Cortical layer 6b mediates state-dependent changes in brain activity and effects of orexin on waking and sleep

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Key words: layer 6b, orexin, sleep homeostasis, cortex, EEG, slow-wave activity, sleep, SNAP25, brain state control

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Abstract

One of the most distinctive features of the mammalian cerebral cortex is its laminar structure. Of all cortical layers, layer 6b (L6b) is by far the least-studied, despite exhibiting direct sensitivity to orexin and having widespread connectivity, suggesting an important role in regulating cortical oscillations and brain state. We performed chronic electroencephalogram (EEG) recordings in mice in which a subset of L6b neurons was conditionally "silenced", during undisturbed conditions, after sleep deprivation (SD), and after intracerebroventricular (ICV) administration of orexin. While the total amount of waking and sleep or the response to SD were not altered, L6b-silenced mice showed a slowing of theta-frequency (6-9 Hz) during wake and REM sleep, and a marked reduction of total EEG power, especially in NREM sleep. The infusion of orexin A increased wakefulness in both genotypes, but the effect was more pronounced in L6b-silenced mice, while the increase in theta-activity by orexin B was attenuated in L6b silenced animals. In summary, we show the role of cortical L6b in state-dependent brain oscillations and global vigilance state control, which could be mediated by orexinergic neurotransmission. Our findings provide new insights in the understanding of abnormal regulation of arousal states in neurodevelopmental and anxiety disorders.

Introduction

Layer 6b (L6b) is the earliest generated and deepest layer of the mouse neocortex and is partially derived from the subplate – a critical structure during cortical development which contributes to the formation of intracortical and thalamocortical networks (McConnell et al., 1989; Allendoerfer and Shatz, 1994; Molnár and Blakemore, 1995; Molnár et al., 1998; Feldmeyer, 2023). While evolutionary conservation of L6b neurons and their primate homologue interstitial white matter neurons (IWN) across many species suggests that these cells are not merely a remnants of a developmental cell population, their functional significance in the adult brain remains unknown (Kostovic and Rakic, 1980; Reep, 2000; Kostović and Judaš, 2010; Duque et al., 2016; Swiegers et al., 2019, 2021; Bhagwandin et al., 2020).

L6b exhibits widespread anatomical connectivity, implying an integrative role across a range of functional brain networks. L6b receives long-range intracortical inputs, enabling direct communication between distant cortical areas (Hoerder-Suabedissen et al., 2018; Zolnik et al., 2020), while being reciprocally innervated by higher order thalamic nuclei and cortical layer 5 (L5) (Zolnik et al., 2024a). Consistent with this notion, it was shown that optogenetic activation of L6b neurons leads to activation of the posterior medial nucleus of the thalamus (Ansorge et al., 2020) and the neocortex (Zolnik et al., 2024a). L5, the major output layer of the cortex, is also bidirectionally communicative with higher order thalamic nuclei (Hoerder-Suabedissen et al., 2018), and has been shown to be important in controlling brain state and

sleep regulation (Krone et al., 2021). L6b neurons furthermore project to cortical layer 1 (L1) (Clancy and Cauller, 1999) and the hippocampus (Ben-Simon et al., 2022), suggesting an additional role in higher order cognitive functions. L6b is therefore uniquely placed to integrate information from both subcortical and cortical regions, convey signals across L1, L5, and higher order thalamus, and promote cortical activation (Hoerder-Suabedissen et al., 2018).

Another important property of L6b neurons is their direct responsiveness to orexin (also known as hypocretin), a powerful wake-promoting neurotransmitter (De Lecea et al., 1998; Sakurai et al., 1998; Bayer et al., 2004; Wenger Combremont et al., 2016a, 2016b; Messore et al., 2024; Zolnik et al., 2024a). Experimental studies have shown that optogenetic activation of orexin neurons promotes awakening (Adamantidis et al., 2007), while optogenetic inhibition promotes sleep (Tsunematsu et al., 2011). The significance of orexin in brain state control is especially apparent in narcolepsy, wherein loss of orexin signalling results in state instability, including sleep attacks and fragmented sleep (Sakurai, 2007). L6b has been postulated to be involved in brain state control by forming an orexin gated feed-forward loop driving brain state control (Hay et al., 2015). In support of this, recent findings with multielectrode array recordings show that cortical activation in prefrontal cortex by orexin is impaired in L6b silenced mice (Messore et al., 2024).

We targeted L6b neurons by using a dopamine receptor 1a (Drd1a)-Cre expressing mouse strain. In this mouse line, Cre is relatively selectively expressed in the lower layers of the cortex (Hoerder-Suabedissen et al., 2018; Zolnik et al., 2024a). Subcortically, sparse expression is found in the hippocampus and the striatum, with denser expression in several midbrain nuclei and in the cerebellum (Hoerder-Suabedissen et al., 2018). Drd1a-Cre cortical expression is specific to excitatory neurons and has been quantified to include 25-40% of L6b neurons in the somatosensory cortex (Hoerder-Suabedissen et al., 2018; Zolnik et al., 2020). These Drd1a-Cre neurons in the primary somatosensory cortex receive predominantly long-range intracortical input (Zolnik et al., 2020), project cortically to L1 and L5, and project subcortically selectively to higher order thalamic nuclei (e.g. lateral posterior (LP) and posterior (Po) nuclei, avoiding first order thalamic nuclei including the dorsal lateral geniculate nucleus, ventrobasal nucleus, and medial geniculate nucleus) (Hoerder-Suabedissen et al., 2018). Drd1a-Cre L6b projections do not exhibit collaterals in the thalamic reticular nucleus (TRN) and form small boutons at their targets (Hoerder-Suabedissen et al., 2018; Casas-Torremocha et al., 2022).

In this study, we continuously recorded EEG/EMG signals across sleep-wake states in ‘L6b silenced’ mice and Cre negative littermate controls (CTL). We achieved L6b silencing by selectively ablating synaptosomal protein of 25 kDa (SNAP25) from orexin-sensitive dopamine receptor 1a (Drd1a)-Cre expressing neurons in L6b in mice from birth (Hoerder-Suabedissen et al., 2018). We found that L6b silenced mice show a slowing of theta-frequency

oscillations during both wakefulness and REM sleep, and an overall decrease in EEG power centred within the spindle frequency range during NREM sleep. Furthermore, during sleep deprivation, slow EEG power increased to a lesser extent in L6b silenced mice, as compared to control animals. Intriguingly, intracerebroventricular (ICV) administration of orexin A resulted in a greater increase in wake duration in L6b silenced compared to control animals; however, at the same time the theta frequency increase observed after orexin B infusion was attenuated in L6b silenced animals, suggesting that activity in L6b has a role in establishing the effects of orexin on cortical activation. Our findings suggest that L6b plays an essential role in state-dependent dynamics of brain activity, vigilance state control and sleep regulation.

Results

The amount of time spent in sleep-wake states is not altered in L6b-silenced mice. In the cortex of L6b silenced mice, Drd1a-Cre expression is most prominent in L6b yet also observed in L6a and occasionally in L5, especially in anterior and medial cortical areas (Fig. 1). No Drd1a-Cre expression was detected in the suprachiasmatic nucleus (Fig. S1).

First, we performed EEG and EMG recordings in control and L6b silenced mice over an undisturbed baseline recording as previously (Yamagata et al., 2021). Electrodes were implanted in the fontal and in the occipital derivation to monitor state-specific brain oscillations including EEG slow-wave activity (SWA, 0.5-4Hz) and spindle-frequency (10-15 Hz) activity, which are both more prominent in anterior derivations in mice (Vyazovskiy et al., 2004; Vyazovskiy and Tobler, 2005a; Blanco-Duque et al., 2024), and theta-activity, which is typically more pronounced in posterior electrodes (Huber et al., 2000)(Fig. 2A). Visual inspection of EEG traces confirmed that the key features of vigilance states were clearly identifiable in both genotypes (Fig. 2B). We then plotted daily profiles of SWA and spectrograms (representative individuals: Fig. 2C), which revealed familiar spectral signatures of waking, NREM, and REM sleep in both control and L6b silenced animals. Specifically, we observed that high SWA (0.5-4 Hz) was prominent during epochs scored as NREM sleep, while theta activity (4-10 Hz) was strongest during epochs scored as REM sleep. High levels of SWA during NREM sleep were most evident in the frontal EEG, reflecting more frequent occurrence of slow waves in anterior brain areas. The theta peak was most pronounced in the occipital EEG as the occipital EEG electrode is closest to the hippocampus in rodents. Plotting individual hypnograms indicated that, in all animals regardless of genotype, sleep occurred mostly during the light phase and wakefulness was more consolidated during the dark period as expected for mice in laboratory conditions (Fig. 2D). This analysis reassured us that functionally silencing neocortical L6b neurons does not result in the emergence of any atypical brain oscillations or states under baseline conditions which may contaminate spectral analyses or compromise our ability to annotate vigilance states using conventional criteria.

Across 24 hours, L6b silenced and control animals spent comparable time in wakefulness (controls 42.5±1.30% vs L6b silenced 41.3±1.82%, $t_{(14)}$=0.5059, p=0.6208), NREM sleep (controls 45.0±0.799% vs L6b silenced 46.6±1.55%, $t_{(14)}$=0.8672, p=0.4005), and REM sleep (controls 7.97±0.710% vs L6b silenced 7.41±0.407%, $t_{(14)}$=0.7262, p=0.4797). Calculating the time course of vigilance states across 24h did not reveal significant differences between genotypes (Fig. 2E).

Next, we addressed whether L6b silencing affects continuity of sleep-wake states. We found that the average duration of individual episodes of wakefulness (controls 14.3±1.29 min vs L6b silenced 14.7±1.43 min, $t_{(14)}$=0.1663, p=0.8703), NREM (controls 4.28±0.200 min vs L6b silenced 4.50±0.298 min, $t_{(14)}$=0.5671, p=0.5796), and REM sleep episodes (controls 1.04±0.0344 min vs L6b silenced 1.10±0.0523 min, $t_{(14)}$=0.9792, p=0.3441) did not differ between genotypes. Likewise, the number of brief awakenings (≤16s) was not altered (controls 51.1±2.04 h^{-1} vs L6b silenced 51.6±3.97h^{-1}, $t_{(12)}$=0.1205, p=0.9061).

