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Article

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Glutamatergic neurons in the lateral periaqueductal gray mediate wakefulness and REM sleep through different pathways

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Abstract

The lateral periaqueductal gray (LPAG) is essential for coordinating active and passive defensive behaviors which rely on heightened arousal, but its impact on sleep–wake regulation remains unknown. Here, by using targeted recombination in active populations transgenic mouse tool along with neuroanatomical approaches, we first show that two different populations of glutamatergic neurons are activated during wakefulness and rapid eye movement (REM) sleep in the LPAG. Fiber photometry showed that most LPAG vesicular glutamate transporter 2 (Vglut2) neurons are preferentially active during wakefulness. Chemogenetic and optogenetic activation of LPAG$^{Vglut2}$ neurons strongly enhanced arousal associated with immobility. The wakefulness- and immobility-promoting effects of LPAG$^{Vglut2}$ neurons are mediated by their projections to the locus coeruleus and ventral gigantocellular reticular nucleus, as supported by optogenetic manipulations. In contrast, chemogenetic inhibition of LPAG$^{Vglut2}$ neurons reduced REM sleep and increased non-REM sleep. Most LPAG neurons activated during REM sleep hypersomnia and showed descending projections to the sublaterodorsal tegmental nucleus. These findings revealed that two different LPAG$^{Vglut2}$ populations of neurons and circuits induce wakefulness associated with immobility and REM sleep.

Keywords: lateral periaqueductal gray; locus coeruleus; rapid eye movement sleep; sublaterodorsal tegmental nucleus; ventral gigantocellular reticular nucleus; wakefulness
Introduction

When confronted with dangerous situations, animals, including humans, use several active and passive coping strategies that rely on defensive behaviors\(^1\)\(^2\). Active defensive responses, such as fight-or-flight reactions, rely on heightened arousal and skeletal muscle activation, which allows the animal to remain alert. In addition, passive defensive responses, including quiescence and freezing, also depend on wakefulness\(^3\). Arousal is the first, necessary step in the activation of the defense cascade in both animals and humans\(^4\). Recent studies showed that the lateral periaqueductal gray (LPAG) is the midbrain structure essential for coordinating the active and passive defensive reactions to threatening stimuli\(^5\)\(^6\)\(^7\). However, whether and how the LPAG participates in sleep–wake regulation remains unclear.

Previous clinical and animal studies showed that LPAG has multiple functions, including defensive behaviors, pain and analgesia, cardiovascular control, breathing, maternal behaviors, and reward\(^8\)\(^9\)\(^10\)\(^11\)\(^12\)\(^13\). The LPAG ensures survival by generating an appropriate response to threats and mediates the fundamental, rigid motor modes of flight or fight, e.g., attack, running, treading, and/or burying\(^14\). Activation of the efferent projections from the lateral parabrachial nucleus to the LPAG drives the escape behaviors\(^15\). Moreover, the auditory cortex controls noise-evoked escape through a direct excitatory projection to LPAG glutamatergic neurons\(^16\). A preferential projection from the vesicular glutamate transporter 2 (Vglut2) neurons of the ventromedial hypothalamus to the LPAG\(^{Vglut2}\) neurons is selectively used during the attack, and it exhibits short-latency and time-locked spikes associated with jaw muscle activity.
during biting. Vesicular gamma-aminobutyric acid (GABA) transporter neurons in the LPAG are required to detect, chase and attack the prey, while LPAG\textsuperscript{Vglut2} neurons are selectively used for the attack in the mouse hunting behavior. These studies suggest that LPAG may have a key role in the defensive system, which relies on heightened arousal. Furthermore, at the neuroanatomical level, the LPAG has functional connections with the central amygdala, hypothalamus, medial prefrontal cortex, lower brainstem, and spinal cord, which are crucial to the sleep–wake cycle. A substantial number of c-Fos positive neurons were observed in the LPAG after REM sleep deprivation and hypersomnia. In contrast to neurons localized in the ventrolateral periaqueductal gray (VLPAG), which are mainly GABAergic, the LPAG neurons do not express glutamate decarboxylase and are therefore not GABAergic. Therefore, we hypothesized that the glutamatergic neurons in the LPAG might play an important role in sleep–wake regulation.

In the present study, by using targeted recombination in active populations (TRAP) transgenic mouse tool and c-Fos immunostaining with in situ hybridization techniques, we observed that two different populations of glutamatergic neurons in the LPAG are active during wakefulness and REM sleep. Then, fiber photometry was used to determine the LPAG glutamatergic neurons activity across the spontaneous sleep–wake cycle. In vivo and in vitro optogenetic and chemogenetic approaches, along with polysomnographic recordings, were used to assess the role of LPAG glutamatergic neurons in wakefulness and rapid eye movement (REM) sleep. Patch-clamp recordings combined with optogenetics were used to investigate the functional connections.
between LPAG glutamatergic neurons and neurons in the locus coeruleus (LC), ventral gigantocellular reticular nucleus (GiV), and sublaterodorsal tegmental nucleus (SLD).

Our results revealed that LPAG glutamatergic neurons differential projections are essential for wakefulness associated with immobility and REM sleep.
Results

The glutamatergic neurons in the LPAG are activated during wakefulness and REM sleep hypersomnia. TRAP2 transgenic mice were used to identify the LPAG neurons that are activated during wakefulness and REM sleep. After 4-hydroxytamoxifen (4-OHT) injection (Supplementary Fig. 1a), tdTomato labeling (so-called TRAPing) was induced in activated neurons during wakefulness or REM sleep. Neurons activated during wakefulness or REM sleep immediately before perfusion expressed c-Fos. Under three conditions (wake-wake, wake-REM sleep rebound [RSR], and RSR-PSR), we investigated the distribution of tdTomato+ and c-Fos+ neurons in the LPAG (Supplementary Fig. 1c, d). The average numbers of tdTomato+ and c-Fos+ neurons in the LPAG of wake-wake mice were 45 ± 4 and 62 ± 8, respectively (Supplementary Fig. 1e), whereas the average number of double-labeled (tdTomato+ and c-Fos+) cells was 16 ± 1. This accounts for approximately 34.7% of the tdTomato+ neurons and 25.3% of the c-Fos+ neurons (Supplementary Fig. 1e). In the wake-PSR mice, the numbers of tdTomato+ cells and c-Fos+ cells in the LPAG were 46 ± 2 and 40 ± 1, respectively (Supplementary Fig. 1e); in this group, the number of double-labeled (tdTomato+ and c-Fos+) neurons was 8 ± 1, which accounted for 21.21% of the tdTomato+ cells and 18.22% of the c-Fos+ cells (Supplementary Fig. 1e). In the PSR-PSR animals, an average of 47 ± 5 tdTomato+ and 48 ± 3 c-Fos+ cells were observed in the LPAG. The number of double-labeled neurons was 9 ± 1, which accounted for 21.21% of the mCherry+ cells and 18.22% of the c-Fos+ neurons (Supplementary Fig. 1e). These findings suggest that the neurons
activated during wakefulness and REM sleep belong to two different populations, excepting 20% of the neurons which are activated during both states.

Then, to determine the distribution and number of LPAG glutamatergic neurons activated during REM sleep deprivation and rebound periods, we quantified the number of neurons that were immunopositive for c-Fos and Vglut2 in all PAG subdivisions in control (RSC), REM sleep-deprived (RSD), and RSR rats (Fig. 1a). The LPAG contained a higher number of c-Fos-Vglut2 double-labeled neurons in RSD and RSR animals compared to RSC ones (Fig. 1b–g). The numbers of c-Fos cells were significantly larger in the LPAG of RSD (65.50 ± 9.10) ($t = 7.762$) and RSR (61.25 ± 10.09) ($t = 8.114$) animals than in RSC animals (Fig. 1g). The double-labeled neurons in RSD and RSR rats constituted 49.35% and 47.83%, respectively, of the c-Fos+ neurons detected in the LPAG (Fig. 1g). These results indicate that half of the neurons localized in the LPAG that are activated during RSD and RSR are glutamatergic.

Previous studies showed that RSD animals spent significantly less time in REM sleep and more time in wakefulness than the RSR and RSC animals$^{19}$; therefore, our results combined with those obtained in the TRAP mice indicate that two different populations of glutamatergic neurons in the LPAG are activated during wakefulness and REM sleep.

**Population activity of LPAG$^{\text{Vglut2}}$ neurons is enhanced during wakefulness.** To study the real-time physiological activities of LPAG glutamatergic neurons during spontaneous sleep–wake cycles, we recorded the LPAG$^{\text{Vglut2}}$ neuron calcium activity using fiber photometry in mice. Cre-dependent adeno-associated virus (AAV)
expressing the fluorescent calcium indicator GCaMP6f (AAV-Ef1α-DIO-GCaMP6f) was unilaterally injected into the LPAg of Vglut2-Cre mice, and an optical fiber was implanted into the LPAg (Fig. 2a). GCaMP6f signals and electroencephalography (EEG)/electromyography (EMG) were simultaneously recorded in freely moving mice within their home cages. We found that most GCaMP6f-expressing cells were located in the LPAg (Fig. 2b). Changes in the activity of the LPAg\(^{Vglut2}\) neurons were significantly and consistently increased during wakefulness compared to REM sleep (\(P < 0.0001\)) and non-REM (NREM) sleep (\(P < 0.0001\)) (Fig. 2c, d). LPAg\(^{Vglut2}\) neurons exhibited significantly lower GCaMP6f activity during NREM sleep, while they displayed medium activity during REM sleep and higher activity during wakefulness (Fig. 2c, d). Notably, LPAg\(^{Vglut2}\) neurons showed significantly increased activity immediately before NREM-to-wake, NREM-to-REM, and REM-to-wake transitions; in addition, these neurons had slightly reduced activities before the wake-to-NREM transition (Fig. 2e). These results revealed that the majority of LPAg\(^{Vglut2}\) neurons are specifically activated during wakefulness, less active during REM sleep and inactive during NREM sleep.

