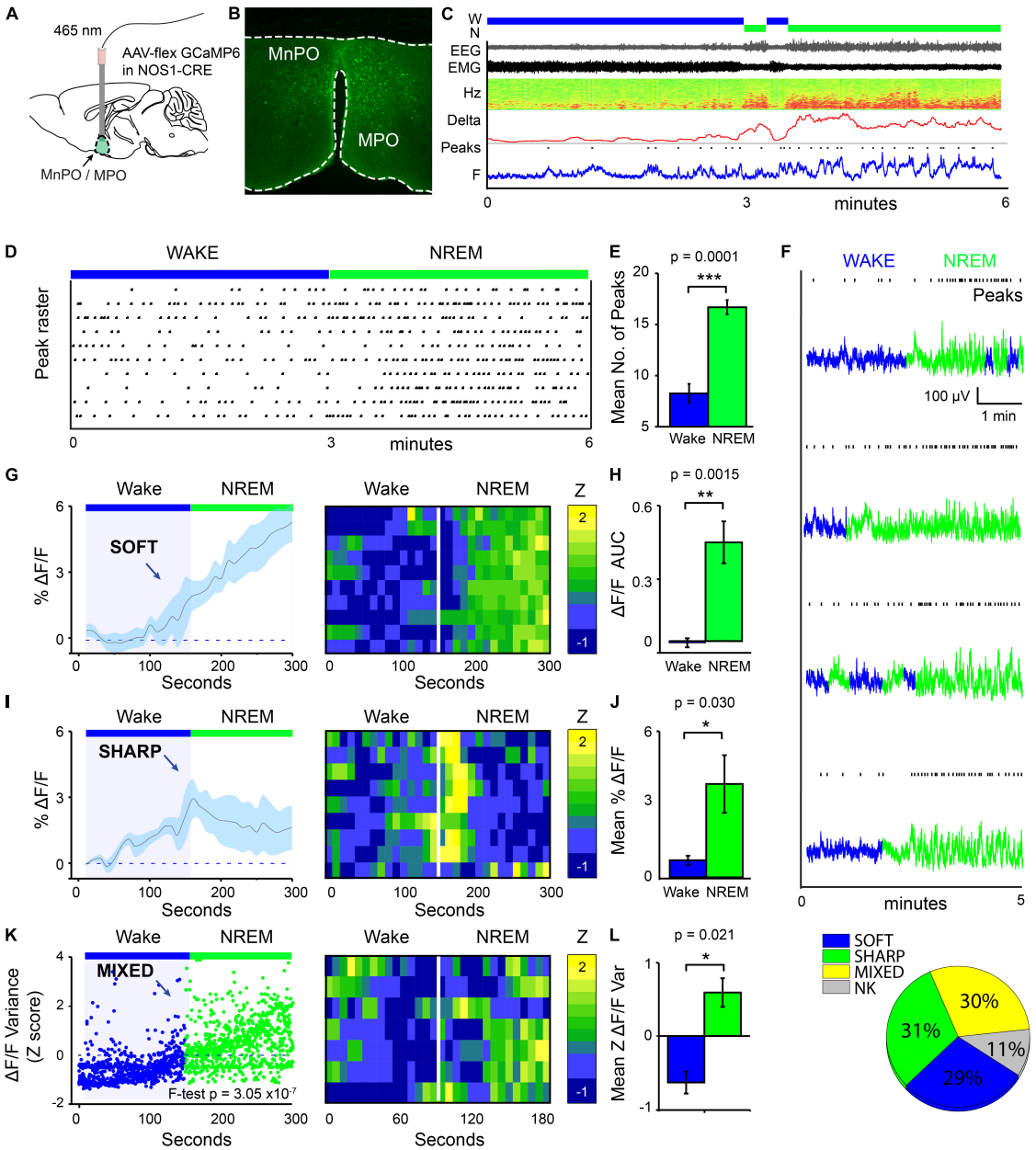

FIGURE 1 | MnPO/MPO hypothalamic NOS1 neurons are more active during sleep. Animals were recorded for 6 h across the light cycle from lights-off to lights-on to facilitate a distribution of sleep states. Transitions are shown over 300 s. **(A)** Schematic for the photometry recording at a 5-mm depth and an example of the GCaMP6 expression site in a *Nos1-MnPO/MPO-GCaMP6s* mouse. **(B)** Expression of GCaMP6s in neurons in the MnPO/MPO hypothalamus as detected by immunocytochemistry with GFP antisera. **(C)** Example transition from wake to NREM over a 6-min interval. Also shown are scored sleep states (Wake, W; NREM, N; REM, R), Filtered EEG and EMG, spectrogram of power in the frequency domain over time (Hz), Delta power (1–4 Hz) with a 5-s root mean square (RMS), raw photometry signal (labeled F) and automated peak counting on the photometry signal (Peaks). **(D)** A raster plot of automated spike counting from calcium photometry signals across wake to NREM transitions. **(E)** Area under the curve (ΔF/F) between wake and NREM for soft transitions in calcium signal (Paired *t*-test, $n = 5$, $p = 0.0001$). **(F)** Raw photometry data with paired automated peak counting for four example transitions over 5 min, colored by sleep state. Wake is shown in blue and NREM is shown in green. Peaks in calcium are marked above each trace. **(G)** The average ΔF/F in calcium signal for soft-type transitions that increase across wake-NREM transitions and ten example transitions represented as a heatmap. **(H)** ΔF/F Area under the curve between wake and NREM for soft transitions (Paired *t*-test, $n = 5$, $p = 0.002$). **(I)** The average ΔF/F for sharp-type increases in calcium signal in wake-NREM transitions before returning to baseline, followed by ten example transitions represented as a heatmap. **(J)** Mean ΔF/F of the calcium signal between wake (baseline) and NREM (peak) for sharp transitions (Paired *t*-test, $n = 5$, $p = 0.004$). **(K)** The *z*-score of ΔF/F variance for mixed-type transitions in calcium signal that increase across wake-NREM transitions (*F*-test of all pooled transitions, $p = 3 \times 10^{-7}$). Shown alongside ten example transitions in the calcium signal represented as a heatmap. **(L)** Quantification of variance in the ΔF/F calcium signal in across mice (Paired *t*-test, $n = 5$, $p = 0.021$). **(M)** Proportion of each transition in calcium signal type (soft increase, sharp increase, mixed increase) found in all wake to NREM transitions. Transitions in calcium signal that could not be classified are labeled NK. The Benjamini-Hochberg procedure was used to account for multiple comparisons at a false discovery rate of 5%. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

more frequent peaks occurred during NREM sleep. Four example photometry traces are shown in **Figure 1F** and color coded by sleep state. Peak counting is shown above each example.