Consistent with EEG/EMG defined vigilance states, passive infrared monitoring of locomotor activity, undertaken in a separate circadian phenotyping study, revealed only minor differences between genotypes. In light-dark conditions, the endogenous period was close to 24h in both genotypes (control 24.0 ± 0.006 h, L6b silenced 24.0 ± 0.01 h, $t_{(11)}$=0.1422, p=0.8895); however, the active phase, Alpha, was slightly longer in L6b silenced animals (control 12.5 ± 0.156 h vs L6b silenced 13.4 ± 0.349 h, $t_{(11)}$=2.463, p=0.0315). The number of activity bouts per day, the duration of activity bouts, and the number of activity counts per bout did not differ with L6b silencing. However, the number of activity counts appeared reduced in L6b silenced animals during the light period (24h: ($t_{(11)}$=2.108, p=0.0588; Light phase: ($t_{(11)}$=2.988, p=0.0124; Dark phase ($t_{(11)}$=1.804, p=0.0987). Constant darkness experiments revealed that neither the intrinsic period nor alpha differed between genotypes (period: control 23.8 ± 0.0605 h vs L6b silenced 23.6 ± 0.0626 h, $t_{(11)}$=1.721, p=0.1133; alpha: control 16.2 ± 0.583 h vs L6b silenced 14.9 ± 0.737 h, $t_{(11)}$=1.457, p=0.1731). The number of bouts per day, the duration of individual bouts, and the number of activity counts per bout were similar between genotypes, but the number of activity counts per day again showed a trend towards lower activity in L6b silenced animals ($t_{(11)}$=2.045, p=0.0656).

Layer 6b silencing leads to state-dependent changes in the EEG. After examining daily sleep-wake amount, time course, and architecture, we investigated the effect of L6b silencing on EEG power spectra. During wakefulness, only minor changes were observed in the frontal derivation. However, differences were more pronounced in the occipital EEG, especially in the higher theta frequency range where power was significantly reduced due to a leftward shift of theta peak frequency (Fig. 3A, 3D, 3E). Higher frequencies were not significantly affected (Fig.

S2, Suppl. T1). During REM sleep, L6b silencing resulted in a reduction of frontal EEG power in the theta and higher frequency ranges (Fig. 3A, Fig. S2, Suppl. T1). In the occipital derivation, we observed a leftward shift of theta-peak frequency in L6b silenced animals (Fig 3D, 3E). During NREM sleep, L6b silencing markedly reduced frontal EEG spectral density across nearly the entire frequency range examined, from 3Hz upward (Fig. 3A, Fig S2, Suppl. T1). No differences in the occipital EEG were apparent across low frequencies, aside from a slight decrease in frequencies above 80 Hz (Fig. S2). To further investigate state-specificity of the differences observed, we examined changes in EEG spectra around these transitions (Fig. 3B). This revealed that the power decrease in NREM sleep was especially prominent in the frontal derivation around the spindle-frequency range (Blanco-Duque et al., 2024), while a shift in theta-peak frequency during REM sleep was especially apparent in the occipital derivation. Importantly, the change in EEG power was not specific to the time interval immediately before state transitions, as normalising EEG spectra in the 32 seconds of NREM preceding NREM-REM transitions to overall NREM spectral power density on baseline day abolished differences between genotypes (Fig. 3C).

Layer 6b silencing affects spectral signatures of sleep pressure during waking and sleep. Next, we investigated the effects of L6b silencing on homeostatic sleep regulation by undertaking 6 hours of sleep deprivation (SD) starting from light onset. This manipulation is usually associated with an increase in EEG indices of cortical arousal (Vyazovskiy and Tobler, 2005b; Vassalli and Franken, 2017; Yamagata et al., 2021), intrusion of sleep-like patterns of activity in wake EEG (Vyazovskiy et al., 2011), and subsequently increased EEG SWA during NREM sleep (Thomas et al., 2020). SD was successful in all animals, with only minimal sleep observed during the 6-hour SD procedure (controls 15.0±3.73 min vs L6b silenced 14.3±3.97 min, $t_{(14)}$=0.1240, p=0.9030). Animals fell asleep soon after the end of SD, where the latency to the first consolidated NREM sleep did not differ significantly between genotypes (controls, 6.75±2.92 min, L6b silenced 11.2±2.56 min, $t_{(14)}$=1.153, p=0.2680). Representative hypnograms of two individual animals during the sleep deprivation experiment are shown in Fig 4A.

First, we compared wake EEG spectra during sleep deprivation between genotypes. In both control and layer 6b silenced animals, EEG theta-activity and higher EEG frequencies appeared to be increased. This indicates a heightened wake “intensity” or level of arousal (Krone et al., 2021; Yamagata et al., 2021) induced by continuous provision of novel objects (Fig. 4B). However, the increase in wake slow frequency power during SD in the occipital derivation was attenuated in L6b silenced animals (Fig. 4C).

In both genotypes, SD was followed by an initial increase in NREM EEG power in the slow-wave frequency range (Fig. 4D), with an expectedly smaller change in the occipital

derivation compared to the frontal derivation. Plotting the time course of NREM sleep SWA across the first 6 h after SD revealed a typical dynamic with higher values of SWA at the beginning of recovery sleep followed by its progressive decline in both genotypes and in both derivations (Fig. 4E). An exponential fit was performed to compare the rate of SWA dissipation after the end of SD in L6b silenced animals to controls. When the time constant was estimated across the 6 hours after the end of SD, there was no significant difference between genotypes in the frontal EEG (controls –0.30 ± 0.084 h-1 vs L6b silenced –0.17 ± 0.023 h-1; $t_{(6.925)}$= 1.451, p= 0.19, unpaired Welch's t test) or occipital EEG (controls –0.12 ± 0.017 h-1 vs L6b silenced –0.12 ± 0.018 h-1, $t_{(10.46)}$= 0.1675, p=0.87) (Fig. 4F,G). However, when the time constant was estimated across the first 2 hours after the end of SD only, there was a significantly lower time constant in L6b silenced animals compared to controls in the frontal EEG (controls -0.57 ± 0.060 h-1 vs L6b silenced -0.28 ± 0.029 h-1, $t_{(8.723)}$=4.286, p=0.0022, unpaired Welch's t test)(Fig. 4G). SWA dissipation in the occipital EEG again did not differ between genotypes (controls -0.33 ± 0.026 h-1 vs L6b silenced -0.34 ± 0.029 h-1, $t_{(10.73)}$=0.2732, p=0.79).

Layer 6b mediates effects of orexin on vigilance states. Finally, we compared effects of intracerebroventricular (ICV) orexin administration between control and layer 6b silenced animals. Histology confirmed that cannulas were positioned in the right lateral ventricle in all animals. Vehicle, orexin A, or orexin B were infused at light onset (Fig. 5A,B). Visual inspection of EEG traces did not reveal any abnormalities or obvious differences between genotypes (Fig. S3), therefore sleep scoring was performed blindly using established criteria (Yamagata et al., 2021). No abnormal oscillatory activities were revealed by subsequent spectral analysis in either genotype or derivation (representative individual examples: Fig. S3).

Sleep architecture was considerably affected by orexin administration. As depicted in individual examples, while vehicle administration had no effect on vigilance states, orexin induced prolonged wakefulness in both genotypes (Fig. 5C, Fig. S4). Plotting the 6-hour time course of vigilance states revealed a pronounced increase in wakefulness and decrease in sleep after both doses of orexin A in both genotypes, while effects of orexin B were less prominent (Fig. 5D). Based on these findings that illustrate that the effects completely dissipate beyond three hours (Fig. 5D) and reports in literature regarding the duration of action of orexin (Piper et al., 2000; Huang et al., 2001; Mieda et al., 2011), the time frame used to analyse sleep architecture was defined as the time from the end of the infusion to three hours post-infusion. During this interval, ORXA administration markedly and dose-dependently increased the time spent in wakefulness (Fig. 5E; ORXA Dose, $F_{(1.316,11.18)}$=27.56; p=0.0001), however, there was no difference between genotypes (ORXA Dose x Genotype, $F_{(2,17)}$=2.597, p=0.10). The amount of NREM sleep and REM sleep decreased (NREM: Vehicle controls 91.1 ± 4.73 min vs L6b silenced 96.3 ± 4.61 min, ORXA controls 65.4 ± 6.08 min vs L6b silenced

51.1 ± 4.43 min, $F_{(1.629,14.66)}$=22.30, p<0.0001; REM: Vehicle controls 15.3 ± 2.75 min vs L6b silenced 14.8 ± 1.78 min, ORXA controls 6.23 ± 2.39 min vs L6b silenced 4.07 ± 0.818 min $F_{(1.057,9.516)}$=21.23, p=0.001). In contrast, ORXB did not have a significant effect on vigilance states (as compared to vehicle: Wake, $F_{(1,9)}$=0.02874, p=0.8691; NREM, $F_{(1,9)}$=0.00898, p=0.9266; REM, $F_{(1,20)}$=1.193, p=0.2878).

The latency to onset of consolidated NREM sleep significantly increased following ORXA infusion in both genotypes (Vehicle: controls 36.2 ± 6.94 min vs L6b silenced 27.7 ± 7.58 min, ORXA controls 91.4 ± 10.5 min vs L6b silenced 109 ± 4.82 min, ORXA Dose, $F_{(2,22)}$=50.10, p<0.0001; ORXA Dose x Genotype, $F_{(2,22)}$=2.376, p=0.1164))(Fig. 5F), as did the average duration of wake episodes (Vehicle: controls 19.8 ± 4.92 min vs L6b silenced 10.6 ± 1.40 min; ORXA: controls 25.8 ± 5.39 min vs L6b silenced 39.7 ± 7.53 min, ORXA Dose, $F_{(2,22)}$=8.995, p=0.0014). Interestingly, the increase in average wake episode duration was significantly greater in L6b silenced animals (ORXA dose x Genotype interaction, $F_{(2,22)}$=4.376, p=0.0251). Lastly, both ORXA and ORXB infusion significantly increased the number of brief awakenings from NREM sleep in both genotypes (ORXA Dose, $F_{(1.460,8.759)}$=7.907, p=0.0149, Mixed-effects model; ORXB Dose, $F_{(7)}$=6.431, p=0.0389, two-way ANOVA). Overall, these results confirm our prediction that ORXA promotes wakefulness, but unexpectedly revealed a somewhat greater effect in L6b silenced animals.