Chemogenetic activation of LPAg\(^{Vglut2}\) neurons promotes wakefulness and immobility. We tested the effect of chemogenetic activation of LPAg\(^{Vglut2}\) neurons by injecting AAV-hSyn-DIO-hM3Dq-mCherry bilaterally in the LPAg of Vgult2-Cre mice (Fig. 3a, b). In vitro electrophysiological experiments showed that current injection in a non-spontaneous-firing hM3Dq-positive Vglut2 neuron induced
membrane depolarization (Fig. 3c). Using whole-cell current-clamp recording, we found that CNO administration induced the depolarization of LPAG$^{\text{Vglut2}}$ neurons and generated action potentials (Fig. 3d). Application of CNO to the LPAG$^{\text{Vglut2}}$ neurons significantly increased the firing frequency and membrane potential (Fig. 3e, f). Furthermore, strong c-Fos expression was found in hM3Dq-expressing (mCherry+) neurons after in vivo injection of CNO, showing strong activation of the LPAG$^{\text{Vglut2}}$ neurons (Fig. 3g–j).

The EEG/EMG recordings showed that CNO injection increased wakefulness and decreased NREM and REM sleep compared to vehicle group administration (Fig. 3k, l). Importantly, chemogenetic activation of LPAG$^{\text{Vglut2}}$ neurons strongly enhanced wakefulness and concomitantly reduced both NREM and REM sleep for 7 h after CNO (1 mg/kg) injection at 09:00 in the morning, compared to the vehicle control (Fig. 3m). The duration of wakefulness during 09:00–16:00 increased by 195.20% ($P < 0.0001$), along with an 87.07% ($P < 0.0001$) decrease in NREM sleep and a 97.11% ($P < 0.0001$) decrease in REM sleep (Fig. 3n). To evaluate the effects of chemogenetic activation on EEG activity, we compared the normalized EEG power spectrum of wakefulness, NREM, and REM sleep (Fig. 3o). Compared to vehicle, CNO administration led to an increase in the EEG activity during wakefulness in the frequency range of 7–11 Hz, corresponding to the theta wave band, and a decrease in the range of 0–2.75 Hz (delta waves) (Fig. 3o). The EEG power spectrum of NREM and REM sleep was compared, but no statistical differences were observed between vehicle and CNO groups (Fig. 3o). These analyses suggest that activation of LPAG$^{\text{Vglut2}}$ neurons induced an increase in
wakefulness with theta oscillations.

To better characterize the sleep–wake behaviors in mice, we measured the EMG amplitude during wakefulness (EMG\textsubscript{Wake}) and compared it between vehicle and CNO groups. Compared to the vehicle group, mice in the CNO group displayed significantly decreased EMG\textsubscript{Wake} levels ($P = 0.0432$) (Fig. 3p). The ratio of the EMG amplitude during wakefulness after treatment (EMG\textsubscript{T-Wake}) and that during normal wakefulness (EMG\textsubscript{N-Wake}) was significantly lower in the CNO group than vehicle group ($P = 0.0083$) (Fig. 3q). Furthermore, we evaluated the effects of the activation of LPAG\textsuperscript{Vglut2} neurons on behavior through the observation of locomotor activity after administering either the vehicle or CNO. Chemogenetic activation of LPAG\textsuperscript{Vglut2} neurons decreased locomotor activity in the open field experiment ($P = 0.0279$) (Fig. 3r, s), suggesting decreased exploratory behaviors. In particular, activation of the LPAG\textsuperscript{Vglut2} neurons by CNO administration was associated with increased immobility time ($P < 0.0001$) (Fig. 3t).

Taken together, the results demonstrate that chemogenetic activation of LPAG\textsuperscript{Vglut2} neurons induced wakefulness, decreased exploratory behavior, and increased immobility.

### Optogenetic activation of LPAG\textsuperscript{Vglut2} neurons promotes wakefulness and immobility.

Next, we performed optrode recording to precisely stimulate the LPAG\textsuperscript{Vglut2} neurons and determine whether it could initiate arousal. AAVs expressing channelrhodopsin-2 (AAV-DIO-ChR2-mCherry) were stereotaxically injected into the LPAG (Fig. 4a–c). Subsequently, whole-cell patch-clamp recordings of the LPAG\textsuperscript{Vglut2}...
neurons were performed during optogenetic activation. Brief blue light pulses (5 ms) induced single action potentials in Vglut2 neurons, which expressed ChR2 (Fig. 4d). Light pulses were able to evoke action potentials with high-frequency fidelity between 1 and 100 Hz (Fig. 4e, f). After the beginning of steady NREM sleep, we performed optical blue light stimulation (473 nm, 5 ms, 30 Hz). Compared to yellow light (589 nm), acute blue light stimulation of the LPAG\textsuperscript{Vglut2} neurons induced instant transitions from NREM sleep to wakefulness (Fig. 4g, j). The probability of transition between each pair of sleep–wake stages indicated that optical blue light activation enhanced the probability of inducing wakefulness, along with a simultaneous decrease in the probability to enter NREM or REM sleep (Fig. 4h–l). Short latencies were also observed for sleep-to-wake transitions during blue light pulses at frequencies ranging from 20 to 50 Hz (Fig. 4m). Acute blue light stimulation of the LPAG\textsuperscript{Vglut2} neurons during wakefulness induced a decrease in the EEG activity in the frequency range of 0.75–4.25 Hz and 5–7.5 Hz, and an increase in the frequency range of 8–13.75 Hz (i.e., theta waves) (Fig. 4n). The EMG\textsubscript{Wake} was determined before and after blue light stimulation. Blue light photostimulation significantly decreased the EMG\textsubscript{Wake} level ($P = 0.0257$) (Fig. 4o). The ratio of the EMG\textsubscript{T-Wake} during wakefulness after treatment with photostimulation and the EMG\textsubscript{N-Wake} that during the normal wakefulness stage was significantly lower in the blue light group than in unstimulated normal counterparts ($P = 0.0164$) (Fig. 4p). Importantly, photostimulation of LPAG\textsuperscript{Vglut2} neurons also promoted immobility ($P = 0.0018$) (Fig. 4q). Sustained activation of LPAG\textsuperscript{Vglut2} neurons via long-term optogenetic stimulation (5-ms pulses at 30 Hz, with 40-s on/20-s off for 60 cycles)
for 1 h consistently maintained wakefulness and reduced NREM and REM sleep, in LPAG\textsuperscript{Vglut2-ChR2} mice compared to the baseline control group (Fig. 4r–t). In addition, the amount of wakefulness was increased by 1.50-fold ($P = 0.0099$), with a 69.81% ($P = 0.0196$) decrease in NREM sleep and a 100.00% ($P = 0.0078$) decrease in REM sleep during 1 h (Fig. 4u).

These results indicate that the optogenetic stimulation of LPAG\textsuperscript{Vglut2} neurons is sufficient for initiating and maintaining wakefulness characterized by a decrease in movements and an increased immobility.

\textbf{Anterograde tracing of LPAG\textsuperscript{Vglut2} neurons.} To identify the neuronal pathway underlying the effect of LPAG\textsuperscript{Vglut2} neurons in the sleep–wake cycle, we used a validated conditional anterograde tract (AAV-hSyn-DIO-hrGFP) tracing approach (Supplementary Fig. 2a). The injection site (Supplementary Fig. 2b, c) was validated by the local expression of hrGFP in the LPAG. The projecting axons of LPAG\textsuperscript{Vglut2} neurons were observed in the subparafascicular thalamic nucleus (SPF) (Supplementary Fig. 2d), precommissural nucleus (PrC) (Supplementary Fig. 2e), superior cerebellar peduncle (scp), sublaterodorsal tegmental nucleus (SLD) (Supplementary Fig. 2f), lateral parabrachial nucleus (LPB) (Supplementary Fig. 2g), locus coeruleus (LC) (Supplementary Fig. 2h), and ventral part of gigantocellular reticular nucleus (GiV) (Supplementary Fig. 2i). The LPAG\textsuperscript{Vglut2} neuronal outputs in the brain are summarized in Supplementary Fig. 2j. These findings indicate that LPAG\textsuperscript{Vglut2} neurons project to the LC, SLD, and GiV, which regulate wakefulness and
REM sleep.

242 **LPAG^{Vglut2}** neurons induce wakefulness and immobility through the LC pathway. To identify the functional role of the projection targets of LPAG^{Vglut2} neurons, we used *in vivo* and *in vitro* optogenetic approaches to precisely activate the LPAG^{Vglut2}-LC pathway. Then, we injected the AAV-DIO-ChR2-mCherry construct into the LPAG with optical fibers targeting the axonal terminals in the LC of Vglut2-Cre mice (Fig. 5a–c). ChR2 expression allowed the photostimulation of cell bodies within the LPAG and the selective activation of terminals from the LPAG that projected to the LC. An optogenetic-assisted electrophysiological method was used to explore the connection between LPAG^{Vglut2} and LC neurons (Fig. 5d, e). Compared to the baseline condition, glutamate blockade with D-APV (25 µM) and NBQX (5 µM), along with GABA receptor blockade (gabazine, 5 µM), reduced the mean values of photostimulation and elicited spontaneous postsynaptic currents in the observed LPAG^{Vglut2} neurons, while GABA receptor blockade did not induce any changes (Fig. 5d). When photostimulation was delivered to the LC, 65% of the recorded neurons in the LC responded (Fig. 5e). The latency between photostimulation delivery and the firing activity of the responding LC neurons is shown in Fig. 5f. It confirms that that LC neurons receive glutamatergic inputs from the LPAG^{Vglut2} neurons. To identify the cell types of the recorded LC neurons, biocytin was added to the recording microelectrode solution for subsequent immunohistochemical staining for tyrosine hydroxylase (TH), which is a marker of norepinephrine (NE) neurons. Then, we
confirmed that LPAG$^{Vglut2}$ neurons mostly targeted TH-positive neurons in the LC (Fig. 5g). Optogenetic blue light activation of the LC at 30 Hz immediately induced transitions from NREM sleep to wakefulness compared to yellow light (Fig. 5h, k). The probability analysis of transition between each pair of sleep–wake stages indicated that optical activation enhanced the probability to enter wakefulness, along with a simultaneous reduction in the probabilities of the switch to NREM and REM sleep (Fig. 5i, j, l, m). Optogenetic blue light stimulation of the LC at 30 Hz led to a decrease in EEG activity in the delta wave frequency range of 0–3.5 Hz, as well as an increase in the theta wave frequency range of 4.5–7.25 Hz during wakefulness (Fig. 5n). Then, the EMG$_{Wake}$ was determined before and after blue light stimulation. Blue light photostimulation significantly decreased the EMG$_{Wake}$ level ($P = 0.0404$) (Fig. 5o). The ratio of the EMG$_{T-Wake}$ during wakefulness after treatment with photostimulation and that the EMG$_{N-Wake}$ during the normal wakefulness stage was significantly lower in the blue light group than in the unstimulated normal counterparts ($P = 0.0365$) (Fig. 5p). Importantly, photostimulation of LPAG$^{Vglut2}$-LC projections also promoted immobility ($P = 0.0392$) (Fig. 5q). Additionally, chronic excitation of the LPAG$^{Vglut2}$-LC projections for 1 h remarkably promoted long-term wakefulness and decreased NREM and REM sleep in LPAG$^{Vglut2}$-ChR2 mice compared to the baseline controls (Fig. 5r–u).