On transitioning to NREM sleep the overall calcium levels in MnPO/MPO NOS1 neurons increased, as did the frequency of peaks in calcium signal. To quantify these changes in calcium signals, we averaged across multiple wake-NREM transitions from multiple mice. These averages contained several profiles: a slower "soft" rising transition in calcium signal with more peaks; and a faster "sharp" profile. Soft transitions from wake to NREM sleep are shown in **Figure 1G**, plotted as ΔF/F and averaged across 5 min of recording, alongside a heat map of ten example transitions. The ΔF/F calcium signal started to rise from the point of transition, and this continued for at least 150 s. This is quantified as the area under the curve in **Figure 1H**. On the other hand, sharp transitions in calcium-induced signals in MnPO/MPO NOS1 were different and anticipated the next NREM sleep transition. These sharp transitions are shown in **Figure 1I** as ΔF/F, alongside a heatmap of ten example transitions. Here, the ΔF/F signal started to rise up to 60 s prior to the start transition and peaked within 30 s of entering NREM sleep, before reducing again by 1 min post-transition. The heatmap shows that these calcium events were time-locked to the wake-NREM transitions. Within 100 s, the calcium signal had almost returned to baseline, despite continuous NREM. This was quantified as the mean amplitude between baseline and the maximum value in **Figure 1J**. Both the soft and sharp transitions in calcium signals had a slow time course that took more than 60 s to complete. The remainder of the calcium signals associated with the transitions from wake to NREM sleep of MnPO/MPO NOS1 neurons could not be classified into soft or sharp profiles. However, when these remaining calcium signals at the wake to NREM transitions were pooled and analyzed by variance, a clear association with the wake to NREM transitions was seen. These "mixed" transitions in calcium signals are shown in **Figure 1K**, plotted as a *Z* score of the ΔF/F variance, alongside a heatmap of ten example transitions. Here, the variance in calcium signal increased across the wake to NREM transitions in four of the five animals measured (**Figure 1L**). Overall, on moving from wake to NREM sleep, approximately 60% of changes in the calcium signal of MnPO/MPO NOS1 were either soft or sharp increases in signal, and 30% were mixed. Approximately 10% of the recordings of calcium signals did not show changes in activity during wake to NREM transitions (**Figure 1M**).

In addition to the increases in calcium signals of MnPO/MPO NOS1 neurons on transition from wake to NREM, we also looked at their calcium signals during transitions from NREM to wake and from NREM to REM sleep (**Figure 2**). We found that NREM sleep was sometimes interrupted with short bouts of wake episodes lasting about one minute (micro-wakes). In these short transitions, a small decrease in the calcium signal of MnPO/MPO NOS1 neurons was seen on entry into wakefulness that continued to decline until the next NREM episode and the calcium signal increased once more (**Figures 2A,B**). In this case, while we noted some clear examples, as shown in **Figure 2C**, there were also large variations between animals and there was insufficient statistical power to infer if these small decreases in calcium signal were significant (**Figure 2B**). The calcium signals in MnPO/MPO NOS1 neurons during the transitions from NREM to REM are shown in **Figure 2D** and shown alongside a heatmap of nine example transitions. As described above, once NREM has commenced the calcium signal tends to decay to a lower baseline (**Figure 1I**). At the transitions of NREM to REM, the average ΔF/F calcium signal remained at a low baseline for at least 30 s into the REM bout (red bar in **Figure 2D**). Following this there was an increase in the signal and plateau that lasted approximately 60 s (**Figures 2D,E**), which continued into micro-wake bouts before reentry into NREM (see examples **Figure 2F**).

Medial Preoptic Nitric Oxide Synthase 1 Neurons Influence Sleep-Wake Structure

Having established that many NOS1 neurons in the MnPO/MPO area are more active during NREM and REM sleep than they are during wake, we next examined their contribution to sleep structure. To do this we reduced synaptic transmission from these cells, using Cre-dependent expression of tetanus-toxin light-chain (GFP-TeLC) (**Figure 3A**). Tetanus-toxin light chain blocks release of neurotransmitter vesicles by cleaving synaptobrevin, a synaptic vesicle protein (Schiavo et al., 1992). *AAV-FLEX-GFP-TeLC* or *AAV-FLEX-GFP* were injected into the MnPO/MPO of *Nos1-Cre* mice to generate *Nos1-MnPO/MPO-GFP-TeLC* and control *Nos1-MnPO/MPO-GFP* mice, respectively (**Figure 3B**). Reducing synaptic transmission from NOS1 neurons produced small but significant alterations to the structure of sleep (**Figure 3C**). These data are quantified for each mouse. Average wakefulness was reduced by almost 25% during the light phase, with a corresponding increase in NREM of approximately 10%. This was followed by an approx. 15–20% decrease in NREM during lights OFF. No clear changes were seen in REM sleep time.