Layer 6b mediates effects of orexin on brain activity. Finally, we investigated whether ORXA and ORXB affect state-dependent EEG oscillations during sleep and wakefulness. For wakefulness, EEG analysis was performed on all artefact-free epochs between the closure of recording boxes and the occurrence of the first consolidated sleep episode (see Methods). Both ORXA and ORXB administration led to a reduction of EEG power between 10-20 Hz and/or increased theta-frequency power (Fig. 6A, 6B). There was a significant Frequency x Genotype interaction for both the frontal and occipital EEG spectra, suggesting that the effects of orexin administration on wakefulness were different between genotypes. Plotting relative EEG spectra revealed a shift of theta-frequency towards lower values in L6b silenced animals after ORXB administration (Fig. 6B).

Next, we analysed EEG spectra during NREM sleep, focusing on the first 3 hours after the onset of the first consolidated NREM sleep episode. Because there were sufficient epochs spent in NREM sleep in the 3-hour time frame after vehicle infusion, a comparison was made between NREM spectra with the same time frame for orexin and vehicle infusion (Fig. S5). Overall, ORXA and ORXB administration led to an increase in EEG power during NREM sleep in the lowest frequency range (<3 Hz) but in L6b silenced mice only (Fig. S5). During REM sleep, only a few scattered frequency bins were significantly affected by orexin administration

or different between genotypes, although there was an indication of a higher absolute theta-peak power in layer 6b silenced mice (Figure S6).

Since orexin promotes wakefulness, administration at light onset can be regarded as pharmacological induction of sleep deprivation. While ORXA administration significantly increased the time spent in wakefulness in the first 3 hours, wakefulness returned to normal levels from the third hour onwards and 24-hour percentages in respective vigilance states were comparable after ORXA and vehicle infusions in both genotypes (Wake, $F_{(2,22)}$=0.7016, p=0.9556; NREM, $F_{(1.674,\ 18.41)}$=1.715, p=0.2092; REM, $F_{(1.686,18.55)}$=2.235, p=0.1409) (Figure S7A). The total 24-hour time spent in REM sleep was reduced in L6b silenced animals compared to controls in both vehicle and orexin conditions (Genotype effect, $F_{(1,11)}$=13.99, p=0.0033). Interestingly, levels of relative SWA during the initial 3 hours after sleep onset following ORXA administration (Fig. S7B) were similar in the frontal derivation but decreased in the occipital derivation in L6b silenced as compared to control animals (Genotype, $F_{(1,11)}$=6.818, p=0.0242).

Discussion

L6b plays a role in regulation of arousal states. In this study, we examined sleep-wake regulation in the 'L6b silenced' mouse model, wherein the synaptic protein Snap25 is chronically ablated from birth in the Drd1a-Cre expressing subpopulation of L6b (Drd1a-Cre; Snap25$^{fl/fl}$;Ai14) across the entire cortical mantle. The distribution of vigilance states, and measures of sleep consolidation and sleep fragmentation did not differ between L6b silenced and control animals at 12 weeks of age. However, during wakefulness, EEG activity in the theta-frequency range, the signature of active or exploratory wakefulness (Vyazovskiy et al., 2006; Vassalli and Franken, 2017), was characterised by a lower peak frequency in L6b-silenced animals, while in REM sleep there was a reduction in theta peak frequency, along with a reduction in gamma-frequency power. We posit that silencing L6b impairs the establishment of an activated brain state, which is required to generate a state of arousal and alertness.

Our findings are in line with previous *in vitro* studies showing that L6b neurons can be activated by orexin, neurotensin, noradrenaline, histamine, and dopamine – all of which are powerful promoters of arousal (Bayer et al., 2004; Wenger Combremont et al., 2016a, 2016b). Notably, data from Zolnik *et al.* shows that optogenetically activating the same Drd1a-Cre+ subpopulation of L6b neurons in head-restrained awake mice can promote conversion from delta oscillations to gamma oscillations, thus promoting a state of cortical activation resembling wakefulness and REM sleep (Zolnik et al., 2024a). Our study of long-term

recordings in freely behaving mice supports this same conclusion; L6b is involved in generating active substates of wakefulness.

Anatomically, L6b is ideally positioned to contribute to the regulation of levels of arousal and vigilance, which are known to exhibit a rich dynamics in both waking and sleep (Andrillon, 2023). L6b receives input predominantly from long-range intracortical projections and projects selectively to higher order thalamic nuclei (Hoerder-Suabedissen et al., 2018; Zolnik et al., 2020, 2024a). Therefore, L6b can integrate inputs from wake-promoting subcortical neuromodulatory areas, as well as information from distant cortical areas and translate this into wide-spread cortical activation with additional relay via higher-order thalamic nuclei. Higher order thalamic nuclei can themselves also be directly activated by orexin (Bayer et al., 2002), and orexinergic activation of L6b can enhance the integration of higher order thalamic inputs in L6a (Hay et al., 2015).

A role of cortical L6b in NREM sleep oscillations. Silencing a subpopulation of L6b not only changed cortical activity during wakefulness and REM sleep, but also during NREM sleep. This was reflected in a reduction in NREM EEG power across a broad frequency range, including delta (0.5-4 Hz), theta (5-10 Hz), sigma (10-15 Hz), and gamma (30-100 Hz) frequencies. Slow wave activity (SWA, 0.5-4 Hz) including slow waves and delta waves is a marker of sleep intensity and reflects the levels of sleep pressure, which builds up as a function of preceding wakefulness duration (Borbély, 1982; Achermann and Borbély, 2003). The reduction of delta power during NREM sleep in L6b silenced animals agrees with the reduction of theta power during wakefulness, since the theta substate of wakefulness drives the build-up of sleep pressure (Vyazovskiy and Tobler, 2005b; Vassalli and Franken, 2017; Yamagata et al., 2021). Activity in the delta frequency band is an expression of the synchronization of neuronal populations in slow waves (Steriade et al., 1993; Vyazovskiy et al., 2009; Thomas et al., 2020), therefore the reduction in delta band power in L6b silenced animals suggests a role for L6b in network synchrony.

Sigma power (10-15 Hz) represents the frequency range of sleep spindles (Blanco-Duque et al., 2024), which are waxing and waning bursts of activity that are thought to originate from interactions between cortex, thalamus, and the thalamic reticular nucleus (TRN) (Steriade et al., 1993; Crunelli et al., 2006). Sleep spindles have been linked to memory consolidation in sleep and are posited to facilitate the transfer of information to long-term storage (Helfrich et al., 2018; Hahn et al., 2019). Thus, the reduction in sigma power in L6b silenced animals suggests that L6b may be involved in these functions. L6b does not directly project to the TRN (Hoerder-Suabedissen et al., 2018), yet there are projections from L6b to L5 (Zolnik et al., 2024a), and subpopulations of L5 innervate segments of the TRN in an area-

specific pattern (Carroll et al., 2022; Hádinger et al., 2023), therefore L6b may indirectly influence sleep spindle generation via L5.

An active role for the neocortex in sleep regulation. We showed recently that state transitions occur less frequently when the entire network excitability is reduced in mice with a mutation in the synaptic vesicle protein VAMP2 (Banks et al., 2020). These findings are broadly consistent with the view that brain state transitions start at the local level and may or may not converge to a 'global' brain state transition (Krueger et al., 2008; Thomas et al., 2020; Andrillon et al., 2024). In the current study, we showed that chronic silencing of a subpopulation of excitatory L6b neurons across the entire cortical mantle causes changes in oscillatory activity during sleep and wakefulness. Mice with a chronically silenced subpopulation of cortical L5 across the whole cortical mantle, however, show a substantial reduction in time spent asleep without clearly affecting global EEG oscillations (Krone et al., 2021). These findings suggest that different brain regions or distinct subpopulations of projection neurons in infragranular layers have differential contributions in the dynamics of wake-sleep states or state intensity.

The fact that chronic silencing of both L5 and L6b markedly affects sleep-wake regulation – one in the time spent in different vigilance states, and the other in state-specific oscillations, respectively – strongly suggests that sleep is not only regulated via subcortical systems forming a 'sleep wake switch' at a global level (Saper et al., 2010), but that the neocortex is also actively involved in regulating its own state, likely through both local and distributed intra and extracortical circuits (Krueger et al., 2008). Recent work has shown that activity in the neocortex is involved in cortical synchrony (Ratliff et al., 2024), regulation of sleep depth and duration (Vaidyanathan et al., 2021), sleep homeostasis (Kon et al., 2024), sleep-preparatory behaviour (Tossell et al., 2023), and REM sleep initiation (Wang et al., 2022; Hong et al., 2023) and progression (Dong et al., 2022), all supporting an active role of the neocortex in brain state regulation.

Yet, our current findings also support an important role of subcortical nuclei in the control of local and global cortical states of arousal. In addition to orexinergic effects, as shown here, this likely extends to other neuromodulatory systems including noradrenaline, dopamine, neurotensin, serotonine, and histamine (Constantinople and Bruno, 2011; Wenger Combremont et al., 2016b, 2016a; Case et al., 2017; Osorio-Forero et al., 2021; Bréant et al., 2022).

Clinical implications. Chronic activation or silencing L6b could potentially be employed to model neuropsychiatric diseases such as autism spectrum disorder (ASD) and schizophrenia, which are often result from abnormalities in neurodevelopment and network connectivity. Notably, in both ASD and schizophrenia, subtle histopathological abnormalities have been

found in the human homologue of L6b – interstitial white matter neurons (Connor et al., 2011; Avino and Hutsler, 2021). Sleep-wake symptoms such as insomnia are common in both conditions (Bailey et al., 1998; Miano and Ferri, 2010; Casanova, 2015; Waite et al., 2016; Duchatel et al., 2019), and specifically, decreases in sleep spindles have been linked to schizophrenia (Blanco-Duque et al., 2024; Ferrarelli, 2024), implying that at least some phenotypical characteristics of these conditions can be modelled with the L6b silenced mouse.

Current results involving orexin are relevant to investigations surrounding narcolepsy. Narcolepsy results from insufficient orexin signalling, probably from an autoimmune process targeting orexin neurons. Narcolepsy patients suffer from pathological intrusions of REM sleep and/or NREM sleep into wakefulness, and fragmentation of sleep and wakefulness (Sakurai, 2007). Importantly, many narcolepsy patients suffer from daytime sleepiness, hypersomnolence, and an inability to focus. This could be a result of impaired executive network function due to impaired orexin signalling in the cortex (Naumann et al., 2001; Lambe and Aghajanian, 2003; Rieger et al., 2003; Lambe et al., 2005).