These results suggested a strong functional contribution of the LPAG$^{Vglut2}$-LC projection in the induction of wakefulness and immobility.
**LPAG^{Vglut2} neurons induce arousal and immobility via the GiV pathway.**

Anterograde tracing results indicated that LPAG^{Vglut2} neurons project to the GiV, which is important in regulating the sleep–wake cycle. To confirm the functional interaction between the downstream target GiV and LPAG^{Vglut2} neurons, we used *in vivo* and *in vitro* optogenetic approaches to activate the LPAG^{Vglut2}-GiV pathway. The AAV-DIO-ChR2-mCherry construct was injected into the LPAG with optical fibers targeting the axonal terminals in the GiV of Vglut2-Cre mice (Fig. 6a–c). Then, an optogenetic-assisted electrophysiological experiment was conducted to determine the role of the LPAG^{Vglut2} projection to the GiV neurons (Fig. 6d, e). Glutamate blockade with D-APV (25 µM) and NBQX (5 µM), along with GABA receptor blockade (gabazine, 5 µM), decreased the mean values of activation and elicited spontaneous postsynaptic currents in the recorded LPAG^{Vglut2} neurons compared to baseline; meanwhile GABA receptor blockade did not induce any change compared to the baseline condition (Fig. 6d). A total of 80% of recorded GiV neurons responded to the photostimulation (Fig. 6e). The latency between photostimulation and the firing of the responding GiV neurons is shown in Fig. 6f. Our results showed that the GiV neurons primarily receive glutamatergic projections from the LPAG^{Vglut2} neurons. Then, we added biocytin to the recording microelectrode solution for subsequent immunohistochemical staining for Vglut2 and glycine and found that LPAG^{Vglut2} neurons targeted both Vglut2- and glycine-positive neurons in the GiV (Fig. 6g).

Optogenetic blue light activation of the GiV at 30 Hz rapidly induced arousal from NREM sleep compared to that using yellow light (Fig. 6h, k). Optical stimulation
increased the probability to induce wakefulness, along with a concomitant reduction in the probability to switch to NREM and REM sleep (Fig. 6i, j, l, m). Optogenetic blue light stimulation of the GiV at 30 Hz enhanced the activity in the frequency range of 4.25–5.75 Hz and reduction in the frequency range of 7–9.25 Hz of theta waves during wakefulness (Fig. 6n). Blue light photostimulation of the LPAG\(^{\text{Vglut2-GiV}}\) pathway significantly decreased the EMG\(_{\text{Wake}}\) level \((P = 0.0256)\) (Fig. 6o). The ratio of the EMG\(_{\text{T-Wake}}\) during wakefulness after treatment with photostimulation and that the EMG\(_{\text{N-Wake}}\) during normal wakefulness was significantly lower in the blue light group than in the unstimulated normal counterparts \((P = 0.0148)\) (Fig. 6p). Furthermore, photostimulation of the LPAG\(^{\text{Vglut2-GiV}}\) projections increased immobility \((P = 0.0068)\) (Fig. 6q). In contrast to LC stimulation, chronic stimulation of LPAG\(^{\text{Vglut2-GiV}}\) projections for 1 h did not induce chronic wakefulness in LPAG\(^{\text{Vglut2-ChR2}}\) mice (Fig. 6r–u).

The present data suggest that GiV is a crucial downstream nucleus for LPAG\(^{\text{Vglut2}}\) neurons to generate wakefulness associated with immobility behavior.

**Chemogenetic inhibition of LPAG\(^{\text{Vglut2}}\) neurons inhibits REM sleep and promotes NREM sleep.** To better understand the role of LPAG\(^{\text{Vglut2}}\) neurons in wakefulness and REM sleep regulation, LPAG\(^{\text{Vglut2}}\) neurons were inhibited with AAV constructs that encoded the engineered Gi-coupled hM4Di receptor (AAV-hSyn-DIO-hM4Di-mCherry) in Vglut2-Cre mice (Fig. 7a, b). An *in vitro* study showed that CNO (5 µM) significantly reduced the number of evoked action potentials, frequencies, and
membrane potentials (Fig. 7c–e), while immunostaining demonstrated that CNO administration suppressed c-Fos immunoreactivity in LPAG neurons expressing mCherry in Vglut2 neurons (Fig. 7f–i). After CNO delivery, the sleep–wake states were measured (Fig. 7j, k). The CNO-injected mice displayed decreased REM sleep and enhanced NREM sleep compared to vehicle-injected mice. The amount of hourly REM sleep was significantly reduced with CNO at 1 mg/kg by 31.12%, 28.57%, and 46.94% during the second, third, and fourth hours, respectively, while the hourly duration of NREM sleep after CNO treatment was increased by 13.82%, 25.78%, and 56.72% during the second, third, and fifth hours, respectively. The hourly duration of wakefulness after CNO injection was reduced by 30.39% and 18.10% during the third and fifth hours, respectively (Fig. 7l). The duration of REM sleep during 15:00–19:00 was reduced by 35.57% ($P = 0.0067$), along with a 14.12% ($P = 0.0014$) increase in NREM sleep and an 11.31% ($P = 0.0284$) reduction in wakefulness (Fig. 7m). CNO injection did not induce any change in the EEG activity during REM sleep, NREM sleep, or wakefulness (Fig. 7n). Chemogenetic inactivation of LPAG$^{\text{Vglut2}}$ neurons did not change the locomotor activity in the open field experiment ($P = 0.2663$) (Fig. 7o, p) or increase immobility ($P = 0.4843$) (Fig. 7q). The EMG amplitude during REM sleep, NREM sleep, and wakefulness were also evaluated after CNO treatment, but there was no significant differences between the vehicle and CNO groups (Supplementary Figs. 3a–f). These findings indicate that the inhibition of LPAG$^{\text{Vglut2}}$ neurons via hM4Di decreases REM sleep and wakefulness, and increases NREM sleep, suggesting an important role of LPAG$^{\text{Vglut2}}$ neurons in the regulation of REM sleep and wakefulness,
LPAG^{Vglut2} neurons activated during REM sleep projects to the SLD. Using combined optogenetic and neuroanatomical approaches, we explored the pathways by which LPAG^{Vglut2} neurons regulate REM sleep. First, the AAV-DIO-ChR2-mCherry construct was injected into the LPAG with AAV-DIO-eGFP targeting the SLD neurons in Vglut2-Cre mice (Fig. 8a). Then, we used an optogenetic-assisted electrophysiological approach to determine the properties of the projection between LPAG^{Vglut2} and SLD neurons. Compared to the baseline condition, glutamate blockade with D-APV (25 µM) and NBQX (5 µM), along with GABA receptor blockade (gabazine, 5 µM), reduced the mean values of photostimulation and induced spontaneous postsynaptic currents in the observed LPAG^{Vglut2} neurons. However, the GABA receptor blockade did not induce any changes (Fig. 8b). After photostimulation delivery, 92.9% of the recorded neurons in the SLD responded (Fig. 8c). We summarize the latency between the photostimulation delivery and the firing event in the responding SLD neurons in Fig. 8d. These findings indicate that the SLD neurons mostly receive glutamatergic inputs from LPAG^{Vglut2} neurons. By adding biocytin to the recording microelectrode solution to enable subsequent immunostaining for Vglut2, we confirmed that LPAG^{Vglut2} neurons mainly target Vglut2-positive neurons in the SLD (Fig. 8e).

Since SLD is located adjacent to the LC, optogenetic stimulation of the LPAG-SLD pathway might simultaneously induce the activation of the LPAG-LC projections.
Therefore, we used a neuroanatomical approach to elucidate the role of LPAG-SLD projections in REM sleep regulation. By injecting a retrograde trace-cholera toxin b subunit (CTb) into the SLD and GiV, we identified the neuronal pathways from the LPAG activated during REM sleep (Fig. 8f). The CTb injection sites were restricted to the SLD or GiV (Fig. 8g, h). To determine whether the LPAG-SLD and LPAG-GiV pathways are activated during REM sleep, we used the flowerpot method for REM sleep deprivation for 72 h, after which rats were allowed to recover for 2 h. Many c-Fos+/CTb+ neurons were found in the LPAG of RSR rats after CTb injection into the SLD (Fig. 8i, j). In contrast, after CTb injection into the GiV, only a few c-Fos+/CTb+ neurons were seldom found in the LPAG of RSR rats (Fig. 8k, l). Within the LPAG, the number of c-Fos+/CTb+ neurons was 1.75 ± 0.22 in GiV-RSR rats and 7.35 ± 0.27 in SLD-RSR rats ($P = 0.0050$) (Fig. 8m). Furthermore, we determined the percentage of double-labeled neurons within the LPAG, and found that 28.37 ± 4.24% of CTb+ neurons were double-labeled in SLD-RSR rats compared to 9.57 ± 1.68% in GiV-RSR rats ($P = 0.0060$) (Fig. 8m). These data suggest that the LPAG*Vglut2*-SLD pathway, but not the LPAG-GiV, is involved in the control of REM sleep.
Discussion

The LPAG is an important component of the periaqueductal gray and it regulates several behaviors, including defensive behaviors, pain processing, chronic social defeat stress, and sensorimotor responses to breathlessness\textsuperscript{22, 23, 24, 25}. These behaviors ensure survival and rely on heightened arousal. In addition, the LPAG is connected to several sleep–wake regulatory nuclei and has been observed to contain many c-Fos+ neurons after REM sleep deprivation and rebound\textsuperscript{8, 18, 26}. In the present study, using fiber photometry, chemogenetics, optogenetics, behavioral tests, polysomnography, and electrophysiological experiments, we demonstrated a prominent contribution of LPAG\textsuperscript{Vglut2} neurons to wakefulness associated with immobility and REM sleep, and we determined the circuits involved (Supplementary Fig. 4).