We assessed whether changes in sleep structure seen in *Nos1-MnPO/MPO-GFP-TeLC* mice affected sleep episode dynamics and/or transitions (**Figure 3D**). There were no changes in the overall number of episodes in wake or NREM sleep for either the light or dark phase of the cycle; however, there was an approximately 45% reduction in REM episodes in the dark phase (**Figure 3D**). Consistent with this result, the number of NREM-REM and REM-wake transitions, but not transitions between wake and NREM sleep, were reduced by approximately 40%. No changes were seen in the light phase (**Figures 3E,F**). Although this was not reflected in the REM sleep amount, it was consistent with less NREM in the dark phase. In addition, we expected the remaining REM sleep to be consequently more consolidated.

Because we did not observe an overall change in the number of wake or NREM episodes during the light or dark phase, we looked at the episode length and number of vigilance states to see if this explained the differences seen in the time spent sleeping (**Figure 4**). In *Nos1-MnPO/MPO-GFP-TeLC* mice we observed reductions of approximately 50% in the frequency of the longest wake episodes (>20 min) in the light phase (**Figure 4A**), although no changes were seen in episode length and number for NREM and REM sleep (**Figures 4B,C**). In the dark phase, the average values for wake did not change, and the data had

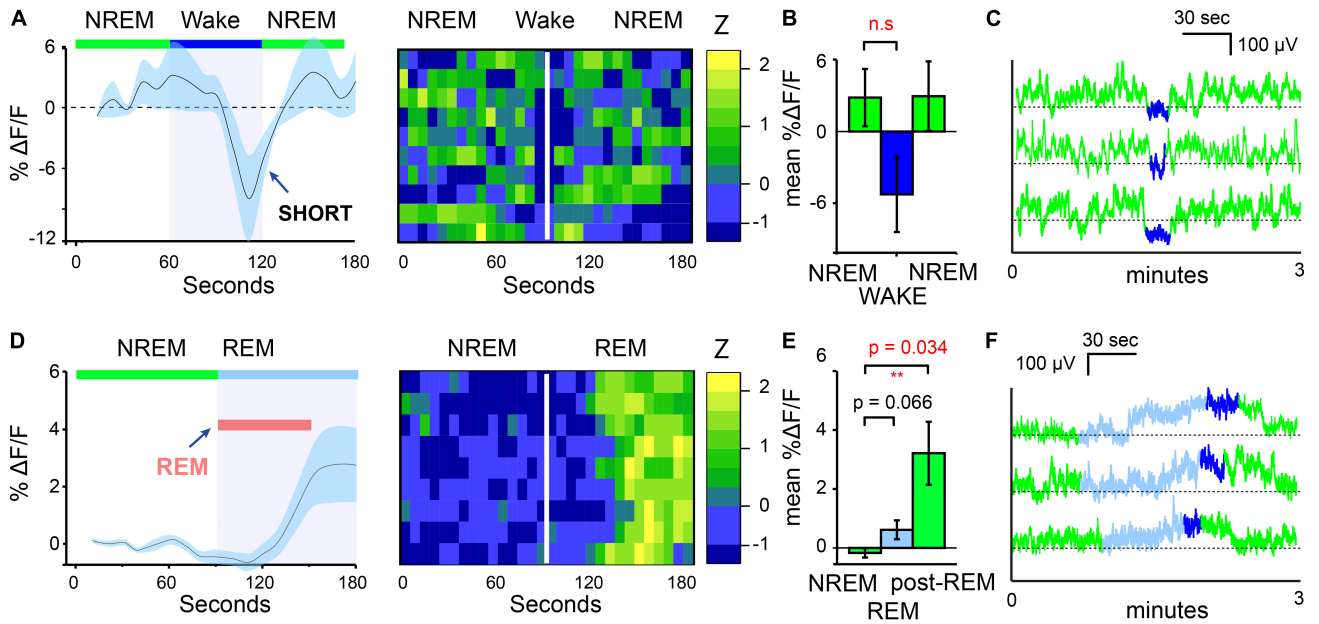

FIGURE 2 | MnPO/MPO hypothalamic NOS1 neurons are active during the later part of REM episodes. Animals were recorded for 6 h across the light cycle from lights-off to lights-on to facilitate obtaining a distribution of sleep states. Transitions are shown over 180 s. **(A)** The average ΔF/F in calcium signal when NREM is interrupted by short bouts of wake. Wake length here is approximated and followed by ten example transitions, represented as a heatmap. **(B)** Mean change in ΔF/F calcium signal between brief wakefulness, prior-NREM and post-NREM for short transitions (Paired *t*-test, $n = 4$, prior-NREM vs. brief wakefulness, $p = 0.063$, post-NREM vs. brief wakefulness $p = 0.076$). **(C)** Examples of NREM to wake transitions from raw photometry data colored by sleep-state (NREM, green; REM, cyan and wake, blue). **(D)** The average ΔF/F for NREM to REM transitions showing no clear changes in signal with a slower increase after approximately 30 s alongside a heat map of nine transitions from NREM to REM sleep. **(E)** Mean change in ΔF/F calcium signal between the end of a NREM period and the later part of a REM episode (Paired *t*-test, $n = 5$, $p = 0.066$) and NREM and post-REM (Paired *t*-test, $n = 5$, $p = 0.034$). **(F)** Examples of calcium signals during NREM to REM to microwake to NREM transitions, colored by sleep-state (NREM, green; REM, cyan; wake, blue). $^{**}P < 0.01$, n.s, not significant.

larger variance (**Figure 4D**). However, while *Nos1-MnPO/MPO-GFP* control mice had a reduced frequency of episodes, >20 min in the dark phase compared with the light phase, the *Nos1-MnPO/MPO-GFP-TeLC* mice instead had an increased frequency of these episodes. From calculating the paired difference for each mouse between the light and dark phase, it was clear that *Nos1-MnPO/MPO-GFP-TeLC* mice were more affected by the light change (**Figure 4D**, inset graph). No further alterations were seen in episode length and number for NREM and REM sleep during lights OFF (**Figures 4E,F**).