The finding that mice with chronically silenced orexin-sensitive L6b neurons show a reduction in theta-frequency oscillations, the hallmark of exploratory wakefulness, suggests that orexin may have a direct effect on the cortex, which could explain problems with focused attention in narcolepsy patients. *In vitro* recording experiments from human interstitial white matter cells have furthermore confirmed orexin responsiveness (Zolnik et al., 2024b). The role of cortical orexin signalling on focussed attention may not exclusively be mediated through L6b neurons or their human homologues, as some subpopulations of L2/3 and L5 neurons can also be activated by orexin in the PFC (Lambe and Aghajanian, 2003; Xia et al., 2005; Song et al., 2006; Li et al., 2010; Yan et al., 2012). More research is needed to investigate how such effects combine and whether there are differential roles of OX1R signalling, which is mostly expressed in PFC, and OX2R signalling, which seems more important in other cortical areas studied so far (Marcus et al., 2001; Bayer et al., 2004). A better understanding of the effects of activation of OX1R and OX2R in neocortex could also have implications for the development of novel pharmacological therapies for insomnia, specifically dual orexin receptor antagonists (DORAs). DORAs are effective in the treatment of insomnia, but cause increased excessive daytime sleepiness compared to placebo, a side effect that may be related to effects on cortical network activity (Na et al., 2024).

L6b likely has multiple functions across different cellular subtypes and cortical areas, and we argue that some of these functions may be disrupted in neurological and neuropsychiatric conditions. Our findings advance our understanding of the role of the cortex in brain-state regulation and open a new avenue for further research into the role of L6b in transthalamic cortico-cortical processing.

Methods

The 'layer 6b silenced' mouse model. In this study, the gene encoding the synaptic protein Synaptosomal Associated Protein of 25 kDa (SNAP25), which mediates regulated synaptic vesicle release (Hanson et al., 1997; Poirier et al., 1998; Sutton et al., 1998), was selectively ablated from a subpopulation of L6b neurons to render these functionally 'silenced' from the first week of postnatal period. While whole-mouse *Snap25* knockout is impossible due to embryonic lethality (Molnár et al., 2002; Washbourne et al., 2002), embryos with selective *Snap25* elimination from specific cortical projection neurons are viable. *Snap25* ablation from L5, L6a, and L6b, respectively, initially results in normal cortical development, with loss of synapses, demyelination, microglia activation, axonal and neuronal degeneration typically observed at later time points (Hoerder-Suabedissen et al., 2019; Korrell et al., 2019; Vadisiute et al., 2022).

Layer 6b can be targeted in the Tg(Drd1a-cre)FK164Gsat/Mmucd mouse (Drd1a-Cre) line, wherein Cre recombinase is expressed from the time of birth (Gerfen et al., 2013; Hoerder-Suabedissen et al., 2018). To generate these mice, Tg(Drd1a-cre)FK164Gsat/Mmucd (*Drd1a-Cre*; MMRRC)) and C57BL6-Snap25tm3mcw (*Snap25fl/fl)* animals were crossed to the tdTomato reporter strain B6;129S6-Gt(ROSA)26Sortm14(CAG-tdTomato)Hze/J (*Ai14*). The confirmation of the loss of regulated synaptic vesicular release from the Cre+ neuronal population was described in our previous publications (Marques-Smith et al., 2016; Hoerder-Suabedissen et al., 2018; Messore et al., 2024).

For baseline and sleep deprivation recordings, adult male L6b silenced animals (n=9), and Cre-negative adult male littermate control animals (n=7) were used following conventional group sizes in similar studies (Yamagata et al., 2021). For the orexin infusion experiments, separate cohorts of n=8 adult male L6b silenced animals and n=5 adult male littermate control animals were used. For the circadian screen, separate cohorts of young adult male L6b silenced (n=6) and control animals (n=7) were used. Genotypes were confirmed by polymerase chain reaction (PCR) through Transnetyx (Transnetyx Inc, Cordova, Tennessee, USA).

Although the age at the start of sleep experiments was comparable between genotypes (controls 11±0.48 weeks vs L6b silenced 10.6±0.39 weeks $t_{(14)}$=0.6966,p=0.4974), L6b silenced animals had a significantly lower body weight (controls 23±0.67 g vs L6b silenced 21±0.47 g, $t_{(14)}$=2.530, p=0.024). A lower body weight compared to Cre negative control animals has also been previously observed in Rbp4-Cre;Snap25$^{fl/fl}$;Ai14 'L5 silenced' mice (Hoerder-Suabedissen et al., 2019; Krone et al., 2021).

Circadian screen. The circadian screen in L6b silenced mice was performed as a part of initial phenotyping in a separate study. Animals were housed in individual cages located inside light-tight chambers to allow control of the light-dark schedule, with food and water *ad libitum*. Home cage activity was assessed using running wheels, wherein rotations were recorded with a 1-minute resolution in ClockLab (ActiMetrics Inc, Wilmette, Illinois, USA, version 6.1.02). Baseline rhythmicity was investigated with a 12h:12h light-dark schedule, with lights on at 5:00AM. After 21 days, the light schedule was changed to constant darkness for 10 days to assess the intrinsic active period duration (alpha). Next, animals were kept in constant light for 16 days to characterise properties of the circadian clock in constant conditions. Lastly, a 6-hour phase-advance protocol was applied, in which lights were turned off at 11:00AM, corresponding to 6h after light onset, then on at 11:00PM. Recordings continued for 10 days.

Surgical procedures and postoperative care. Surgical implantation of EEG/EMG recording electrodes was carried out as previously described (Krone et al., 2021; McKillop et al., 2021). Bregma was localized and the positions of the electrodes were marked, with the frontal EEG 2 mm anteroposterior (AP), +2 mm to the right of the midline (ML), occipital EEG -3.5mm AP and +2.5mm ML. Holes were created with a microdrill at the marked positions and above the cerebellum for the reference electrode. Next, the EEG/EMG headstage was positioned with the bone screws into the holes. The headstage was secured with dental cement (Super bond, Prestige Dental Products Ltd, Bradford, UK). EMGs were implanted into the nuchal muscles and secured to the headstage with acrylic dental cement (Simplex Rapid, Associated Dental Products Ltd, Swindon, UK). The headstage weight was <10% of presurgical body weight in all animals. Postoperative analgesia was provided through jellies mixed with oral Metacam (meloxicam, 5 mg/kg, Boehringer Ingelheim Ltd., Bracknell, UK) as needed.

For the orexin infusion experiment, surgeries were extended with the unilateral implantation of a cannula in the right lateral cerebral ventricle. To insert the cannula, an additional hole was drilled (AP -0.3mm, ML +0.9mm, DV -2.3mm; coordinates based on (Suzuki et al., 2005)) in the skull, and the guide cannula (C315G/Spc, Plastics1, Protech International Inc., Boerne, Texas, USA) was inserted into the holder in the stereotactic frame and slowly positioned atop the dura. The cannula was then lowered to 2.3 mm below the dura. Silicone elastomer (Kwik-Sil, World Precision Instruments Inc., Sarasota, FL, USA) was applied to close the craniotomy. The guide cannula was secured with dental cement (Super bond, Prestige Dental Products Ltd, Bradford, UK), and a dummy cannula (C315DC/Spc, Plastics1) was inserted into the guide cannula to prevent infection and bore occlusion. Dummies were manually moved in the cannulas every few days to habituate animals to the procedure and to ensure clean cannula bores.

Recording setup. After recovery from surgery, animals were transferred to the recording room. Animals were housed in individual custom-made Plexiglas cages (20x32x35cm^3) with bedding and nesting material under a controlled light-dark cycle (9AM light on, 9PM lights off). Food was supplied *ad libitum* on the floor of the cage and water was provided in a bottle. Two Plexiglas cages were placed in each sound-attenuated recording chamber (Campden Instruments, Loughborough, UK). Recording chambers were lit with an LED strip attached to the top of the chamber (light levels 120-180 lux), which was connected to a timer on the same light-dark schedule as the room. Chambers were ventilated through a fan mounted on the side wall of the chamber which was electrically shielded with aluminium foil. A hole in the top of the chamber allowed for the insertion of EEG/EMG cables and positioning of webcams (Logitech, C270, Lausanne, Switzerland) for remote monitoring. Room temperature and humidity were maintained at 20 ± 1°C and 60 ± 10%, respectively.

Preparation of orexin solutions. ORXA (Orexin A (human, mouse, rat) Trifluoroacetate salt, 4028262.0500, lot no 1068668, Bachem AG, Switzerland) was dissolved in sterile saline to a concentration of 0.3 nmol/µl or 0.15 nmol/µl, and ORXB (Orexin B (human) Trifluoroacetate salt, 4028263.0500, lot no 1000056801, Bachem AG) to a concentration of 0.3 nmol/µl under sterile conditions in a flow cabinet. Doses were determined based on previous studies involving orexin infusion in mice (Mobarakeh et al., 2005; Suzuki et al., 2005; Mieda et al., 2011) and the reported dissolvability of orexin according to product data sheets.

Cannula system. Each mouse had a separate tubing system consisting of an internal needle (C315I/SPC, Plastics1) inserted into the implanted guide canula, which was connected to a Hamilton syringe (SYR 5 µl 75N, Hamilton company, Nevada, USA) via transparent tubing (C313CT, C313C, Plastics1). The Hamilton syringe was placed in a microinjector pump (Pump 11, Elite, Harvard Apparatus, Massachusetts, USA). Tubing was front-filled with 3-4 µl drug solution and back-filled with saline, and marks were made to allow monitoring of fluid movement. Infusion success was therefore verified by the movement of the Hamilton plunger and the fluid level of the drug solution as compared to markings made preceding the infusion.

Experimental timeline baseline and sleep deprivation recording. After a day of habituation, animals were connected to EEG/EMG cables and signals were checked using the Synapse recording software setup (Synapse, Tucker Davis Technologies, Florida, USA). After a few days, a baseline recording was undertaken. Typically, experiments lasted 1-3 weeks with 2-3 days in between experimental days other than those which involved baseline recordings.

For sleep deprivation (SD), animals were kept awake from light onset for 6 hours, which is when mice in laboratory conditions are predominantly asleep. At light onset (9AM),

the chambers were opened, and nests were removed from the cages. Animals were continuously observed by an experimenter for the full 6 hours, and when an animal seemed to be falling asleep based on posture and/or online EEG/EMG traces, a novel object was introduced into the cage. This is a common SD procedure in rodents which keeps animals awake in an ethologically relevant and less-stressful manner than other SD methods (McKillop et al., 2018). After 6 hours, objects were taken out of the cages, nests were reintroduced, and chambers were closed.