Muscle tone, which is modulated across sleep–wake states, is maximized during wakefulness, reduced during NREM sleep, and absent during REM sleep\textsuperscript{27, 28}. However, chemogenetic and optogenetic activation of the LPAG\textsuperscript{Vglut2} neurons in our study induced wakefulness with immobility. The excitation of the LPAG\textsuperscript{Vglut2} neurons also induced an increase in the EEG power of theta waves, which depend on the hippocampus\textsuperscript{29}. We propose that the dominant effect of the induction of wakefulness, covered up the REM sleep-promoting effects of LPAG\textsuperscript{Vglut2} neurons. Indeed, REM sleep is decreased and NREM sleep is increased when all LPAG\textsuperscript{Vglut2} neurons are inhibited, indicating that two population of LPAG\textsuperscript{Vglut2} neurons are co-distributed and play roles in wakefulness or REM sleep. These neurons could be candidate targets for the treatment of sleep disorders, including REM sleep behavior disorder and narcolepsy.
The immobility behavior reported in our study could correspond to the classical
defense-related immobile state named freezing. Indeed, freezing mainly refers to the
absence of physical movement as reflected on EMG recording and automated scoring
of video recording\textsuperscript{30}. We did not define the immobility behavior in our present study as
freezing, because freezing is a defensive state of immobility characterized by the
cessation of all movements accompanied by the parasympathetic-induced heart rate
deceleration (i.e., bradycardia)\textsuperscript{31} and we cannot determine with our analysis whether
the immobility induced correspond to a freezing. We did not monitor the heart rate and
other parameters of mice during chemogenetic or optogenetic manipulation. In addition,
despite being immobilized, the muscle tone is high during freezing, which is not in line
with the low muscle tone intensity observed in our study. Furthermore, general
immobility can be identified as an absence of movements, whereas a sleep state is also
seen as a state of immobility. The EMG of neck muscle recording is useful to
discriminate the defense-related immobility from sleep\textsuperscript{32}. In our study, the EMG
amplitude during the stimulation was less than that in spontaneous wakefulness and we
therefore defined it as a state of immobility.

Our study showed that LPAG\textsuperscript{Vglut2} neurons project to different several brain regions.
We further showed that the LPAG\textsuperscript{Vglut2} neurons promote arousal and immobility via the
LC\textsuperscript{NE} connections. These results are consistent with those of previous studies, which
showed that LC received a strong projection from PAG\textsuperscript{33, 34}. LC is the most dorsal of
the ten noradrenergic nuclei located in the pontine brainstem\textsuperscript{35}. These LC\textsuperscript{NE} neurons are
critical to induce wakefulness\textsuperscript{36, 37, 38}. In the state of wakefulness, tonic LC\textsuperscript{NE} activity
correlates with the state and covaries with the levels of arousal. In addition to tonic
discharge rates, LC neurons generate phasic bursts when exposed to novel or
significant stimuli. An increased EEG frequency, as well as behavioral markers of
attention and alertness, reflect the phasic LC activity. Furthermore, LC neurons
exhibit vigilant state-dependent changes in their firing properties: they are highly active
during wakefulness, they are much less active during NREM sleep, and they are almost
silent during REM sleep. Deactivated LC neurons may promote NREM and REM
sleep, while activated neurons in the LC may participate in the maintenance of
wakefulness. LC neurons projecting to the lateral amygdala receive synaptic
input from orexin neurons in the lateral hypothalamus, which plays an important role
in the regulation of passive defensive behaviors. Combined with previous findings,
our data provide an enhanced understanding of the roles of LPAG-LC neurons
in the sleep–wake cycle. They also suggest that these neurons increase arousal while
ensuring the expression of an appropriate behavioral response. This study suggests that
the pathway involved in fear generalization is the one that is sometimes seen in
conditions such as posttraumatic stress disorder.

Another projection from LPAG to GiV neurons is responsible for the
effects on the initiation of wakefulness and immobility. GiV is also known as the
magnocellular reticular nucleus located in the ventromedial medullary reticular
formation. The glycineric premotoneurons in the GiV are REM-on neurons, and the
descending REM-on glutamatergic neurons in the SLD induce muscle atonia through
their excitatory projections to the GiV inhibitory neurons. Optogenetic
activation of glutamatergic neurons in the ventral medulla, which mainly includes Giv, induces wakefulness. Furthermore, GiV is involved in motor control and this region also receives projections from VLPAG neurons to induce passive defensive behaviors.

Our results showed that the LPAG neurons project to the GiV neurons expressing either glycine or Vglut2, and the optogenetic activation of GiV neurons initiated wakefulness, without maintaining the state, owing to the dual effects of these two neuron types. LPAG neurons may have initiated wakefulness via the projection to GiV glutamatergic neurons, while they promoted immobility with low muscle tone intensity similar to the muscle atonia during REM sleep through the GiV inhibitory neurons.

We also found that LPAG neurons are also involved in REM sleep regulation. Trap and c-Fos staining experiments demonstrated the role of LPAG neurons in the regulation of REM sleep. Chemogenetic inhibition of LPAG neurons decreased REM sleep, although chemogenetic and optogenetic activation of these neurons promoted wakefulness, likely masking the effect on REM sleep. Projections to the SLD derived from the LPAG neurons are likely responsible for REM sleep onset and maintenance. There is an anatomical connection between the LPAG and SLD and GiV.

Both the glutamatergic neurons in the SLD and glycinergic neurons in Giv are crucial REM-on neurons. The activation of REM-on glutamatergic neurons from the SLD initiates and maintains REM sleep. Descending REM-on glutamatergic SLD neurons induce muscle atonia via their excitatory projections to the glycinergic premotoneurons located in the GiV. When we injected CTb in the SLD and GiV, we confirmed the
existence of a strong LPAG-SLD pathway but only a weak LPAG-GiV pathway
activated during REM sleep rebound. Hence, it seems that the LPAG–SLD pathway,
but not the LPAG–GiV pathway, is likely involved in the induction of REM sleep by
LPAG^{Vglut2} neurons.

In our present study, we found that mostly different neurons in the LPAG were
activated during wakefulness and REM sleep in the TRAP mice. Therefore, two
different populations of glutamatergic neurons are active during wakefulness and REM
sleep in the LPAG. In future studies, these two different subpopulations should be
studied separately by means of the discovery of specific markers and the use of Cre
mice.

In conclusion, we found that LPAG^{Vglut2} neurons are essential for controlling the
wakefulness associated with immobility via the LC and GiV pathways. Another
population of LPAG^{Vglut2} projecting to the SLD would regulate REM sleep. Our results
suggested that LPAG^{Vglut2} neurons constitute a potential therapeutic target for the
treatment of neuropsychiatric disorders with sleep–wake abnormalities.
Methods

Animals. Vglut2-Cre transgenic mice (males, 22–26 g) obtained from Jackson Laboratory (Bar Harbor, ME, USA) and Sprague Dawley rats (males, 240-280 g) obtained from Charles River Laboratories (Wilmington, MA, USA) were placed separately in Plexiglas cylinders with an outer diameter of 30 cm and a height of 40 cm. The Fos2A-iCreER/+ transgenic mice (TRAP2) for activity-dependent genetic labeling were kindly provided by Dr Li-Qun Luo from Stanford University and crossed with R26Ai14/+ mice (Ai14) to produce double heterozygous (TRAP2; Ai14, TRAP-RED) mice52,53 (Supplementary Fig. 1a). The animals were housed at a temperature of 22 °C, humidity of 60%, and illumination of 100 lux under a 12/12 h light/dark cycle (lights on: 07:00). Food and water were provided ad libitum. Animal care and experiments were performed in accordance with the institutional guidelines and regulations of Chinese and European governments. The experimental protocols were approved by the Animal Care and Use Committee of Fudan University, China and the University of Lyon 1, France.

Chemicals and drug administration. CNO (C0832, Sigma-Aldrich, St. Louis, MO, USA) saline solution (0.1 mg/ml) was used for the in vivo experiments, and the volume was determined by body weight (10 ml/kg). For in vitro experiments, a stock CNO solution was produced using artificial cerebral spinal fluid (ACSF) and diluted to 5 µM. A stock 4-OHT (H6278, Sigma-Aldrich) ethanol solution (20 mg/ml) was produced by adding the correct quantity of ethanol, and putting it onto a vortex mixer, followed by
a shaker at 37°C for 15 min or until completely dissolved. An aluminum foil was used to minimize the exposure to light. A working solution (10 mg/ml) was prepared by adding corn oil (C8267, Sigma-Aldrich) to the stock solution and allowing the ethanol to evaporate using a speed-vac for 2–3 hr. The drug was administered intraperitoneally depending on the time (Supplementary Fig. 1a).

Viral injections and fiber implantations. The viruses were packaged into the AAV serotype 2/9 vectors with titers of almost $1–5 \times 10^{12}$ genomic copies/mL. AAV-EF1α-DIO-GCaMP6f, AAV-hSyn-DIO-hM3Dq/hM4Di-mCherry, AAV-EF1α-DIO-ChR2-mCherry, and AAV-lox-stop-hrGFP were purchased from Shanghai Taitool Inc. (Shanghai, China) or BrainVTA, Co., Ltd. (Wuhan, China).