Medial Preoptic Nitric Oxide Synthase 1 Neurons Contribute to Theta Power During REM Sleep

We analyzed the sleep-state specific power spectra of *Nos1-MnPO/MPO-GFP-TeLC* mice compared with *Nos1-MnPO/MPO-GFP* control mice, following normalization to WAKE power within each mouse. During the lights-on phase, NREM sleep was not associated with changes in power (**Figures 5A,B**); however, REM sleep did show significant changes (**Figure 5C**). Specifically, there was an increase in delta power, normally associated with NREM sleep, of approximately 30%, as well as a corresponding decrease in theta power (**Figure 5D**). This difference was in the 2–4 Hz range of delta power referred to as the δ2 band (Hubbard et al., 2020). Theta (6–9 Hz) power was reduced by approximately 20% but no changes in the higher frequencies (10–14 Hz) were seen. During the dark phase (**Figures 5E,F**), NREM showed an approximately 15% reduction in theta power. This contrasted with no change in this band during the light phase. For REM sleep in the dark phase (**Figures 5G,H**), differences between *Nos1-MnPO/MPO-GFP-TeLC* and *Nos1-MnPO/MPO-GFP* mice mirrored those seen in the light phase, with an approximately 25% increase in δ2 power as well as a 20% decrease in theta power.

Medial Preoptic Nitric Oxide Synthase 1 Neurons Reduce Body Temperature

Using implanted temperature loggers, we measured the core body temperature at 2-min resolution in *Nos1-MnPO/MPO-GFP-TeLC* and *Nos1-MnPO/MPO-GFP* mice (**Figure 6**). We produced a typical 24-h period in temperature change by first averaging over 7 days for each mouse (5040 measurements) before comparing distributions across groups. The temperature distribution of *Nos1-MnPO/MPO-GFP-TeLC* mice shifted to warmer temperatures compared with those of control mice (**Figure 6A**). The cumulative distribution illustrated that the most significant change was in the probability of observing core temperature between 35.5 and 36°C, but without a change in the minimum or maximum temperatures (**Figure 6B**). Furthermore, during the