Experimental procedure orexin infusion. In the orexin infusion experiment, animals were moved into the Plexiglas cages in the recording room 5-7 days after recovering from surgery. Animals were connected to EEG/EMG cables a day later, then allowed to habituate to the new environment for several days before experiments commenced. The first day involved a baseline recording, wherein animals were only briefly disturbed for inspection and handling at 9AM. On each infusions day, chambers were opened at 9AM and animals were connected to the cannula system. Next, 2 µl of orexin or vehicle were administered at a rate of 1 µl/min within 55 min of light onset. After the infusion was complete, the system was left in place for an additional 5 minutes to allow the remaining solution to diffuse from the internal needle, and then the dummy cannulas were re-inserted, and mice were left undisturbed. The higher dose of ORXA was expected to give the strongest effect, so this infusion and a vehicle infusion were prioritised for the first two infusions in counterbalanced order. Additional infusions were administered longitudinally, with ORXB being counterbalanced with the lower dose of ORXA for the third and fourth infusion. Infusion order was swapped for one individual animal for technical reasons.

Data acquisition. EEG/EMG head stages were custom-made from 8-pin 90 degrees connectors (Pinnacle Technology Inc, Kansas, USA, model 8415-SM) to which stainless steel wires were attached that were wrapped around a stainless-steel bone screw (Fine Science Tools, 19010-10, InterFocus Ltd, Cambridge, UK) for the EEG electrodes; for the EMG electrodes, stainless steel wires were folded into a loop that was soldered into a smooth blob, and then attached to the 8-pin connector. Connections were conductivity-tested and electrically isolated with a layer of dental cement (Simplex Rapid dental acrylic cement (Associated Dental Products Ltd, Swindon, UK).

Data was acquired and stored locally using a 128-channel neurophysiology recording system with an RZ2 processor (Tucker Davis Technologies (TDT), Florida, USA) connected to a computer installed with TDT Synapse software (Tucker Davis Technologies). Custom-made EEG/EMG cables connected the headstage to a splitter-box in a configuration for analogue referencing (frontal-cerebellar, occipital-cerebellar, a backup derivation frontal-

occipital, and EMG1-EMG2). EEG/EMG signals were preamplified by a PZ5 Neurodigitizer system (TDT), sampled at 1017.3 Hz (for baseline/sleep deprivation experiment recordings) or 305 Hz (orexin infusion experiment recordings), and processed with an anti-aliasing filter at 45% of the sampling rate.

Signal processing. Blinded raw data from the circadian screen was analysed in Clocklab, then further processed in MATLAB (v2022a, The MathWorks Inc., Massachusetts, USA) and GraphPad Prism (v.9.3.1, GraphPad Software, Inc., California, USA). EEG and EMG signals were processed offline using custom-written MATLAB scripts. This involved first resampling signals to 256 Hz, then filtering to 0.3 Hz-100 Hz for EEG signals and 3-100 Hz for EMG signals using a Chebyshev type II filter. Resampled and filtered signals were then converted into European Data Format (.edf) files and blinded for manual assignment of vigilance states in 4-seconds epochs in SleepSign (Kissei Comptech Co Ltd, Nagano, Japan). Vigilance states were scored as non-rapid eye movement (NREM) sleep, rapid eye movement (REM) sleep, or Wakefulness (W) based on visual inspection of frontal and occipital EEG and EMG traces. Brief episodes of movements during NREM sleep (≤ 16 seconds) and REM sleep (≤ 8 seconds) were scored as movement rather than wakefulness, and not considered a termination of sleep episodes. Minimum NREM and Wake episode duration was defined as 1 minute and minimum REM episode duration was defined as 16 seconds. Epochs with large movement and other noise were scored as artefacts of the respective vigilance state, which were included in vigilance state analysis but not spectral analysis. For spectral analysis, Fourier transforms were calculated from raw data using the pwelch function in MATLAB.

Excluded data. i) Circadian screen. During the 12h:12h light-dark schedule, the first two days were excluded for all animals due to insufficient entrainment, and the last day was excluded for all animals because the data was incomplete. For both the constant dark and the constant light paradigm, one day was excluded for all animals due to missing data. For the constant light condition, data from one control animal was excluded for technical reasons. ii) Baseline and sleep deprivation recording. EEG and EMG channels of low quality (excessive noise and artifacts) were excluded from analysis (the occipital EEG channel in one control animal for both the baseline and SD day, and the occipital EEG channel of one additional control animal and a L6b silenced animal were excluded for the SD day only)). In two (different) animals, EMG signals were of insufficient quality to reliably score bouts of movement, so these animals were excluded from analysis of brief awakenings. Epochs involving large movements and other noise were scored as artefacts of the respective vigilance state, which were included in vigilance state analysis but not EEG power spectral analysis. The percentage of artefact epochs across the 24-hour file was comparable between genotypes (controls = 2.80±1.43%,

L6b silenced = 2.09±0.729%, $t_{(14)}$=0.4706, p=0.65). iii) Orexin infusion experiment. For spectral analysis, all individual outputs were inspected for technical quality; if less than 140 4-second epochs contributed to the spectrum, the dataset was excluded. The number of animals contributing to resulting spectra after exclusion criteria were applied are stated in respective figure legends.

Statistics. Statistical analyses were conducted using GraphPad Prism. Statistical significance was defined as $p \leq \alpha = 0.05$. Differences between L6b silenced and control animals across all metrics involving a single value per animal were evaluated using unpaired two-tailed t-tests, except for NREM latency which was tested with a Mann Whitney U test. When there were multiple values per animal, genotype groups were compared using two-way Analyses of Variance (ANOVA) or with a Mixed Effects ANOVA when there were missing values. Namely, comparisons of relative spectra employed two-way ANOVAs, but that of relative occipital EEG wake spectra after ORXB infusion involved Mixed Effects analysis; frequency bins <1 Hz and <0.75 Hz were excluded due to artifacts in two L6b silenced animals, respectively, which led to missing values. The Geisser-Greenhouse method was applied to correct for non-sphericity (F-test). Significant two-way interactions were followed up with post-hoc comparisons, applying the Bonferroni correction (0.05/number of observations) in all instances except for post-hoc comparisons of EEG spectra (no correction was applied, as adjusting for 119 frequency bins would be too conservative). Post hoc differences in frequency bins are only reported if two or more consecutive bins showed a difference (a range of >0.5 Hz). For EEG spectral analysis, data from individual animals were either log-transformed or normalised to baseline or vehicle spectra before statistical testing. To investigate the acute effects of orexin, sleep architecture following orexin infusion was analysed starting at the time of each infusion and ending 3 hours thereafter (2700 epochs of 4 seconds each). Two doses of ORXA (0.3 nmol and 0.6 nmol) were administered, but as the effects of the lower dose appeared to be consistent with the effects of the higher dose, subsequent descriptions for some analyses focus on the higher dose of ORXA.

Histology. After experiment completion, animals were deeply anaesthetised with a lethal dose of pentobarbitone and perfused transcardially with 0.1 M Phosphate Buffered Saline (PBS), followed by 4% formaldehyde (F8775; Sigma-Aldrich) in 0.1 M PBS. Head stages (including cannulas) were removed, then brains were extracted for post-fixation in 4% PFA for 24 hours at 4 °C before being transferred to 0.05% PBS-azide (PBSA) for long-term storage at 4 °C. Brains were sliced into 50µm-thick coronal sections using a vibroslicer (Leica VT1000S). Free floating sections were collected and stored in PBSA. For verification of cannula locations, nuclei were stained with 1:1000 4′,6- diamidino-2-phenylindole (DAPI, Invitrogen, D1306) for

visualisation of tissue structure. For determination of Drd1a cell density (Fig. 1), Sections were incubated with primary antibodies (rabbit α-Cplx3 (1:1000, Synaptic Systems #122302), mouse α-Tbr1 (1:500, ProteinTech #66564-1-LG) in 5% donkey serum, 0.3% Triton-X, 0.1M PBS) at 4°C for 48hrs. After 3x10 min washes, sections were incubated with secondary antibodies (donkey α-Rb (1:500, Invitrogen #A21206, 488nm), donkey α-mouse (1:500, Invitrogen #A32787, 647nm) in 5% donkey serum, 0.3% Triton-X, 0.1M PBS) for 2 hrs at room temperature. After another set of 3×10min washes, sections were incubated with 1:1000 4′,6-diamidino-2- phenylindole (DAPI) for 30mins at room temperature and washed 2×10min in 0.1M PBS. Sections were mounted using mounting medium (Fluorsave, VWR, Lutterworth UK) on microscope slides (Thermoscientific superfrost, RF12312108, Loughborough, UK) with cover slips prior to imaging (VWR coverglasses, 24 x 50mm, 531-0146, Lutterworth UK).

Imaging. Brain sections were first imaged with an epifluorescence microscope mounted with a camera (Leica Digital Module R, (DMR) with DFC500) or laser scanning confocal microscope (Zeiss LSM710) to confirm cannulas successfully penetrated the lateral ventricle. Images were processed in Fiji/ImageJ (FIJI, Schindelin et al 2012, 2.9.0 v1.54b) using background subtraction, brightness, and contrast adjustments, and merging of images from different fluorescent channels. Further processing, such as adding labels and indicating histological or anatomical boundaries, were carried out in Inkscape (Inkscape Project. (2020). *Inkscape*. Retrieved from https://inkscape.org). Images for cell quantifications were obtained using 20X spinning-disk confocal microscopy (Olympus SpinSR SoRa) and pre-processed by maximum-intensity z-stack projection, auto-brightness thresholding, and background subtraction using a custom Fiji/ImageJ .ijm script. Outputs were imported into QuPath (v0.4.2) (Bankhead et al., 2017) for cell segmentation. Resulting detections and downsampled images were additionally processed using another custom Fiji/ImageJ.ijm script (i.e., binary masks application, pixel inversion, and file type conversion to RGB .pngs), merged for atlas registration (2017 CCFv3) (Wang et al., 2020), and analysed using the QUINT pipeline (Yates et al., 2019). Software versions employed as a part of the QUINT pipeline include QuickNII (RRID:SCR_016854) (Puchades et al., 2019), VisuAlign (v0.8 RRID:SCR_017978), and Nutil (generating object reports with minimum pixel size = 4, point cloud density = 4, without object splitting, and extracting all coordinates) (Groeneboom et al., 2020). A custom MATLAB script (R2022b) re-formatted Nutil outputs for statistical analysis and graphical representation using GraphPad Prism.

