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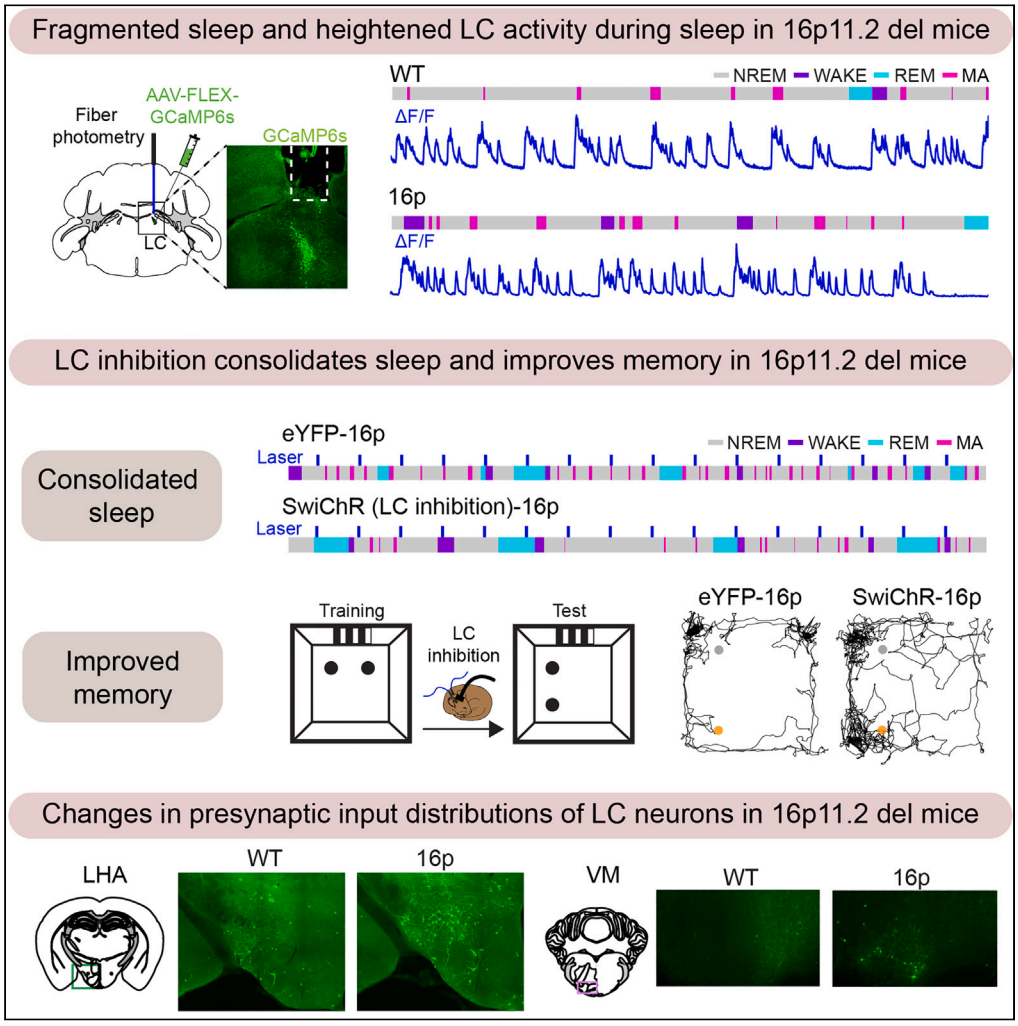

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Highlights

16p11.2 deletion mouse model of autism spectrum disorder exhibits fragmented sleep

The activity of LC neurons is heightened during sleep in 16p11.2 deletion mice

Inhibiting LC consolidates sleep and restores memory in 16p11.2 deletion mice

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Circuit mechanism underlying fragmented sleep and memory deficits in 16p11.2 deletion mouse model of autism

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SUMMARY

Sleep disturbances are prevalent in children with autism spectrum disorder (ASD). Strikingly, sleep problems are positively correlated with the severity of ASD symptoms, such as memory impairment. However, the neural mechanisms underlying sleep disturbances and cognitive deficits in ASD are largely unexplored. Here, we show that non-rapid eye movement sleep (NREMs) is fragmented in the 16p11.2 deletion mouse model of ASD. The degree of sleep fragmentation is reflected in an increased number of calcium transients in the activity of locus coeruleus noradrenergic (LC-NE) neurons during NREMs. In contrast, optogenetic inhibition of LC-NE neurons and pharmacological blockade of noradrenergic transmission using clonidine consolidate sleep. Furthermore, inhibiting LC-NE neurons restores memory. Finally, rabies-mediated screening of presynaptic neurons reveals altered connectivity of LC-NE neurons with sleep- and memory-regulatory regions in 16p11.2 deletion mice. Our findings identify a crucial role of the LC-NE system in regulating sleep stability and memory in ASD.