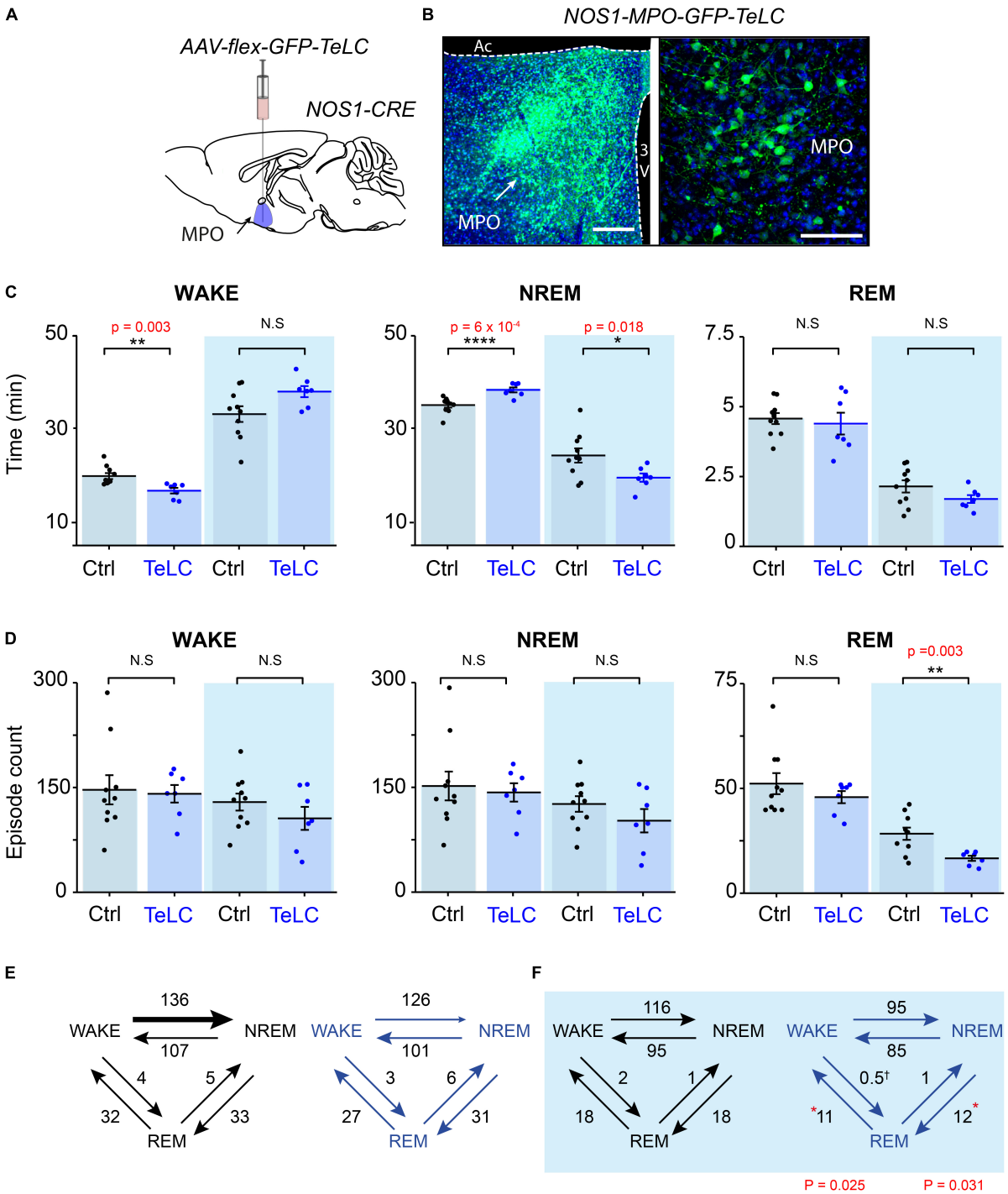

FIGURE 3 | Reducing transmitter release from NOS1 neurons in MnPO/MPO hypothalamus alters sleep amounts and the number of sleep episodes in a manner dependent on the light-dark cycle. **(A)** Schematic of the stereotaxic injection of *AAV-flex-GFP-TeLC* into the MnPO/MPO area of *Nos1-CRE* mice to generate *Nos1-MnPO/MPO-GFP-TeLC* mice. **(B)** Example histology from MnPO/MPO showing expression of the GFP-TeLC protein as detected with a GFP antibody; left picture, lower magnification view, scale bar is 200 μm, right picture, higher magnification view, scale bar 100 μm. **(C)** Quantification of sleep states for each mouse in the 12-h light or dark periods shown as average time in vigilance state per hour. Wakefulness in the light phase (two-tailed *t*-test, $n = 7$ and $n = 10$, $p = 0.003$), NREM in the light phase (two-tailed *t*-test, $n = 7$ and $n = 10$, $p = 0.0006$), NREM in the dark phase (two-tailed *t*-test, $n = 7$ and $n = 10$, $p = 0.018$). **(D)** The number of episodes of wake, NREM and REM between light and dark. REM in the dark phase (two-tailed *t*-test, $n = 7$ and $n = 10$, $p = 0.003$). **(E)** Analysis of sleep transitions between sleep states in the light phase. No differences were observed between groups. **(F)** The transitions between *Nos1-MnPO/MPO-GFP-TeLC* mice and *Nos1-MnPO/MPO-GFP* mice in the dark phase. Transitions from NREM to REM ($p = 0.025$) and from REM to wake ($p = 0.031$), from two-tailed *t*-test, $n = 7$ and $n = 10$. Multiple comparisons were accounted for using the Benjamini-Hochberg procedure at a false discovery rate of 5%. $^*P < 0.05$, $^{**}P < 0.01$, N.S, not significant.