Mice were anesthetized with pentobarbital sodium (50 mg/kg, i.p.) and placed onto a stereotaxic apparatus (RWD Life Science, Shenzhen, China). A midline scalp incision was made to expose the skull followed by unilateral or bilateral craniotomy directly above LPAG (AP: – 4.1 mm, ML: ± 0.35 mm, DV: – 2.3 mm). Then a glass pipette was carefully lowered into the LPAG for virus microinjection using a compressed air system and nitrogen gas with 20–40 psi pulses. After the injection was administrated, the glass pipette was held in place for 10 min, allowing the virus to diffuse. The animals were placed on a heated surface until they fully recovered from anesthesia, then, the mice were returned to their home cage.

For the fiber photometry recording, Vglut2-Cre mice were microinjected with AAV-EF1α-DIO-GCaMP6f into the left side of the LPAG at a volume of 30 nL. After the
injection, a ceramic ferrule and optic fiber assembly (inner diameter: 200 µm, numerical aperture (NA): 0.37; Newdoon, Shanghai, China) was implanted using the following coordinates (AP: −4.1 mm, ML: ± 0.35 mm, DV: −2.2 mm) such that the fiber tip was located on top of the left LPAG. To activate or inhibit the LPAG\textsuperscript{Vglut2} neurons with DREADDs, Vglut2-Cre mice 30 nL of either AAV-hSyn-DIO-hM3Dq-mCherry or AAV-hSyn-DIO-hM4Di-mCherry on both sides of the LPAG. For the activation of the LPAG\textsuperscript{Vglut2} neurons and their pathways with optogenetics, 30 nL of AAV-EF1α-DIO-ChR2-mCherry was bilaterally microinjected into the LPAG in Vglut2-Cre mice. Then the mice were unilaterally implanted with optical fibers (inner diameter: 200 µm, NA: 0.37; Newdoon) above the LPAG (AP: − 4.1 mm, ML: ± 0.35 mm, DV: − 2.2 mm), LC (AP: − 5.4 mm, ML: ± 0.8 mm, DV: − 3.7 mm), or GiV (AP: − 6.7 mm, ML: ± 0.3 mm, DV: − 4.3 mm). To explore the output patterns of the LPAG\textsuperscript{Vglut2} neurons, Vglut2-Cre mice were unilaterally microinjected with 200 nL of AAV-lox-stop-hrGFP in the LPAG. Mice were allowed to rest for at least 3 weeks to recover completely after being injected with the virus and allow viral expression before further experiments. In all experiments, the animals with incorrect injection sites were excluded from further analyses.

**EEG/EMG electrode implantation surgery.** EEG recordings and analysis were performed as previously described\textsuperscript{54, 55, 56}. Under pentobarbital sodium anesthesia (50 mg/kg, i.p.), two stainless steel screws attached to a Teflon-coated stainless-steel wire (1 mm diameter) were inserted through the skull into the cortex and served as EEG
electrodes; two Teflon-coated stainless-steel wires were placed bilaterally into the trapezius muscles and served as EMG electrodes. All wires were attached to a microconnector as well as to the skull with dental cement.

Male Sprague-Dawley rats were implanted with EEG and EMG electrodes under anesthesia with ketamine (Virbac Santé Animale, Carros, France; 100 mg/kg, i.p.) and xylazine (Bayer, 50 mg/kg, i.p.). Lidocaine (Xylovet, Ceva Santé Animale, Libourne, France) was subcutaneously injected for analgesia. Three stainless steel screws were fixed in the parietal and frontal bones, and a unipolar EEG recording was fixed on the cerebellum bone. Two wire electrodes were inserted into the neck muscles for bipolar EMG recordings. All leads were connected to a miniature plug (Plastics One Inc., Torrington, CT, USA), which was glued to the skull.

**Polygraphic recordings and analysis.** Cortical EEG and neck EMG signals were amplified, filtered (Biotex, Kyoto, Japan; EEG, 0.5–30 Hz; EMG, 20–200 Hz) and digitized at a sampling rate of 128 Hz using VitalRecorder (Biotex). Using a standard algorithm, sleep-wake stages were categorized offline into 4-second epochs of wakefulness, NREM or REM sleep by SleepSign (Biotex). Then, the epochs were visually examined and corrected if necessary. EEG power density was calculated using fast Fourier transform with a 0.25 Hz resolution, and presented as a percentage of the total power across 0–25 Hz. Based on the protocols of previous studies, we used the original EMG data and mean values from SleepSign (Biotex) recordings to quantify the nuchal muscle tone. For chemogenetic activation manipulation, we analyzed the muscle
tone during 10:00–13:00 after administration of vehicle or CNO. For optogenetic inactivation manipulation, we analyzed the muscle tone during 60-s stimulation or interval period, eliminating the first and last 4 seconds to avoid the change in muscle tone caused by the transition of the vigilance stage. To reduce the individual differences in mice, the mean EMG values were normalized by baseline mean EMG value\textsuperscript{48, 58}. To avoid muscle tone alterations induced by the transition of the vigilance stage, we only included the wakefulness episodes that lasted for more than 48 s and excluded the first and last 8 seconds in the quantification process.

After the surgery, rats were allowed 1 week of recovery in the Plexiglas cylinder. Then, a cable was connected to the plug on the rats with the other end attached to a slip-ring commutator to allow free movement of the rats in the cylinder. Another 3 days of habituation were allowed before data collection. EEG and EMG signals were digitized through a CED interface using Spike2 software (Cambridge Electronic Design, Cambridge, UK). Vigilance stages were categorized accordingly.

**Protocols for REM sleep deprivation and rebound.** In the TRAP experiment, REM sleep deprivation was performed using an automated method involving the online signal analysis software Sleepscore\textsuperscript{59, 60} (Viewpoint, New South Wales, Australia). The vigilance state was automatically labeled for each 1-s epoch based on several discriminant parameters of EEG/EMG signals. When a REM sleep episode was detected, a signal was transmitted to a stimulation box through Matlab, and the animal was awakened by vertical shaking of the cylinder floor via an electromagnet\textsuperscript{61, 62}. The
48-hour automatic REM sleep deprivation was highly efficient in mice with only 2.2% of remnant REM sleep\textsuperscript{59}. REM sleep deprivation started at 10:00, when the proportion of REM sleep was high. After 48 h of REM sleep deprivation, mice were returned to their home cylinder and allowed to sleep \textit{ad libitum} during a 2-h recovery period (Supplementary Fig. 1a). RSR-RSR animals were injected with 4-OHT after 2 h of RSR. One week later, the animals were subjected to REM sleep deprivation and perfused after 2 h of RSR. In W-RSR or W-W animals, mice were placed in a white open-field box (base, 45 × 45 cm) containing several toys for 2 h. The animals were monitored using video and polysomnography. Whenever the mice fell asleep, they were awakened by slow repositioning of the toys or the gentle touch of a soft tissue. One week later, W-W animals underwent the same procedure, whereas the W-RSR animals were deprived of REM sleep for 48 h and allowed to recover for 2 h before perfusion.

REM sleep deprivation was performed using the flowerpot method\textsuperscript{63}. Rats were divided into three groups: control, REM sleep deprivation for 3 days, and recovery after REM sleep deprivation for 3 days. Animals in the control group were kept in the standard cage throughout the experiment. At 12:00 A.M., REM sleep deprived rats were placed in a Plexiglas cylinder containing three platforms (6.2 cm diameter, 7–12 cm height), which were surrounded by water (2 cm) for 75 h. Rats with REM sleep rebound syndrome received the same treatment for 72 h, and were allowed to recover in a cylinder with a dry bed of woodchips. They were euthanized 2 h after the first REM sleep episode. During the periods of deprivation, food and water were available \textit{ad libitum}, and cylinders were cleaned daily.
**Fiber photometry.** The calcium transients of Vglut2 neuronal cell bodies in the LPAG of freely moving mice were recorded by optical fiber photometry as previously described. In short, to induce fluorescent signals, a 488-nm laser (OBIS 488LS, Coherent Inc., Santa Clara, CA, USA) beam was reflected from a dichroic mirror (MD498, Thorlabs Inc, Newton, NJ, USA), focused by an objective lens (Olympus, Tokyo, Japan), and coupled through a fiber collimation package (F240FC-A, Thorlabs Inc) into a patch cable which was connected to the ferrule of an upright optical fiber implanted in the mouse via a ceramic sleeve (125 μm O.D.; Newdoon). GCaMP6 fluorescence was bandpass filtered (MF525–39, Thorlabs Inc, New Jersey, USA) and collected by a photomultiplier tube (R3896, Hamamatsu, Higashi-ku, Hamamatsu City, Shizuoka, Japan). An amplifier (C7319, Hamamatsu) was used to convert the current output from the photomultiplier tube into voltage signals, which was further filtered through a low-pass filter (40-Hz cut-off; Brownlee 440, Santa Clara, CA, USA). The photometry voltage traces were downsampled to match the EEG/EMG sampling rates using a Power 1401 digitizer and Spike2 software (Cambridge Electronic Design). Photometry data were exported as Matlab mat files from the Spike 2 software for further analysis. The value of the photometry signal ($\Delta F/F$) was calculated as $(F–F_0)/F_0$, where $F_0$ is the mean fluorescent signal. The average $\Delta F/F$ values were calculated for all sleep–wake states. To analyze the state transitions, we identified each state transition and aligned $\Delta F/F$ in a ± 50-s window around each point that was calculated. The average peak of the $\Delta F/F$ in different sleep stages was compared$^{64, 65}$. 
**Optogenetic manipulations during polygraphic recordings.** For *in vivo* light manipulations, light pulses were generated by a laser stimulator (SEN-7103, Nihon Kohden, Tokyo, Japan) and output through an isolator (ss-102J, Nihon Kohden). A rotating optical joint (FRJ_FC-FC, Doric Lenses, Quebec, Canada) was used to reduce the torque on the implant on animals and attached to one end of the optical fiber. Light-pulse trains (5-ms duration each) were programmed and carried out during the light period, when mice were inactive. Chronic photostimulation lasted for 1 h, with 5-ms pulses at 30 Hz followed by rest for 20 s after every 40 s (60 cycles). EEG/EMG recordings during the same period of the previous day served as control. The parameters of the sleep–wake cycle were analyzed. Power intensities of blue or yellow light at the optical fiber tip were measured using a power meter (PM10, Coherent) and calibrated to 3–7 mW.