INTRODUCTION

Many children with autism spectrum disorder (ASD) suffer from sleep disturbances including delayed sleep onset, frequent night awakenings, and short sleep episodes.[1–3] Strikingly, sleep disturbances worsen ASD symptoms, especially cognitive capabilities.[4–6] Yet, the neural mechanisms underlying sleep problems and the resulting cognitive impairment in ASD remain poorly understood.

Chromosomal copy-number variations (CNVs) are associated with an increased prevalence of ASD; in particular, CNVs in chromosomal region 16p11.2 increase the risk for ASD.[7–11] 16p11.2 CNVs are associated with both sleep disturbances and cognitive impairment.[11–13] In particular, 16p11.2 hemideletion (16p11.2 del/+) mice display sleep disturbances and cognitive deficits[14–18]; the underlying mechanisms, however, are largely unknown.

Recent studies demonstrated that locus coeruleus noradrenergic (LC-NE) neurons are rhythmically activated during non-rapid eye movement sleep (NREMs) in synchrony with an infraslow rhythm fluctuating on a ~1-min timescale (infraslow σ rhythm), which is reflected in the electroencephalogram (EEG) σ power and the frequency of sleep spindles.[19–21] The activation of LC-NE neurons during NREMs tends to overlap with microarousals (MAs) while their activity decreases before transitions to REMs, suggesting that the infraslow oscillations of the LC-NE activity during NREMs coordinate the onset of MAs and REMs.[19–24] The activity of LC neurons and NE levels are increased during NREMs after learning, suggesting that the activation of LC-NE neurons during NREMs is involved in memory consolidation.[25,26] Activating LC-NE activity during post-learning sleep impairs spatial learning and memory performance, while suppressing their activity improves memory.[21,23] However, the extent to which LC-NE neurons contribute to sleep disturbances and memory deficits observed in ASD remains unknown.

By employing fiber photometry, optogenetic, and pharmacological manipulations, spatial object recognition task, and mono-synaptically restricted rabies virus tracing, we investigated the role of LC-NE neurons in sleep disturbances and memory impairment in the 16p11.2 deletion mouse model of ASD.

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RESULTS

16p11.2 del/+ mice exhibit fragmented sleep and impaired phase-coupling of sound-evoked arousals with infraslow σ rhythm

We performed electroencephalogram (EEG) and electromyography (EMG) recordings in 16p11.2 del/+ mice and wild-type (WT) littermates to examine their sleep architecture throughout 24 h recordings (Figure 1A). The overall time spent in NREMs, REMs, and wakefulness was not significantly different between 16p11.2 del/+ mice and WT littermates (Figures 1B, S1A, and S1B; t tests, p = 0.995, 0.334, and 0.388 for NREMs, REMs, and wake; detailed statistical results are shown in Table S1). However, the number of MAs during NREMs was significantly higher in 16p11.2 del/+ mice, resulting in a decreased duration and increased frequency of NREMs episodes compared with WT mice (Figures 1B and 1C; t tests, p = 0.020, 0.011, and 0.009 for MAs, NREMs duration, and frequency). MAs are defined as short (<20 s) wake-like episodes during sleep, characterized by desynchronized EEG and activated EMG.[20,21,24,27–29] Given their short duration, MAs have not been scored in previous sleep studies on 16p11.2 del/+ mice, which used a more coarse binning for sleep annotation than the 2.5 s in our study.[16,17] Accordingly, examining the distribution of the duration of NREMs bouts, we found that 16p11.2 del/+ mice have an increased proportion of short NREMs bouts compared with WT mice (Figure 1D; mixed ANOVA, duration p = 5.702e-40, genotype p = 1.000, interaction p = 7.109e-7). MAs preferentially occur at the descending phase of the infraslow σ rhythm, where mice are most susceptible to wake up in response to external stimulation.[20,21,30,31] We therefore examined the infraslow σ rhythm of 16p11.2 del/+ and WT mice. The strength of the infraslow σ rhythm was significantly decreased compared with that of WT mice (Figure 1E; t test, p = 0.029). The number of sleep spindles, a major contributor to EEG σ power, was not affected in 16p11.2 del/+ mice (Figure S1C; t test, p = 0.887), suggesting that decreased strength of infraslow σ rhythm in 16p11.2 del/+ is caused by a weakened clustering of sleep spindles. Finally, we confirmed that the sleep fragmentation and weakened infraslow σ rhythm phenotype in 16p11.2 del/+ mice was also conserved when they are crossed with dopamine β-hydroxylase (DBH)-Cre or GAD2-Cre mice used in the remainder of this study (Figures S1D–S1K). Thus, 16p11.2 del/+ mice have an increased number of MAs resulting in sleep fragmentation, and their infraslow σ rhythm is weakened.