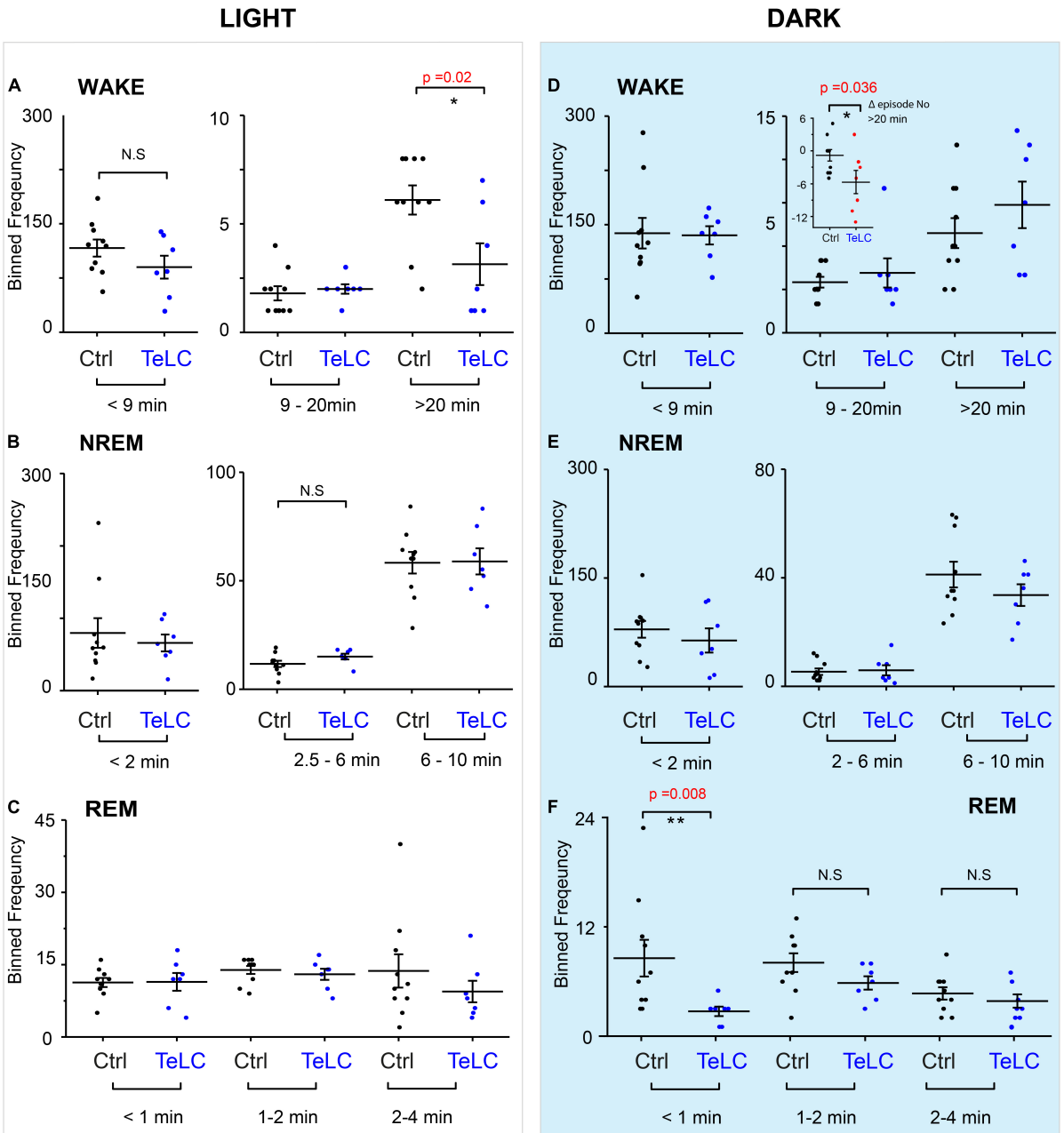

FIGURE 4 | Distribution of sleep episode lengths is reduced for *Nos1-MnPO/MPO-GFP-TeLC* mice during wakefulness in the light period but increased in wakefulness in the dark period. **(A)** Wakefulness in the light period binned by episode length. No differences are seen in bins less than 20 min. Episodes greater than 20 min in *Nos1-MnPO/MPO-GFP-TeLC* mice ($n = 7$ TeLC and $n = 10$ GFP, $p = 0.02$). **(B)** NREM in the light period binned by episode length. No differences are seen in bins less than 10 min ($n = 7$ TeLC and $n = 10$ GFP). **(C)** Light phase REM. No differences are seen in bins less than 4 min ($n = 7$ TeLC and $n = 10$ GFP). **(D)** Dark phase wakefulness. No differences are seen between groups within each light cycle ($n = 7$ TeLC and $n = 10$ GFP). Inset graph, between the light periods *Nos1-MnPO/MPO-GFP* and *Nos1-MnPO/MPO-GFP-TeLC* groups as a paired difference (d, inset graph, $n = 7$ TeLC and $n = 10$ GFP, $p = 0.036$). **(E)** Dark phase NREM. No differences are seen in bins less than 10 min ($n = 7$ TeLC and $n = 10$ GFP). **(F)** Dark phase REM. No differences are seen in bins greater than 1 min. Episodes of less than 1 min are significant between *Nos1-MnPO/MPO-GFP-TeLC* and *Nos1-MnPO/MPO-GFP* mice ($n = 7$ TeLC and $n = 10$ GFP, $p = 0.008$). Multiple comparisons were accounted for using the Benjamini-Hochberg procedure at a false discovery rate of 5%. $^*P < 0.05$, $^{**}P < 0.01$, N.S, not significant.

dark phase, while *Nos1-MnPO/MPO-GFP* control mice dropped their core temperature, *Nos1-MnPO/MPO-GFP-TeLC* control mice did not, both during the middle of the dark phase and prior to the next lights-on period (**Figures 6C,D**); furthermore, during the siesta period *Nos1-MnPO/MPO-GFP-TeLC* mice spent 36% less total time in NREM sleep compared with control mice, although there were no significant changes in NREM sleep episode count or mean length (**Figure 6E**).

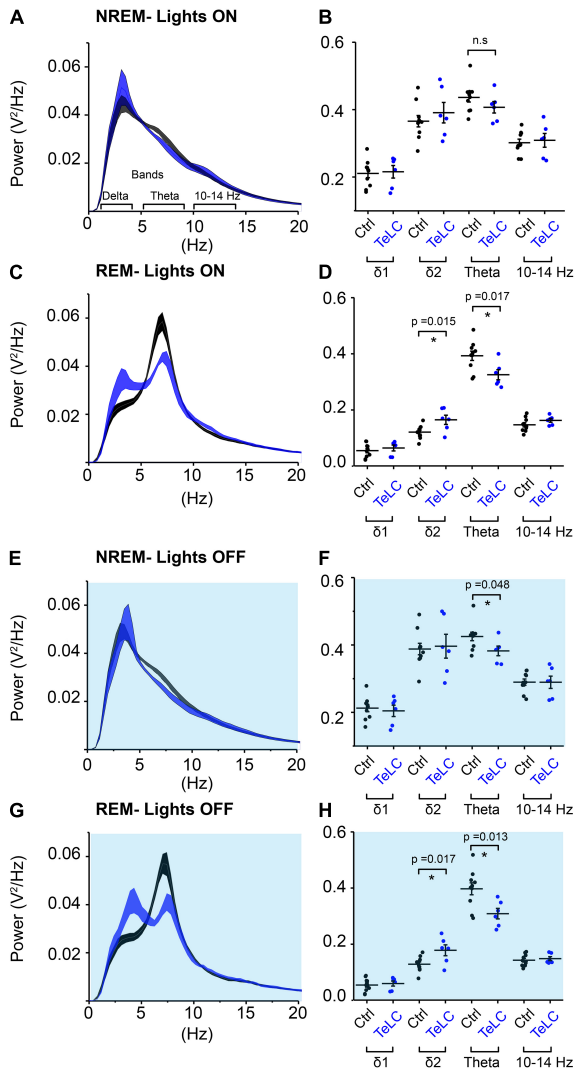

FIGURE 5 | NOS1 neurons in MnPO/MPO contribute to theta power during REM sleep. **(A)** Normalized power for NREM during the light period shown as a power spectrum. Different frequency bands are also illustrated. **(B)** For NREM during the light period, data for individual mice are shown for relevant frequency bands. **(C)** REM during the light period. **(D)** Quantification of spectral differences during REM in the light period for individual mice show more δ2 in the TeLC condition (*t* test, $n = 6$, TeLC; $n = 10$, GFP; $p = 0.015$) alongside reduced theta (*t* test, $n = 6$, TeLC; $n = 10$, GFP; $p = 0.017$). **(E)** Normalized power for NREM in the dark period shown as a power spectrum. **(F)** Data for individual mice are shown for relevant frequency bands (*t* test, $n = 6$, TeLC; $n = 10$, GFP; $p = 0.048$). **(G)** REM during the dark period. **(H)** Quantification of spectral differences during REM in the light period for individual mice (δ2 between groups, *t* test, $n = 6$, TeLC; $n = 10$, GFP; $p = 0.017$). Theta between groups (*t* test, $n = 6$, TeLC; $n = 10$, GFP; $p = 0.013$). Multiple comparisons were accounted for using the Benjamini-Hochberg procedure at a false discovery rate of 5%. $^*P < 0.05$, n.s, not significant.