Acknowledgements

St John's College Research Centre Grant on L6b to EM and ZM

ZM and Ed Mann MRC Grant; VV and ZM BBSRC grant

Medical Research Council (UK) grant MR/S01134X/1 (VV)

EM was funded by the O'Sullivan Family Graduate Scholarship

LG was funded by a WT studentship.

MM was funded by a Rhodes Scholarship.

SW was funded by an NC3Rs studentship (NC/S001689/1)

AC was funded by a BBSRC studentship (BB/M011224/1)

HA was funded by the Wellcome trust through a postdoctoral fellowship (206500/Z/17/Z).

LK was supported by the Wellcome Trust through a doctoral studentship (203971/Z/16/Z) and postdoctoral fellowship (224083/Z/21/Z).

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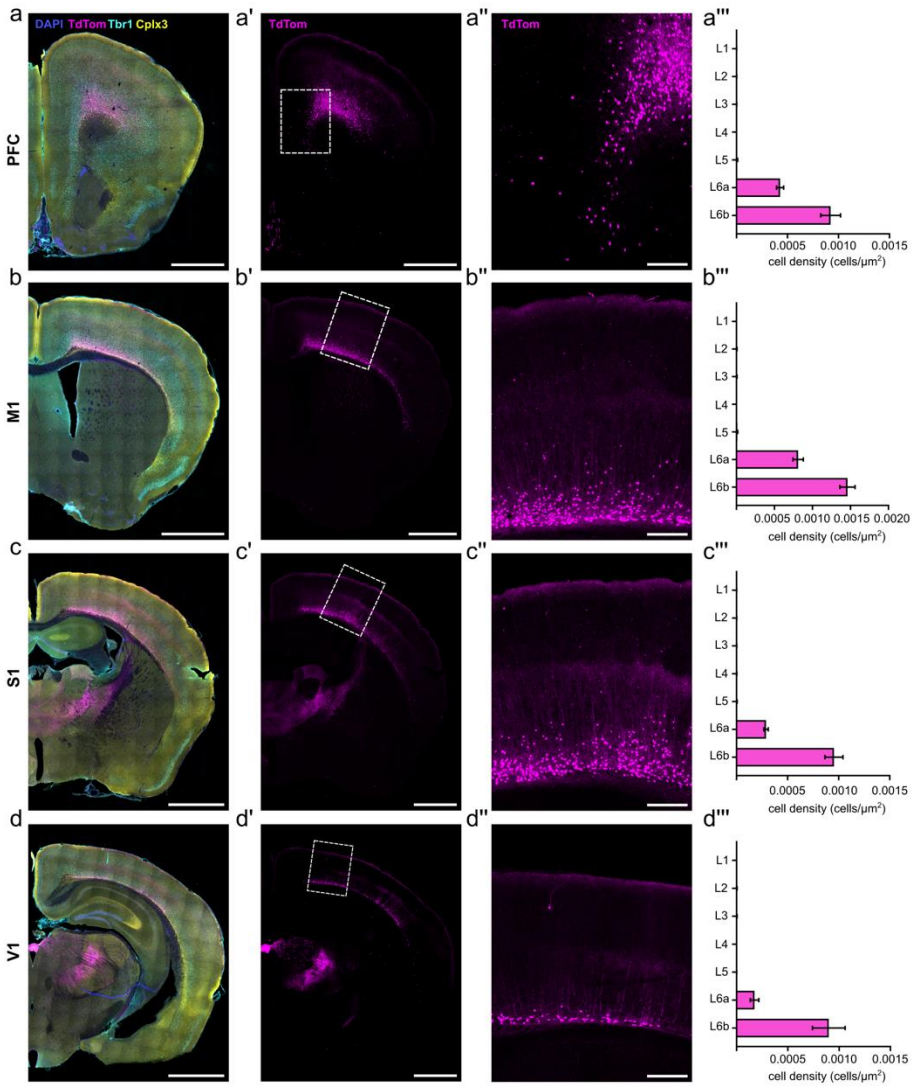

Figure 1. Drd1a Cre positive cells are prefentially localized in L6b across the entire cortical mantle. Drd1a (TdTom) distributions across prefrontal cortex (a), primary motor cortex (b), primary somatosensory cortex (c) and primary visual cortex (d) in a single example animal, with cell densities averaged across multiple animals.

(a,b,c,d) Hemisection for reference, with DAPI staining for tissue structure (blue), Drd1a-Cre+ cells identified by TdTom expression (magenta). Tbr1 immunostaining (cyan) labels layer 6 and is used to identify the layer 5-6 boundary. Cpxl3 immunostaining (yellow) labels L6b and is used to distinguish L6a and L6b.

(a',b',c',d') Hemisection for reference with only the Drd1a-TdTom channel shown. White boxes indicate the cortical region of interest enlarged in (a'',b'','c'',d'').

(a'',b'',c'',d'') Magnified image of the cortical region of interest.
(a''',b''',c''',d''') Laminar cell densities of Drd1a-Cre;TdTom positive cells. Greatest densities are found in L6b, followed by L6a, with sparse to no Drd1a expression in upper layers. More anterior cortical areas PFC and M1 exhibit greater Drd1a cell densities compared to the more posterior cortical areas S1 and V1 (Cortical layer, $F_{(6,126)}=261.3$, $p<0.0001$; Brain region, ($F_{(4,126)}=45.95$), $p<0.0001$; Cortical layer x brain region, $F_{(24,126)}=21.45$, $p<0.0001$). Data represented as mean ± SEM.
Scale bar a-d and a'-d', 1000 µm. Scale bar a''-d'', 200 µm.
Experimental replicates, PFC (n=5), M1 (n=6), S1 (n=6), V1 (n=3). For each animal, 3 technical replicates were used.

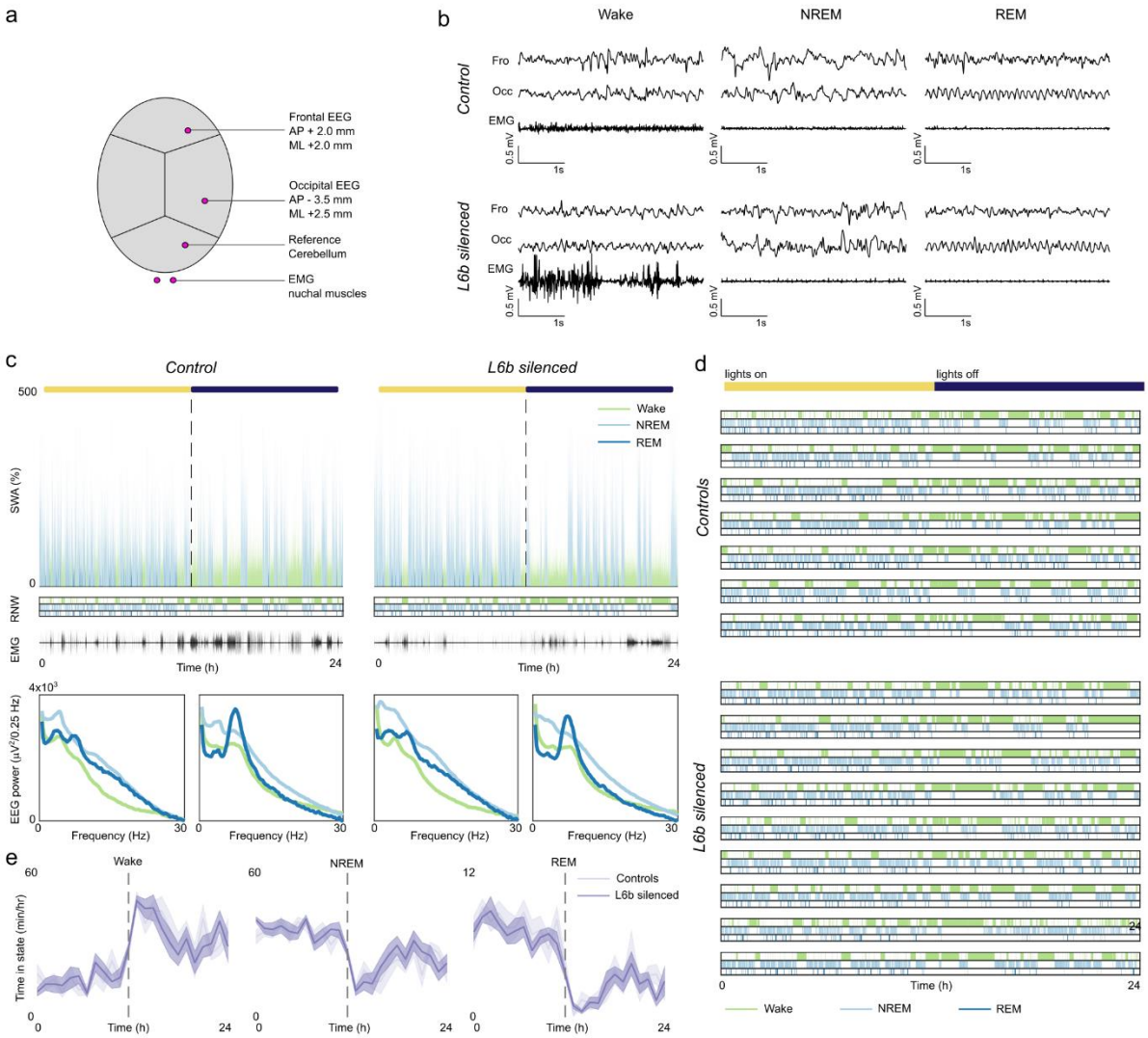

Figure 2. Daily sleep-wake architecture is unchanged in L6b silenced animals.

a. Position of electrodes. The frontal and occipital EEG (expressed as mm distance from Bregma in midline (ML) and anteroposterior (AP) directions) were referenced against a cerebellar screw. Two EMGs were implanted in the nuchal musculature and referenced against one other.

b. Representative frontal EEG, occipital EEG and EMG traces during the respective vigilance states in a L6b silenced and a control animal show similar patterns of activity, allowing blinded scoring of vigilance states.

c. 24-hour profiles of slow wave activity (SWA) and EMG activity and vigilance-specific spectrograms in a representative L6b silenced animal and control animal. The bar on top represents the duration of the light phase (yellow) and dark phase (dark blue).
d. Hypnograms for all individual animals.
e. Daily time course of wakefulness, NREM and REM sleep were comparable in L6b silenced and control animals. Controls n=7, L6b silenced n=9.