**Behavioral tests.** The open field test (OFT) is commonly used to test locomotor activity in mice. In a quiet and sound-proof room with dim light (∼ 20 lux), individual mice were placed in a 50 × 50-cm arena with black surface and enclosed by 40 cm high walls and were allowed to move freely for 15 min. The activities of the test subjects were recorded using an automatic video tracking system (Labmaze V3.0, Beijing Zhongshi Dichuang Technology Development Co., Ltd., Beijing, China). The OFT was performed 30 min after CNO or vehicle treatment. After each trial, the instrument was wiped clean with a 75% alcohol solution to remove traces left by the previous mouse.
Rearing was defined by the mouse lifting both of its forelimbs off the floor. When rearing happened along the walls of the arena, the mouse typically leaned against the walls.

For chemogenetic and optogenetic manipulation, the immobility time was recorded by a video camera positioned on the front side of the recording chambers. The data were analyzed using Labmaze V3.0 software (Beijing Zhongshi Dichuang Technology Development Co., Ltd) at a 5% immobility threshold.

**Histological procedures.** Under deep anesthesia, the animals were transcardially perfused with 4% paraformaldehyde. Brains were removed, post-fixed and dehydrated in 30% graded phosphate-buffered sucrose solution. Then, the brains were coronally sliced into 30-μm sections on a cryostat. For *in situ* hybridization experiments, continuous floating sections were collected in RNase-free cryoprotectant solution and stored at −20°C for use. As previously mentioned, the free-floating sections were stained by immunohistochemistry. In brief, brain sections were rinsed in PBS, incubated at room temperature in 0.3% hydrogen peroxide in PBS (0.1 M) for 30 min, and incubated at room temperature for 1 h in 3% normal donkey serum and 0.3% Triton X-100 in PBS (PBST). The primary antibody, which was rabbit anti-c-Fos (1:10000; Millipore, Darmstadt, Germany), was double stained, diluted in PBST, and reacted with brain slices overnight at 4°C. On the next day, the sections were rinsed in PBS and incubated for 2 h in biotinylated anti-rabbit secondary antiserum (1:1000; Jackson ImmunoResearch, Laboratories, West Grove, PA, USA). The brain slices were coated
with the avidin-biotin-peroxidase complex (1:1000; Vector Laboratories, Burlingame, CA, USA) for 1 h. The immunoreactive cells were promoted through the reaction with 0.04% diaminobenzidine tetrahydrochloride and 0.01% hydrogen peroxide which was enhanced by nickel. This procedure was conducted on the same section to detect the expression of mCherry, which used the rat anti-mCherry (1:10000; CLONTECH Laboratories, Inc., Palo Alto, CA, USA) as the primary antibody and anti-rat secondary antiserum without nickel for the visualization. Finally, the brain sections were mounted on glass slides and observed under the microscope (Olympus BX51, Tokyo, Japan). A digital camera (DP72, Olympus) captured the images.

In the c-Fos immunohistochemistry combined with Vglut2 in situ hybridization experiments, the recombinant plasmid (pCRII-TOPO, Invitrogen, Carlsbad, CA, USA) containing the Vglut2 cDNA was linearized by Not I and Hind III (New England Biolabs, pswich, MA, USA), and transcribed using SP6 and T7 RNA polymerases (for antisense and sense riboprobes, respectively). A non-radioactive RNA labeling kit (Roche Diagnostic, Rotkreuz, Switzerland) was used for the transcription of riboprobes according to the manufacturer’s instructions. The digoxigenin-labeled riboprobes were stored in a hybridization buffer at -20 °C. As described previously, brain sections were incubated for 18 h with a rabbit antiserum to c-Fos (1:3000 for Vglut2, Merck, Darmstadt, Germany) in PBST at room temperature. Then, the brain sections were successively incubated at room temperature with a biotinylated goat antirabbit IgG solution (1:1000, Vector Laboratories) and an ABC-HRP solution (1:1000, Elite kit, Vector Laboratories) both for 90 min each. These sections were submerged for almost
15 min in a 0.05 M Tris-HCl buffer, which contained 0.025% 3,3-diaminobenzidine-4 HCl (DAB, Sigma-Aldrich) and 0.003% H_2O_2. Brain sections were rinsed for three times in PBST between steps and incubated in PBST containing 10 mM dithio-threitol (DTT, Sigma-Aldrich) twice for 10 min and in a standard saline citrate solution (SSC 2X) for 10 min. All buffers except DTT contained 0.2% RNase inhibitor (Sigma-Aldrich). Brain sections were subjected to the hybridization buffer consisting of 150 mM NaCl, 8 mM Tris-HCl, 1 mM Tris-Base, 6 mM NaH_2PO_4, 5 mM Na_2HPO_4, 5 mM EDTA, 50% formamide, 10% dextran sulphate, yeast tRNA (Sygma type III, 1 mg/mL, Sigma-Aldrich), 0.02% ficoll, and 0.02% polyvinyl pyrorolidone-containing 0.5 g/mL of the digoxigenin-labeled probe overnight at 65°C. Sections were rinsed twice at 55°C for 20 min in the complex of SSC 1X, 50% formamide and 0.1 % Tween-20. Then, the sections were treated at 37°C for 15 min with 10 µg/µl RNase A in Tris 10 mM-containing 1 mM EDTA and 500 mM NaCl. After rinsing with PBST three times, sections were incubated overnight with an anti-digoxigenin antibody conjugated to alkaline phosphatase (1:2000, Roche Diagnostic) in PBST containing 0.2% blocking agent (Roche Diagnostic). Then, they were rinsed twice in PBST, once in PBS 10 mM, and once in a buffer containing 1 M Tris-HCl, 1 M NaCl, 500 mM MgCl_2, and 1% Tween-20. Each wash lasted 10 min. Sections were then incubated at 37°C in the same buffer containing nitroblue tetrazolium and 5-bromo-4-chloro-3-indolyl-phosphate (20 µl/mL, Roche Diagnostic). After 4 h of incubation in the revelation buffer, sections were washed in PBST twice for 10 min each. Finally, the sections were mounted on glass slides, dried, and covered with Vectamount (Vector Laboratories). In the absence
of primary antibodies (anti-c-Fos and anti-digoxigenin) or with the sense probe, the control group ensured the labeling specificity.\textsuperscript{19, 48}

**Immunostaining analysis.** The atlas of Swanson served as a reference for the structures (Swanson 1992). Double- or single-labeled sections were drawn using an Axioscope microscope (Zeiss, Oberkochen, Germany), equipped with a motorized X–Y-sensitive stage and a video camera, which was connected to a computerized image analysis system (Mercator, Explora Nova, La Rochelle, France). On both sections (–5.6 and –8.6 mm to Bregma), the number of double-labeled Fos-Vglut2 neurons was quantified at the LPAG level, and ImageJ software (National Institutes of Health, Bethesda, MD, USA) was used to measure optical density.\textsuperscript{18, 19}

**In-vitro electrophysiology.** The brain slices (280-µm coronal sections) from male Vglut2-Cre mice were prepared with a vibratome (VT-1200, Leica Microsystems, Wetzlar, Germany) under general anesthesia. Almost 25 ml of ice-cold (4 °C) oxygenated cutting solution was perfused in a Vglut2-Cre mouse who developed unconsciousness, followed by decollation as soon as possible. The mouse brain was carefully and rapidly removed and placed into ice-cold oxygenated cutting solution, trimmed, translated, and fixed on an ice-cold plate infiltrated with ice-cold oxygenated cutting solution. The slices included four brain regions: the LPAG, LC, GiV, and SLD. The ingredients of the cutting solution (a) and ACSF (b) included the following: (a) 213 mM sucrose, 10 mM glucose, 2.5 mM KCl, 1.25 mM NaH2PO4, 26 mM NaHCO3, 3
mM MgSO$_4$, 0.1 mM CaCl$_2$ and 0.4 mM Ascorbic Acid, at 4 °C. (b) 25 mM glucose, 119 mM NaCl, 2.5 mM KCl, 1.25 mM NaH$_2$PO$_4$, 26 mM NaHCO$_3$, 1 mM MgCl$_2$ and 2 mM CaCl$_2$, at 32 °C. Brain sections were incubated in ACSF at 32 °C for the first 30 min and then shifted to room temperature for the next 30 min. Notably, the solutions used for the experiments were fresh and contained 95% O$_2$ and 5% CO$_2$. The electrodes were backfilled with the internal potassium gluconate solution to record the electrophysiological activity of the targeted neurons. These electrodes had a resistance range of 4–7 MΩ. The potassium gluconate internal solution (c) contained the following:

(c) 130 mM K-gluconate, 10 mM Hepes, 10 mM KCl, 0.5 mM EGTA, 4 mM ATP-Mg, 0.5 mM GTP-Na, and 10 mM phosphocreatine (pH = 7.5). Short-term (50 ms) injected currents (20 pA) were used to test the input resistance. All electrodes used in the experiments were backfilled with the internal solution of potassium gluconate.

For whole-cell patch-clamp recording, the slices were placed into the recording chamber filled with oxygenated ACSF (liquid dropped at 1–2 ml/min). Using infrared differential interference contrast and a fluorescence microscope, the neurons with good shape and specific fluorescence were targeted. The current-clamp and voltage-clamp recording modes were used to record the voltage (mV) and currents (pA), respectively.