DISCUSSION

In this study we have discovered that many NOS1 cells in the midline PO hypothalamus are naturally sleep-active, although there seemed to be several different categories of responses and probably several different types of cell. Based on their calcium signals, the fast and transient "sharp" activations of these NOS1 cells from wake to NREM and the slower, prolonged "soft" transitions from wake to NREM may represent two populations of sleep-active NOS1 neurons. However, the cells are not active throughout NREM sleep, but instead are most active at the transitions from wake to NREM and the subsequent first part of NREM. They also become active during the later parts of REM sleep. The "sharp transition" subgroup is more likely to play a role in sleep onset, whereas the other groups appear to be following with their activity after NREM and REM sleep are established. The cells are not silent during wake periods, but their activity is intermittent. A minority (10%) of these NOS1 cells show no change in their calcium signals at the transitions of the vigilance states, again suggesting subtypes of cells. We used TeLC expression in NOS1 neurons to disrupt their synaptic activity, which in turn disrupted the sleep-wake profile of the mice in a manner that varied with the light or dark phases of the 24-h cycle. In the dark phase, mice with TeLC expressed in MnPO/MPO NOS1 neurons showed a reduction of time in NREM sleep and a loss of the shortest REM episodes; NREM to REM and REM to wake transitions were also reduced. REM sleep was accompanied by increased delta power and decreased theta power, possibly suggesting functional disruption of REM sleep. In the lights-on phase, however, there was an increase in NREM sleep, but REM sleep was unchanged. Overall, the mice were chronically warmer.

Our new results are consistent with our previous work on these cells. We have previously shown that a subset of MnPO/MPO glutamate/NOS1 neurons, when activity-tagged following an external warm-stimulus to the mice, could on reactivation induce NREM sleep and concomitant body cooling (Harding et al., 2018). Similarly, a GABAergic MnPO/MPO population, tagged in the same manner, could only induce sleep (Harding et al., 2018). As we did not observe overlap in these populations by immunohistochemistry, we suggested a model of external warmth-triggered sleep with a NOS1/glutamate (MnPO/MPO) population signaling to a downstream GABAergic population in MPO (Harding et al., 2020). Thus MnPO/MPO NOS1 neurons can sense changes in temperature although we do not know if this is direct sensing or through afferents from the skin. In *Nos1-MnPO/MPO-GFP-TeLC* mice, contrary to the expectation that NREM sleep would be unchanged or reduced during the light phase, these mice had an increase in the light phase, and a subsequent reduction in this state during the dark phase. So, it is possible that these changes in sleep-wake states result from the altered thermoregulation, or the effect is complex because of the likely multiple subtypes of cell. The effects on REM sleep (selective for the dark phase) were unanticipated, but perhaps not surprising given that REM sleep is partly controlled by unknown cell types in the MPO area (Suntsova and Dergacheva, 2004; Gvilia et al., 2006), and we have presumably influenced a NOS1 cell subtype involved in REM production. Alternatively, the reduction in REM sleep when NOS1 neurons are blocked might be linked to the reduction of NREM sleep, which both happen in the dark phase, and not as a result of a

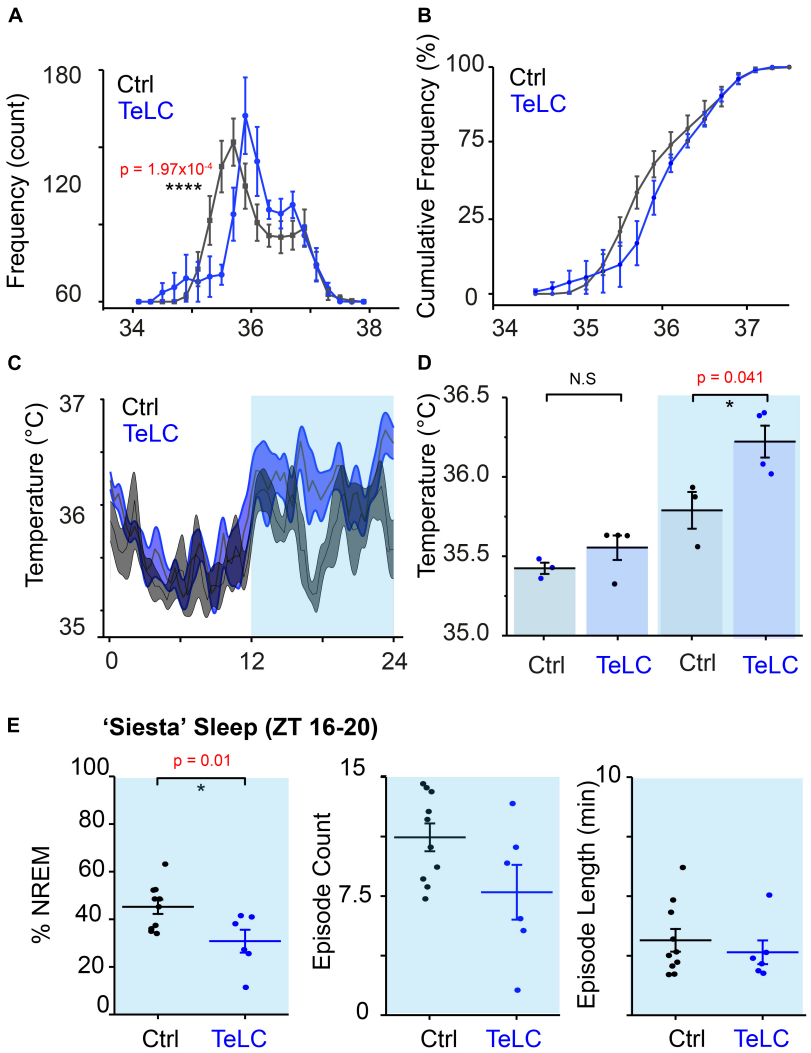

FIGURE 6 | NOS1 neurons in MnPO/MPO hypothalamus act to reduce body temperature. **(A)** Aggregated temperature data from 7 days of recording for each mouse averaged by ZT time at 2-min intervals to produce a 'typical' day histogram, averaged for each group (rmANOVA, $n = 4$, TeLC; $n = 6$, GFP; temperature × group at 35.4–35.6°C, $p = 1.97 \times 10^{-4}$). **(B)** Cumulative frequency of temperature distribution. **(C)** Temperature profile over 24 h. **(D)** Quantification of 24-h temperature profile in the light and dark phase (two-tailed t test assuming unequal variance, $n = 4$, TeLC; $n = 3$, GFP; $p = 0.041$). **(E)** Percentage sleep and episode count and episode length a 'siesta' period between ZT 16–20 (two tailed t-test, $n = 7$ and $n = 10$, $p = 0.01$). *$P < 0.05$, N.S, not significant.