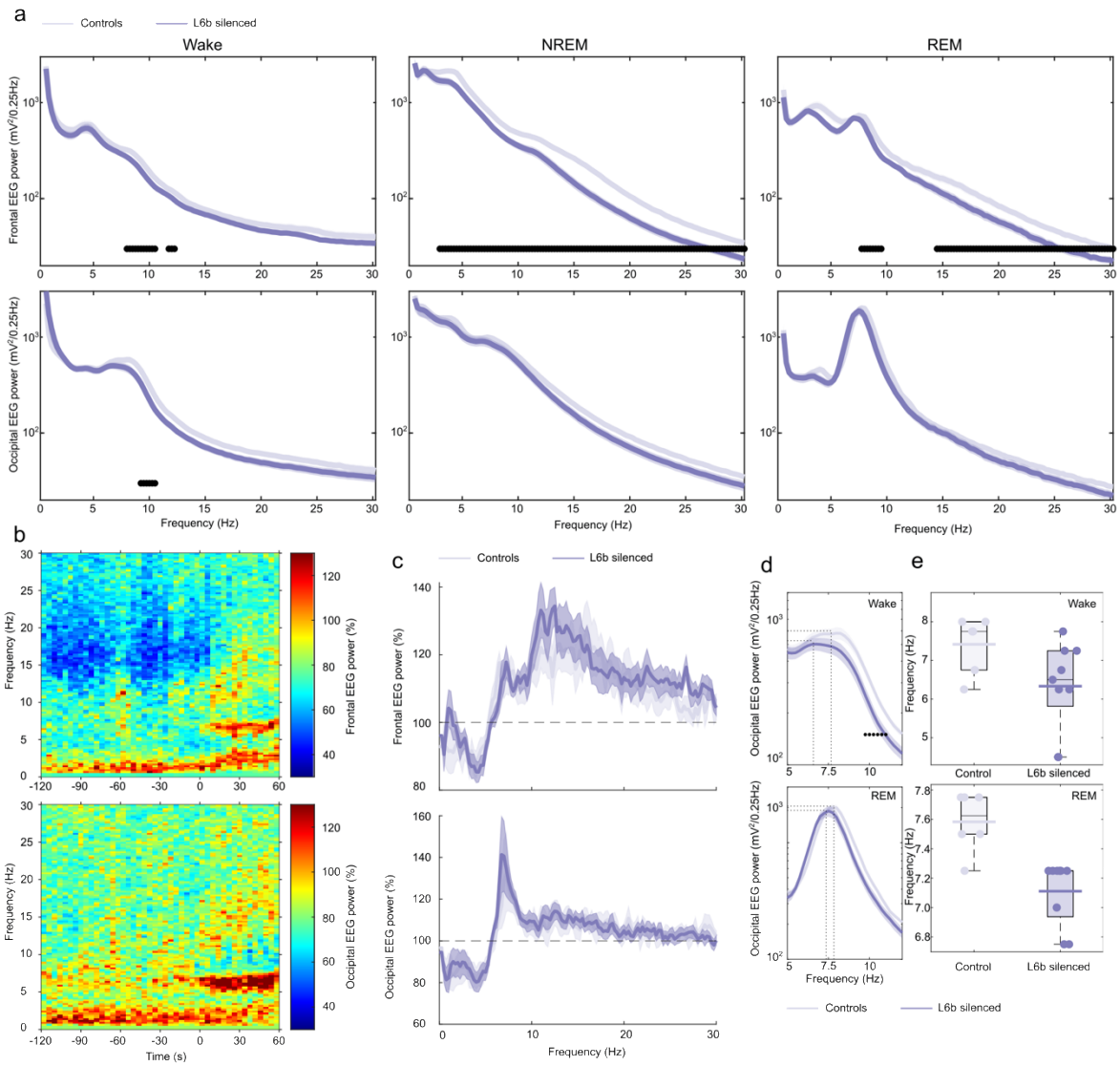

Figure 3. Vigilance state specific EEG spectra are changed in L6b silenced animals.

a. EEG spectral power in the frontal and occipital derivation during Wake, NREM and REM sleep in L6b silenced and control animals. Filled dots show bins with significant differences between genotype groups after comparison with multiple t tests in 0.25 Hz bins. Frontal EEG, Controls n=7, L6b silenced n=9. Occipital EEG, Controls n=6, L6b silenced n=9.

b. EEG spectral power in the 2 minutes preceding and 32 seconds following NREM-REM transitions averaged across all NREM-REM transitions across 24 hours, with average power in L6b silenced animals relative to control animals in percentages.

Top, frontal EEG, controls n=6, L6b silenced n=9. Bottom, occipital EEG, controls n=6, L6b silenced n=9.

c. EEG spectral power during NREM sleep in the 32 seconds preceding the NREM-REM transition relative to the EEG power in NREM sleep across 24 hours, in the frontal (top, controls n=7, L6b silenced n=9) and occipital (bottom, controls n=6, L6b silenced n=9) EEG.
d. Enlarged representation of the occipital EEG spectral power shown in (a) during wakefulness (top) and REM sleep (bottom). Filled dots mark significant genotype differences in 0.25-Hz bins. Controls n=6, L6b silenced n=9. Dotted lines illustrate the EEG spectral power and frequency of the theta peak.
e. Peak theta frequency in the occipital EEG during wakefulness (top) and REM sleep (bottom) for control and L6b silenced animals. Controls n=6, L6b silenced n=9.

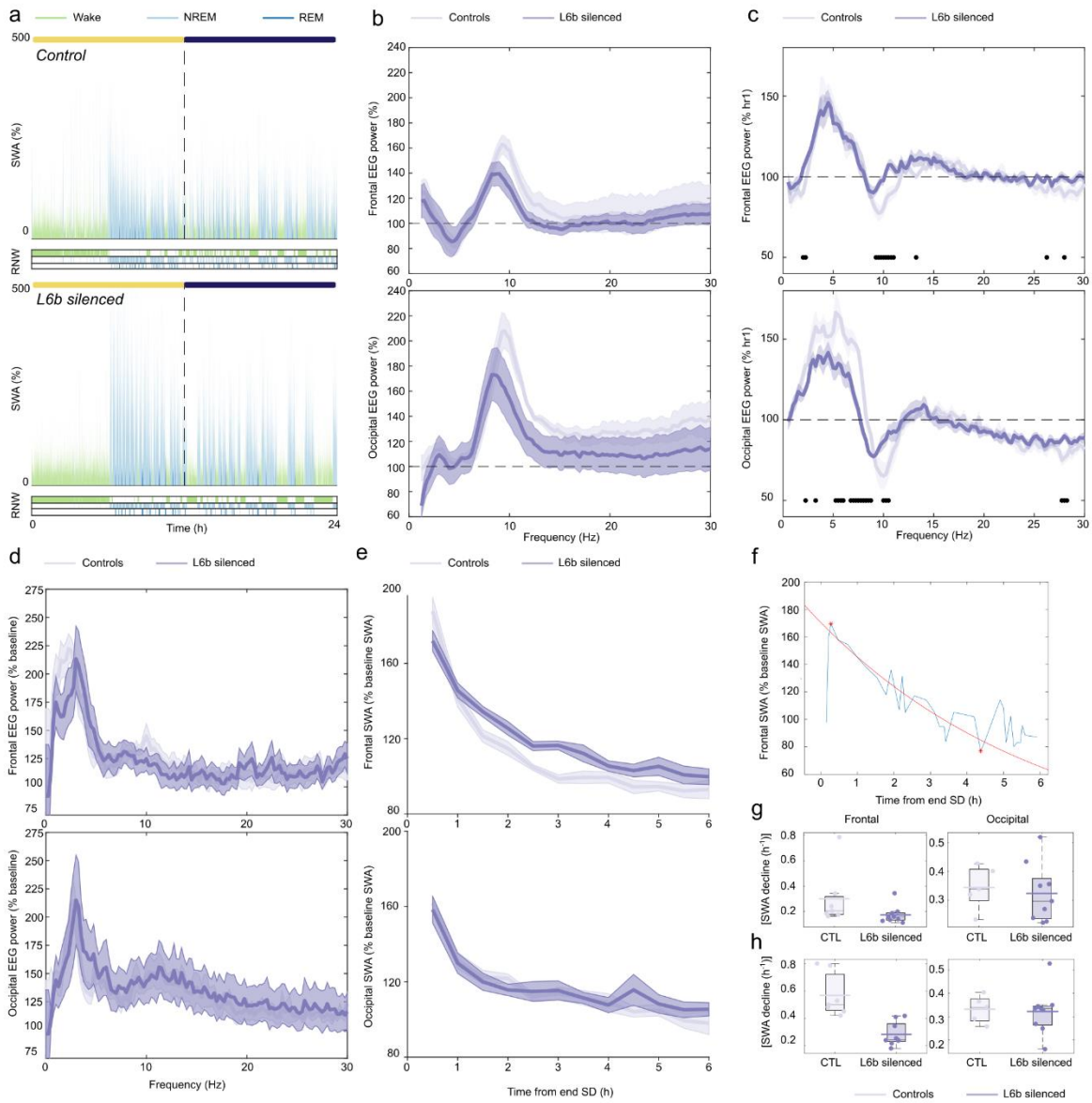

Figure 4. The response to sleep deprivation is altered in L6b silenced animals.

a. 24-hour profiles of SWA (0.5-4.0 Hz) after sleep deprivation in a representative control and L6b silenced animal, with hypnograms plotted below. The bar on top marks the duration of the light phase (yellow) and dark phase (dark blue).
b. EEG power during sleep deprivation in L6b silenced and control animals, normalised to wakefulness spectra on baseline day.
c. EEG spectral power in the sixth (final) hour of sleep deprivation normalised to the first hour of sleep deprivation. Filled circles represent bins with significant genotype differences after comparison with multiple t tests.

d. Spectral power in the frontal EEG during the first 30 minutes of NREM sleep following sleep deprivation.
e. Time course of SWA (0.5-4.0 Hz) during NREM sleep across the six hours following sleep deprivation.
f. The rate of SWA decline was approximated with an exponential fit, the method is shown for the frontal EEG from a representative individual animal.
g. Absolute exponent coeffects for an exponential fit across the six hours following sleep deprivation.
h. Absolute exponent coeffects for an exponential fit across only the first two hours following sleep deprivation.