For the in vitro optogenetic experiments, the AAV-hSyn-DIO-ChR2-mCherry virus was microinjected into the LPAG of the Vglut2-Cre male mice. The virus required at least 3 weeks to affect the somas and fibers of the LPAG$^{Vglut2}$ neurons. Then, we tested the activity of the affected LPAG$^{Vglut2}$ neurons with 473-nm light in the designed protocols, including the electrophysiological properties and fidelity. To verify the feasibility of the
neural pathway from the LPAG to the LC, GiV or SLD, 473-nm light was applied onto the LPAG$^{Vglut2}$ fibers in the LC, GiV or SLD, respectively, which was confirmed by the existence of postsynaptic currents after optogenetic activation. To identify the neural origin of LPAG$^{Vglut2}$ inputs, the postsynaptic currents recorded from the projected neurons were perfused sequentially with ACSF, gabazine (SR, 5 µM), 2-amino-5-phosphonopentanoic acid (D-APV, 25 µM), and 2,3-dihydroxy-6-nitro-7-sulfamoylbenzo[f]quinoxaline-2,3-dione (NBQX, 5 µM). Note that the inhibitory postsynaptic currents (IPSCs) were blocked by gabazine to demonstrate the differences in excitatory postsynaptic currents (EPSCs), while D-APV and NBQX were applied to block the glutamatergic currents (or EPSCs) induced by AMPA and NMDA receptors to illustrate the influence on IPSCs. All drugs were diluted with ACSF. Data were acquired, filtered (low-pass filtered at 1 kHz), and digitized (at 4 kHz) in the recording system using p-Clamp 10.3 software (Axon Ins., Washington, DC, USA), Multi-Clamp 700B Amplifier (Axon Ins.) and Micro1401 (CED Ins., Cambridge, UK). Neurons with Ra of over 30 MΩ or a floating range over 20% were excluded. To record the morphological data from mice, the solution containing biocytin was selected. The brain slices with the recorded neurons were post-fixed overnight after whole-cell recording. The brain slices were rinsed with 0.01 M PBS 3–5 times, and then incubated with streptavidin in 0.01 M PBST (1:1000) at 4 °C for 12 h. Then the sections were rinsed with 0.01 M PBS 3–5 times at room temperature and incubated with other antibodies if other labeling was needed. Finally, the antifade solution was applied. Fluorescence images were obtained using an Olympus IX71 microscope or a Leica confocal system.
**Statistical analysis.** Data are expressed as mean ± standard error of the mean (SEM).

Two-way repeated-measures analysis of variance (ANOVA) was used to compare multiple data between the groups. Paired and unpaired *t*-tests were used for single-value comparisons. One-way ANOVA was used to compare more than two groups, and the *post-hoc* Tukey test was applied for multiple pairwise comparisons. A two-tailed *p*-value < 0.05 was considered statistically significant. We used SPSS software (version 16.0; IBM, Corp., Armonk, NY, USA) for data analysis.
Data availability

The data that support the findings of this study are available from the corresponding authors upon reasonable request.
Code availability

The custom-written analysis code is available from the corresponding author upon reasonable request.
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Author contributions

Z.L.H, P.L. and Y.Q.W conceived the study. L.L., Y.Q.W., and S.A. performed the behavioral experiments. L.L., and Y.Q.W. performed the anatomical experiments. J.B.J. performed the electrophysiological experiments. Y.Q.W, L.L., P.L. and Z.L.H. wrote the paper.
Competing interests

The authors declare no competing interests.
References


Fig. 1 Lateral periaqueductal gray (LPAG) neurons expressing c-Fos during rapid eye movement (REM) sleep deprivation (RSD) and REM sleep rebound (RSR) are glutamatergic. a Scheme for the rats submitted to 72-h protocol of control (RSC), RSD, and RSR using the flowerpot method. b–e Photomicrographs showing c-Fos (brown nuclear stained) and Vglut2 (blue diffuse cytoplasmic stained) double-stained neurons in the LPAG of the RSD (b, c) and RSR (d, e) rats. c and e show the area inside the rectangle in b and d at a higher magnification. The red arrowheads indicate the double-labeled neurons in the LPAG. Scale bars: 100 μm in b and d and 20 μm in c and e. f Schematic distribution of single c-Fos+ (black dots) and double-labeled (c-Fos+/Vglut2+, red dots) neurons on a coronal section in representative animals under RSC, RSD, and RSR conditions. g Histogram showing the numbers of single c-Fos+ and the percentage of double-labeled (c-Fos+/Vglut2+) neurons within the LPAG and neighboring areas under each experimental condition. Values are presented as means ± standard error of mean (SEM) (n = 4). ** p < 0.01 compared to RSC animals, *** p < 0.01 compared to RSD animals, using one-way ANOVA followed by Tukey’s post hoc test.
Fig. 2 Population activity of LPAG