REM-specific mechanism. This idea is consistent with a lower theta power during REM, which may indicate reduced REM sleep propensity.

Initially, given that *nos1* gene expression in the MPO hypothalamus has a highly restricted expression pattern, as detected by both *in situ* hybridization and immunohistochemistry (see e.g., **Figure 4E** in Harding et al., 2018), we anticipated that *nos1* expression would be a pragmatic and useful marker for functional manipulation of a unique subset of cells. Unfortunately, this has turned out not to be the case. While many of the NOS1 neurons in MPO studied by calcium photometry have clear sleep-active patterns, it has become apparent since we started our work that multiple subtypes of NOS1 neuron exist in the PO area, including NOS1/VGLUT2, NOS1/VGAT, NOS1/galanin neurons and others (Moffitt et al., 2018). The bidirectional changes in sleep when TeLC is expressed in MnPO/MPO NOS1 neurons likely reflect the reduced synaptic transmitter release from multiple subtypes of NOS1 cell in MnPO/MPO. For example, activation of glutamate (VGLUT2) neurons in the PO area induces wakefulness (Vanini et al., 2020), so if this particular subset were to express the *nos1* gene, TeLC expression in them might reduce wakefulness; on the other hand, we have shown previously that *nos1*-expressing GABA cells induce NREM sleep (Harding et al., 2018); thus TeLC expression in NOS1 cells might promote wakefulness (Harding et al., 2018). Further progress to dissect this circuitry requires intersectional genetics. Nevertheless, it remains striking that the majority of MnPO/MPO NOS1 cells have most of their activity during the transitions from wake to NREM sleep, and during the later parts

of REM sleep episodes. Targets for NOS1 neurons could include GABAergic and galaninergic neurons in the LPO area that are involved in NREM sleep induction and maintenance (Kroeger et al., 2018; Ma et al., 2019), as well as uncharacterized long-range targets. Although we always used the same coordinates, we did not attempt to distinguish NOS1 cells in the small and neighboring MnPO and MPO areas.

Expressing TeLC in MnPO/MPO NOS1 neurons raised the average body temperature of the mice. This would be consistent with effects on temperature mediated by BDNF/PACAP or TRPM2 expressing neurons in the MPO area (Song et al., 2016; Tan et al., 2016; Harding et al., 2018); these neurons could co-express NOS1. There are also glutamatergic wake-promoting neurons in the PO that are associated with mild body cooling of approximately 1°C that could also have a role (Vanini et al., 2020), and may also express the *nos1* gene. However, unlike the effects on temperature produced by BDNF/PACAP or TRPM2 cells, the increases we see appear to be associated with the light phase of the dark-light cycle, specifically in the siesta period (ZT16-20) and the period before the dark-to-light transition. Thus, MnPO/MPO NOS1 cells are driving down temperature at the same time as NREM sleep is initiated, consistent with our earlier work (Harding et al., 2018). Overall, this may support a larger hypothesis on optimization of sleep for energy reallocation (Harding et al., 2020).

In summary, we have found that the activity pattern of some MnPO/MPO NOS1 cells is quite striking, being rather selective at the boundary between wake to NREM transitions and the later part of REM sleep, and that synaptic transmission from PO NOS1 neurons likely contributes to NREM and REM sleep organization, as well as chronic body cooling. We write "likely" because we have not formally shown that TeLC expression reduced transmitter release in these neurons, and we have not identified post-synaptic targets of MnPO/MPO NOS1 cells. We currently think that MnPO/MPO NOS1 neurons probably have both short local outputs and long-range connections where transmitters could be released. A further caveat is that NO itself is likely to be part of the signaling system from these cells. We did not address this because NO release from cells is independent of vesicle release. But as NOS1 synthase is calcium-dependent (Knowles and Moncada, 1994), periods of elevated calcium seen in NOS1 neurons at the wake to NREM transitions and during NREM sleep will result in NO release from these cells, and NO could well be influencing sleep structure and temperature regulation. We further speculate that NOS1 neurons, possibly using NO release, may have a role in controlling vasodilation specifically in the context of sleep. We should bear in mind that we have only looked at male mice, and because the PO area is sexually dimorphic, NOS neurons could differ in their effects between the sexes. Given the rather precise calcium activity of some MnPO/MPO NOS1 cells at the boundary of wake to NREM transitions, further dissection will likely reveal part of a regulatory circuit controlling sleep induction/maintenance and the simultaneous lowering of body temperature.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The animal study was reviewed and approved by Imperial College AWERB Committee and United Kingdom Home Office.

AUTHOR CONTRIBUTIONS

WW, NF, and EH conceived the project. EH with input from WW and NF designed the experiments. EH, WB, RZ, XY, RY, and LL performed the experiments. RZ and LL were supervised by EH for this work. BH and TC provided technology for EEG recordings. FM helped with the manuscript and data analysis. AV provided the Neurologgers. EH performed the data analysis and produced the figures. NF and WW contributed to the data analysis and supervised the project. EH with NF and WW wrote the manuscript. All authors contributed to the article and approved the submitted version.

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