Frontal EEG, controls n=7, L6b silenced n=9. Occipital EEG, controls n=6, L6b silenced n=8.

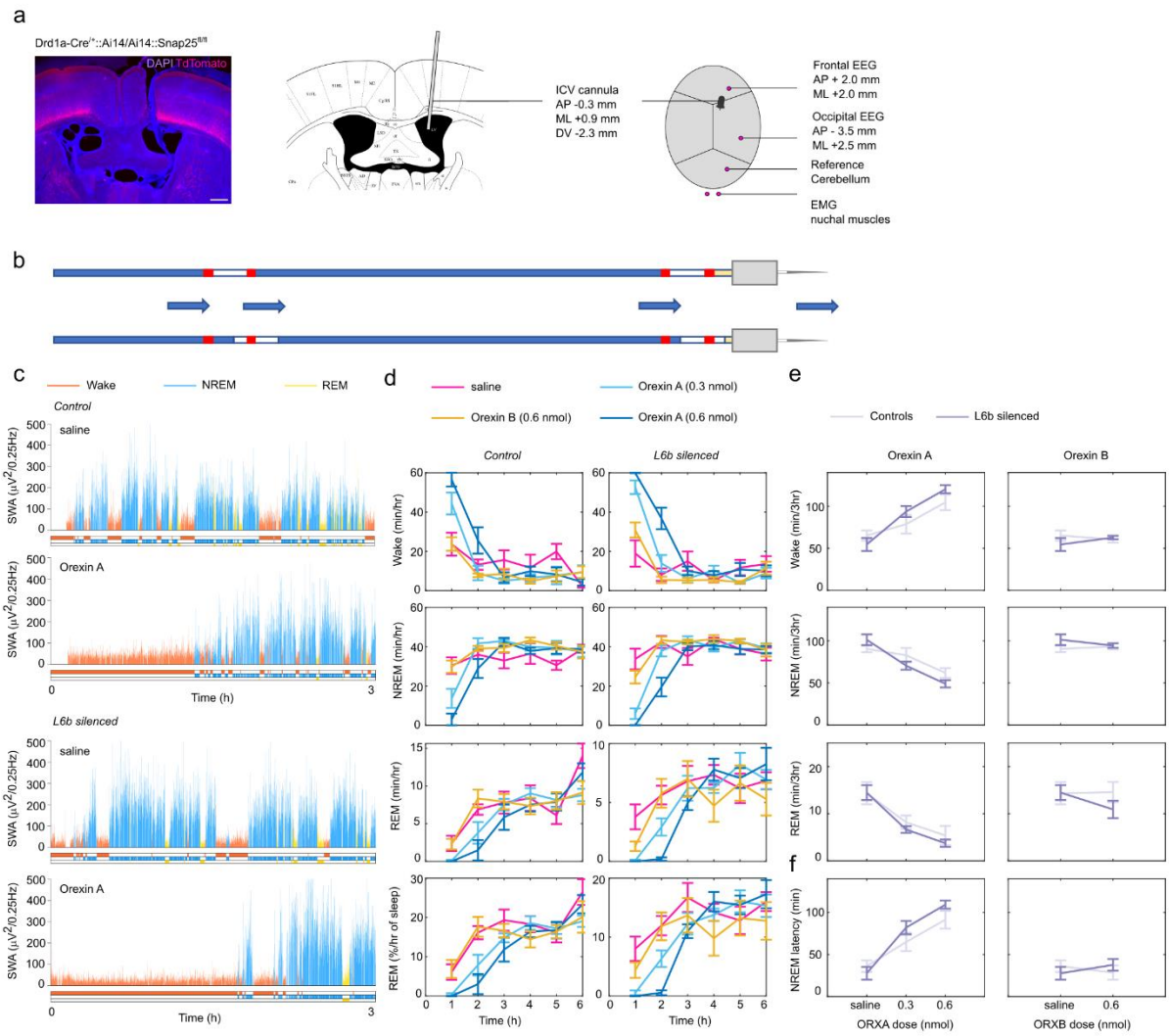

Figure 5. Intracerebroventricular infusion of orexin A promotes wakefulness in L6b silenced and control animals

a. Histological and schematic overview of the intracerebroventricular infusion canula and EEG/EMG implant, with position of the cannula and electrodes defined as coordinates from bregma in the anterioposterior (AP) direction and midline (ML) direction, and cannula dorsoventral position (DV) from the surface of the dura.
b. Schematic overview of the infusion procedure with tubing front-filled with orexin or vehicle solution and backfilled with saline.
c. Time course of SWA in the 3 hours following infusion of vehicle and 0.6 nmol orexin A in a representative control and L6b silenced animal.

d. Time course of vigilance states in the first 6 hours after infusion of vehicle (saline), a lower dose of orexin A (0.3 nmol), a higher dose of orexin A (0.6 nmol), orexin B (0.6 nmol) in L6b silenced and control animals. Controls n=4, L6b silenced n=7.
e. Total amount of wake, NREM and REM in the first 3 hours after infusion of orexin A or orexin B. Controls n=4, L6b silenced n=7.
f. The latency to consolidated NREM sleep was increased after infusion of orexin A (left column) but not ORXB (right column). Controls n=4, L6b silenced n=7.

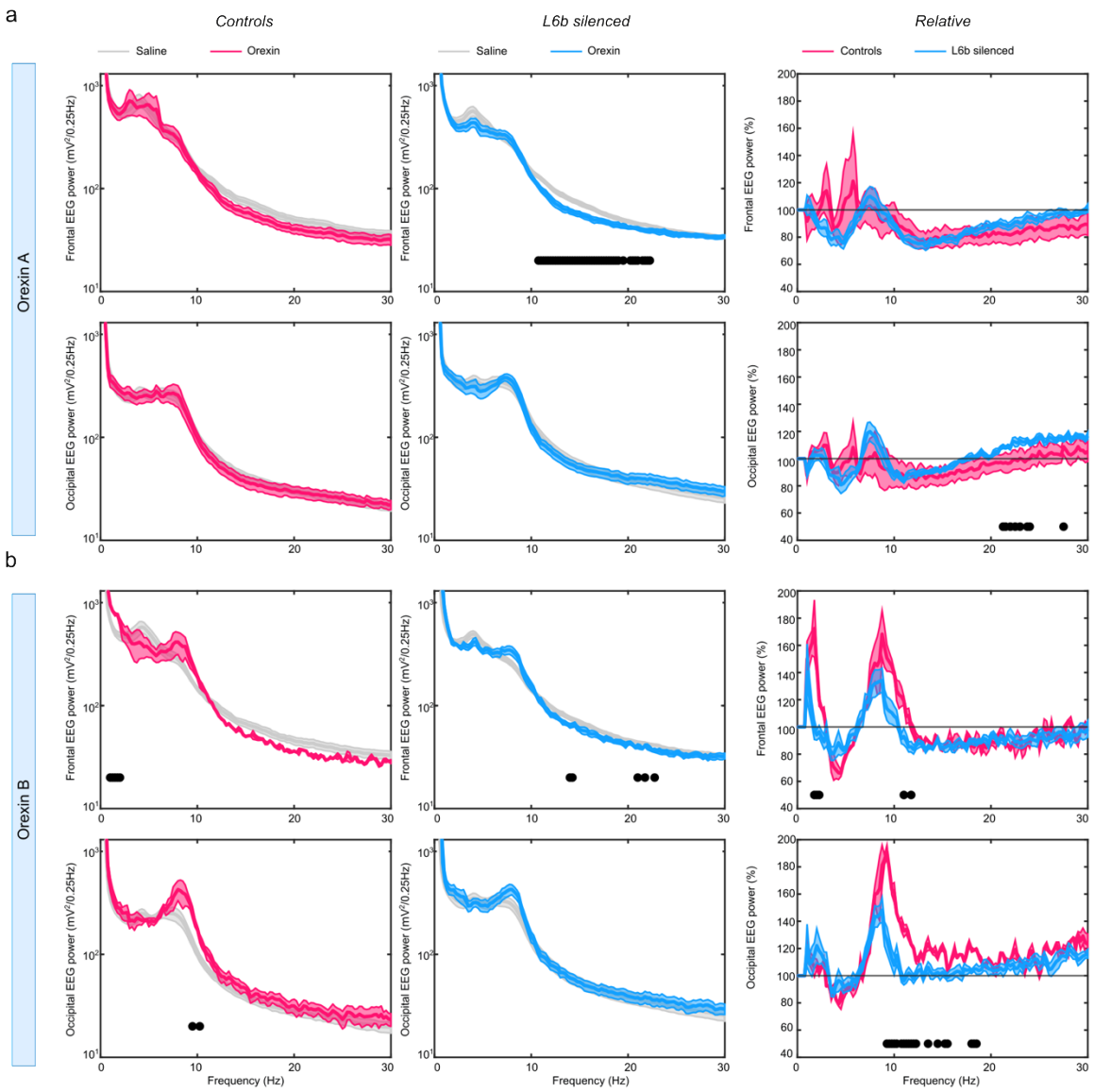

Figure 6. Orexin A and B have different effects on EEG wake spectra in L6b silenced than in control animals.

a. Effects of orexin A (0.6 nmol) infusion on frontal and occipital EEG power. The left column shows absolute EEG power in control animals after vehicle (grey) and orexin (magenta) infusion (n=5). The middle column shows absolute EEG power in L6b silenced animals after vehicle (grey) and orexin (cyan) infusion (n=8). The right column shows a comparison of EEG power after orexin infusion relative to vehicle infusion between control (magenta, n=5)) and L6b silenced (cyan, n=8)) animals.

Filled circles depict significantly differences between genotypes in 0.25-Hz bin power spectra with two-sided unpaired t tests.

b. Effects of orexin B (0.6 nmol) infusion on the frontal and occipital EEG power spectrum. The left column shows absolute EEG power in control animals after vehicle (grey) and orexin (magenta) infusion (n=3). The middle column shows absolute EEG power in L6b silenced animals after vehicle (grey) and orexin (cyan) infusion (n=6). The right column shows a comparison of EEG power after orexin infusion relative to vehicle infusion between control (magenta, n=3)) and L6b silenced (cyan, n=6)) animals. Filled circles depict significantly differences between genotypes in 0.25-Hz bin power spectra with two-sided unpaired t tests.