Vglut2 neurons across sleep–wake states. a Schematic of in vivo fiber photometry recordings. b Unilateral viral targeting of AAV-EF1α-DIO-GCaMP6f into the LPAG of Vglut2-Cre mouse and the tip of the fiber optic located above the LPAG. Scale bar: 200 µm. c Representative fluorescent traces, relative electroencephalography (EEG) power, and EEG/electromyography (EMG) traces across spontaneous sleep–wake states. d ΔF/F peaks during non-REM (NREM) sleep, wakefulness, and REM sleep. The fluorescent peak value was normalized by mean ΔF/F peaks in NREM sleep (n = 3 mice, 11 sessions per mouse, **p < 0.01, one-way ANOVA followed by Tukey’s post hoc test). e Fluorescent signals aligned with
sleep–wake transitions. Upper panel: Individual transitions with color-coded fluorescent intensities. Lower panel: Average calcium transients from the transitions expressed as mean ± SEM.
Fig. 3 Chemogenetic activation of LPAG\textsuperscript{Vglut2} neurons promotes wakefulness and immobility. a Vglut2-Cre mice were infused with AAV expressing hM3Dq in a Cre-dependent manner under the control of the Vglut2 promoter. b Drawings of
superimposed AAV-injected areas in the LPAG of Vglut2-Cre mice \((n = 8\), indicated with different colors). c Membrane potential changes induced by current injection in a non-spontaneous firing neuron. Resting membrane potential: \(-70\) mV. d Typical example of membrane potential recorded from an hM3Dq-expressing Vglut2 neuron during the application of clozapine-N-oxide (CNO) to brain slices. Bath application of CNO (5 \(\mu\)M) produced depolarization and firing. e, f CNO induced a significant increase in firing frequency and depolarization in eight Vglut2 neurons. Individual and average values of the firing frequency (e) and membrane potential (f) are shown on the scatter plot. g-j Typical samples of two LPAG-hM3Dq mice injected with either vehicle (g, h) or CNO (i, j). The full line rectangle areas in g and i were magnified (h, j) to demonstrate the co-expression of hM3Dq fusion protein mCherry and c-Fos, which is indicated by red arrows. k, l Typical examples of one LPAG-hM3Dq mouse each injected (i.p.) with vehicle (k) and CNO at 1 mg/kg (l). m Time course of wakefulness, NREM sleep and REM sleep in Vglut2-Cre mice expressing hM3Dq after administration of vehicle or CNO. n The total time spent in wakefulness, NREM sleep, and REM sleep in Vglut2-Cre mice expressing hM3Dq for 7 h after administration of vehicle or CNO. o EEG power density for 7 h after administration of vehicle or CNO. The power of each 0.25-Hz bin was averaged and normalized by calculating the percentage of each bin from the total power (0–25 Hz). The horizontal bars indicate statistical differences \((p < 0.05)\). p Quantification and comparison of the EMG\(_{\text{Wake}}\) (the EMG amplitude during wakefulness) between vehicle and CNO groups. q The ratio of EMG\(_{\text{T-Wake}}\) (the EMG amplitude during wakefulness after treatment with vehicle or CNO) and EMG\(_{\text{N-Wake}}\) (the EMG amplitude during the normal wakefulness stage) between the vehicle and CNO groups. r The locomotor activity in Vglut2-Cre mice expressing hM3Dq for 15 min after administration of vehicle or CNO. s A representative trajectory plot of a Vglut2-Cre mouse after administration of vehicle or CNO. t Mean immobility time in Vglut2-Cre mice expressing hM3Dq for 15 min after administration of vehicle or CNO. Data are presented as mean \(\pm\) SEM \((n = 8–12)\). Each dot indicates data from a single mouse. * \(p < 0.05\), ** \(p < 0.01\), compared to the vehicle
group, using repeated measures ANOVA followed by a paired $t$-test ($m$), a paired $t$-test ($e, f, n, o-q$), and one-way ANOVA followed by Tukey’s post hoc test ($r, t$).
Fig. 4 Optogenetic activation of LPAG^Vglut2 neurons induces wakefulness and immobility. a Vglut2-Cre mice injected with AAV-DIO-ChR2-mCherry into the LPAG. b Representative images of mCherry immunofluorescence in the LPAG. Scale bar: 500 µm. c Drawings of superimposed AAV-injected areas in the LPAG of Vglut2-Cre mice (n = 6, indicated with different colors). d The electrophysiological properties of the recorded Vglut2 neuron. e The responses of ChR2-expressing Vglut2 neuron in the LPAG to 5-ms blue light (473 nm) photostimulation at 30 Hz. f Fidelity responses of the recorded ChR2-expressing Vglut2 neurons in the LPAG to blue light pulses at frequencies up to 100 Hz. g, j Representative EEG/EMG traces and heat map of EEG power spectra showing acute photostimulation (30 Hz/5 ms) of blue (g) and yellow (j)
light applied during NREM sleep in a ChR2-mcherry mouse. h, i, k, l Sleep stages after blue (h, i) and yellow (k, l) light stimulation in ChR2-mCherry mice. m Latencies of transitions from NREM sleep (S) to wakefulness (W) after photostimulation at different frequencies. n EEG power density before and after the photostimulation in ChR2-mCherry mice. The power of each 0.25-Hz bin was averaged and normalized by calculating the percentage of each bin from the total power (0–25 Hz). The horizontal bars indicate statistical differences (p < 0.05). o Quantification and comparison of the EEG power density before and after the photostimulation in ChR2-mCherry mice. p The ratio of EMG<sub>Wake</sub> (the integral muscle tone intensity during wakefulness) before and after the photostimulation in ChR2-mCherry mice. q Mean immobility time during base and blue light stimulation in ChR2-mcherry mice. r-t Time course of wakefulness (r), NREM sleep (s), and REM sleep (t) during a semi-chronic optogenetic experiment (30 Hz/5 ms, 40 s on/20 s off) before and after the photostimulation in ChR2-mCherry mice. u Total amounts of each stage before and after the photostimulation in ChR2-mCherry mice. Data are presented as mean ± SEM (n = 6). Each dot indicates data from a single mouse. * p < 0.05, ** p < 0.01, compared to yellow light (m) and the base group (n-u), using repeated measures ANOVA followed by a paired t-test (r-t) or paired t-test (m-q, u).
Fig. 5 Optogenetic stimulation of the LPAG$^{Vglt2}$-LC pathway induces wakefulness and immobility. a Schematic drawing shows ChR2-expressing LPAG and the targeted downstream nuclei, the locus coeruleus (LC). b Representative images of the mCherry immunofluorescence in LPAG and the terminals in the LC from the LPAG$^{Vglt2}$ neurons. Scale bar: 500 µm. c Diagrams of superimposed AAV-injected areas in LPAG and the terminals in the LC of Vglut2-Cre mice ($n = 4$, indicated with different colors). d Typical traces representing the inward current elicited by photostimulation of ChR2-
expressing LPAG axons at the LC under different conditions. e The proportion of recorded LC neurons that did and did not respond to the photostimulation of ChR2-expressing LPAG terminals. f The latency between the delivery of photostimulation and the firing event of the responding LC neurons are summarized. g Typical example of a connected biocytin-labeled neuron that was tyrosine hydroxylase (TH)-positive. Scale bar: 20 µm. h, k Representative EEG/EMG traces and heat map of EEG power spectra showing acute photostimulation (30 Hz/5 ms) of the LPAG\textsuperscript{Vglut2}-LC pathway with blue (h) and yellow (k) light applied during NREM sleep in a ChR2-mcherry mouse. i, j, l, m Sleep stages after blue (i, j) and yellow light (l, m) stimulation of the LPAG\textsuperscript{Vglut2}-LC pathway in ChR2-mCherry mice. n EEG power density before and after photostimulation of the LPAG\textsuperscript{Vglut2}-LC pathway in ChR2-mCherry mice. The power of each 0.25-Hz bin was averaged and normalized by calculating the percentage of each bin from the total power (0–25 Hz). The horizontal bars indicate statistical differences ($p < 0.05$). o Quantification and comparison of the EMG\textsubscript{Wake} (the integral muscle tone intensity during wakefulness) before and after photostimulation of the LPAG\textsuperscript{Vglut2}-LC pathway in ChR2-mCherry mice. p The ratio of EMG\textsubscript{T-Wake} (the EMG amplitude during wakefulness before and after treatment with blue light) and EMG\textsubscript{N-Wake} (the EMG amplitude during normal wakefulness stage) before and after photostimulation of the LPAG\textsuperscript{Vglut2}-LC pathway in ChR2-mCherry mice. q Mean immobility time during base and blue light stimulation of the LPAG\textsuperscript{Vglut2}-LC pathway in ChR2-mCherry mice. r, s, t Time course of wakefulness (r), NREM (s) and REM sleep (t) during semi-chronic optogenetic experiment (30 Hz/5 ms, 40 s on/20 s off) before and after photostimulation of the LPAG\textsuperscript{Vglut2}-LC pathway in ChR2-mCherry mice. u, Total amounts of each sleep stage before and after photostimulation of the LPAG\textsuperscript{Vglut2}-LC pathway in ChR2-mCherry mice. Data are presented as the mean ± SEM. Each dot indicates data from a single mouse ($n = 4$). * $p < 0.05$, ** $p < 0.01$, compared to the base group (n-u), using repeated measures ANOVA followed by a paired $t$-test (r-t) or paired $t$-test (n-q, u).
Fig. 6 Optogenetic stimulation of the LPAG\textsuperscript{Vglut2}-GiV pathway induces wakefulness and immobility. a Schematic drawing shows ChR2-expressing LPAG and the targeted downstream nuclei, the ventral gigantocellular reticular nucleus (GiV). b Representative images of the mCherry immunofluorescence in LPAG and the terminals in the GiV from the LPAG\textsuperscript{Vglut2} neurons. Scale bar: 500 µm. c Diagrams of superimposed AAV-injected areas in LPAG and the terminals in the GiV of Vglut2-Cre mice (\(n = 4\), indicated with different colors). d Typical traces representing the inward
current elicited by photostimulation of ChR2-expressing LPAG axons at the GiV under different conditions. e, The proportion of recorded GiV neurons that did and did not respond to photostimulation of ChR2-expressing LPAG terminals. f The latency between the delivery of photostimulation and the firing event of the responding GiV neurons are summarized. g Typical example of a connected biocytin-labeled neuron that was Vglut2- and glycine-positive. Scale bar: 20 µm. h, k Representative EEG/EMG traces and heat map of EEG power spectra showing that acute photostimulation (30 Hz/5 ms) of the LPAGVglut2-GiV pathway with blue (h) and yellow (k) light applied during non-rapid eye movement (NREM) sleep in a ChR2-mCherry mouse. i, j, l, m Sleep stages after blue (i, j) and yellow light (l, m) stimulation of the LPAGVglut2-GiV pathway in ChR2-mCherry mice. n EEG power density before and after photostimulation of the LPAGVglut2-GiV pathway in ChR2-mCherry mice. The power of each 0.25-Hz bin was averaged and normalized by calculating the percentage of each bin from the total power (0–25 Hz). The horizontal bars indicate statistical differences (p < 0.05). o Quantification and comparison of the EMGWake (the EMG amplitude during wakefulness) before and after photostimulation of the LPAGVglut2-GiV pathway in ChR2-mCherry mice. p The ratio of EMGf-Wake (the EMG amplitude during wakefulness before and after treatment with blue light) and EMGN-Wake (the EMG amplitude during the normal wakefulness stage) before and after photostimulation of the LPAGVglut2-GiV pathway in ChR2-mCherry mice. q Mean immobility time during base and blue light stimulation of the LPAGVglut2-GiV pathway in ChR2-mCherry mice. r-t Time course of wakefulness (r), NREM sleep (s), and REM sleep (t) during semi-chronic optogenetic experiment (30 Hz/5 ms, 40 s on/20 s off) before and after photostimulation of the LPAGVglut2-GiV pathway in ChR2-mCherry mice. u Total amounts of each sleep stage before and after photostimulation of the LPAGVglut2-GiV pathway in ChR2-mCherry mice. Data are presented as mean ± SEM. Each dot indicates data from a single mouse (n = 4). * p < 0.05, ** p < 0.01, compared to the base group (n-u), using repeated measures ANOVA followed by a paired t-test (r-t) or paired t-test (n-q, u).
Fig. 7 Chemogenetic inhibition of LPAG\textsuperscript{Vglut2} neurons decreases REM sleep and increases NREM sleep. a Vglut2-Cre mice were infused with AAV expressing hM4Di in a Cre-dependent manner under the control of the Vglut2 promoter. b Diagrams of superimposed AAV-injected areas in the LPAG of Vglut2-Cre mice (n = 8, indicated
with different colors). c Typical traces of an hM4Di-expressing LPAG Vglut2 neuron bath applied with CNO) at a concentration of 5 µM. d, e Firing frequency (d) and membrane potential (e) from CNO (5 µM) application to the electrophysiological bath in five Vglut2 neurons. f, g, h, i Typical samples of two LPAG-hM4Di mice injected with either vehicle (f, g) or CNO (h, i). The full line rectangle area in f and h were magnified (g, i) to show the co-expression of hM4Di fusion protein mCherry and c-Fos, which is indicated by red arrows. j, k Typical examples of one LPAG-hM4Di mouse injected (i.p.) with vehicle (j) and CNO at 1 mg/kg (k). l Time course of REM sleep, NREM sleep, and wakefulness in Vglut2-Cre mice expressing hM4Di after administration of vehicle or CNO. m The total time spent in REM sleep, NREM sleep, and wakefulness in Vglut2-Cre mice expressing hM4Di for 3 h after administration of vehicle or CNO. n EEG power density for 3 h after administration of vehicle or CNO. The power of each 0.25-Hz bin was averaged and normalized by calculating the percentage of each bin from the total power (0–24.75 Hz). o The locomotor activity in Vglut2-Cre mice expressing hM4Di for 15 min after administration of vehicle or CNO. p A representative trajectory plot of a Vglut2-Cre mouse after administration of vehicle or CNO. q Mean immobility time in Vglut2-Cre mice expressing hM4Di for 15 min after administration of vehicle or CNO. Data are presented as mean ± SEM (n = 6–10). Each dot indicates data from a single mouse. * p < 0.05, ** p < 0.01 compared to the vehicle group, using repeated measures ANOVA followed by a paired t-test (l) or a paired t-test (m).
**Fig. 8** LPAG$_{\text{Vglut2}}$ neurons were activated during REM sleep through the projections to the SLD. **a** Schematic of the experiment. AAV-ChR2 was injected into the LPAG of Vglut2-Cre mice, and the response was recorded in the SLD. **b** Typical traces representing the inward current elicited by photostimulation of ChR2-expressing LPAG axons at the SLD under different conditions. **c** The proportion of recorded SLD neurons that did and did not respond to the photostimulation of ChR2-expressing LPAG terminals. **d** The latency between delivery of photostimulation and the firing event of the responding SLD neurons are summarized. **e** Typical example of a connected biocytin-labeled neuron that was Vglut2-positive. Scale bar: 20 μm. **f** Scheme of the injection of CTb in rats that were submitted to 72-h protocol of REM sleep rebound (RSR). **g, h** Photomicrographs illustrating representative injection sites in the SLD (g) and the GiV (h). **i-l** Low- (i, k) and high-power (j, l) photomicrographs showing the LPAG neurons active after RSR and projecting to the SLD (i, j) and GiV (k, l). **j** and **l** show the area inside the rectangle in **i** and **k** at a higher magnification. Arrows indicate double-stained neurons (c-Fos+/CTb+). **m** Histogram showing the numbers of total c-Fos+ and CTb+ neurons and the percentage of double-labeled neurons within LPAG for each experimental condition (SLD-RSR rats, n = 4; GiV-RSR rats, n = 4). **p <
0.01 compared to GiV-RSR animals, using one-way ANOVA followed by Tukey’s post hoc test.
Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

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