During NREMs, mice are more susceptible to wake up in response to acoustic stimuli during the descending phase of the infraslow σ rhythm, whereas they tend to sleep through during the ascending phase.[30] Because 16p11.2 del/+ mice exhibit a weakened infraslow σ rhythm, we investigated whether the phase tuning of sound-evoked arousals is altered in 16p11.2 del/+ and WT mice. Sounds (65 dB, 20 s) were randomly presented every 4–20 min, while performing sleep recordings in WT and 16p11.2 del/+ mice (Figure 1F; representative recordings are from a 16p11.2 del/+ mouse). Both groups of mice woke up during ∼ half of the trials in response to the tone, suggesting that the probability to be awakened by an external sound is not altered in 16p11.2 del/+ mice (Figure 1G). In WT mice, the EEG σ power started slowly decreasing ∼20 s before the tone onset in sound-evoked arousal trials, while it increased in sleep-through trials (Figure 1H; two-way repeated measures [rm] ANOVA, time p = 0.507, wake p = 0.040, interaction p = 0.033). In contrast, in 16p11.2 del/+ mice, the time course of the EEG σ power was similar in both arousal and sleep-through trials (Figure 1I; two-way rm ANOVA, time p = 0.883, wake p = 0.370, interaction p = 0.248). Thus, 16p11.2 del/+ mice exhibit an impaired phase tuning of sound-evoked arousals with the infraslow σ rhythm.

Increased activation of LC-NE neurons during NREMs in 16p11.2 del/+ mice

Previous studies showed that MAs during NREMs closely overlap with calcium transients in LC-NE neurons.[20,21,24] To test whether an increased number of calcium transients may contribute to the heightened MA frequency in 16p11.2 del/+ mice, we monitored the population activity of LC-NE neurons using fiber photometry. To express genetically encoded calcium indicators in LC-NE neurons, we crossed 16p11.2 del/+ mice with DBH-Cre mice and injected adeno-associated viruses (AAVs) with Cre-dependent expression of GCaMP6s (AAV-FLEX-GCaMP6s) into the LC of 16p11.2 del/+ x DBH-Cre or 16p11.2 +/+ (WT) x DBH-Cre mice (Figure 2A). In both groups, the LC-NE neurons were highly active during wakefulness, less active during NREMs, and almost silent during REMs (Figures 2B and 2C; one-way rm ANOVA, p = 8.259e-29, 1.736e-21; pairwise t tests with Bonferroni correction, p = 7.631e-17, 9.336e-13 for REMs vs. Wake, 6.854e-15, 1.723e-11 for Wake vs. NREMs, 1.125e-11, 2.314e-8 for REMs vs. NREMs in WT and 16p). During NREMs, the calcium transients of LC-NE neurons were often accompanied by MAs (Figure 2B). The number of LC-NE calcium transients and the probability that they coincide with MAs was significantly higher in 16p11.2 del/+ x DBH-Cre mice compared with WT x DBH-Cre mice (Figure 2D; t tests, p = 0.032, 0.048 for Ca^{2+} transients and overlap with MAs), suggesting that the heightened LC-NE activity underlies the increased frequency of MAs and the resulting sleep fragmentation in 16p11.2 del/+ x DBH-Cre mice.

Elevated activity of LC-NE neurons in novel environment leads to fragmented NREMs and reduced REMs in 16p11.2 del/+ mice

In children with ASD, exposure to a novel environment worsens sleep efficiency.[32,33] As a behavioral paradigm to test the impact of a novel environment on sleep, we placed the same 16p11.2 del/+ and WT mice in a novel or familiar cage, while measuring their sleep using EEG/EMG recordings. To measure the overall sleep architecture and the activity of LC-NE neurons in a new or familiar environment, 16p11.2 del/+ x DBH-Cre mice and WT x DBH-Cre mice were injected with AAV-FLEX-GCaMP6s followed by an implantation of an optic fiber targeting the LC as well as EEG and EMG electrodes (Figure 3A). The number of MAs during NREMs was significantly increased in 16p11.2 del/+ x DBH-Cre in the novel cage compared with the familiar cage resulting in decreased duration of NREMs episodes (Figures 3B and S2A; mixed ANOVA, genotype p = 3.865e-4, 2.055e-5, condition p = 0.002, 0.002, interaction p = 0.005, 0.063; pairwise t tests with Holm correction, p = 0.003, 0.003 for MAs and NREMs duration). In contrast, WT x DBH-Cre mice did not change the number of MAs regardless of whether they were

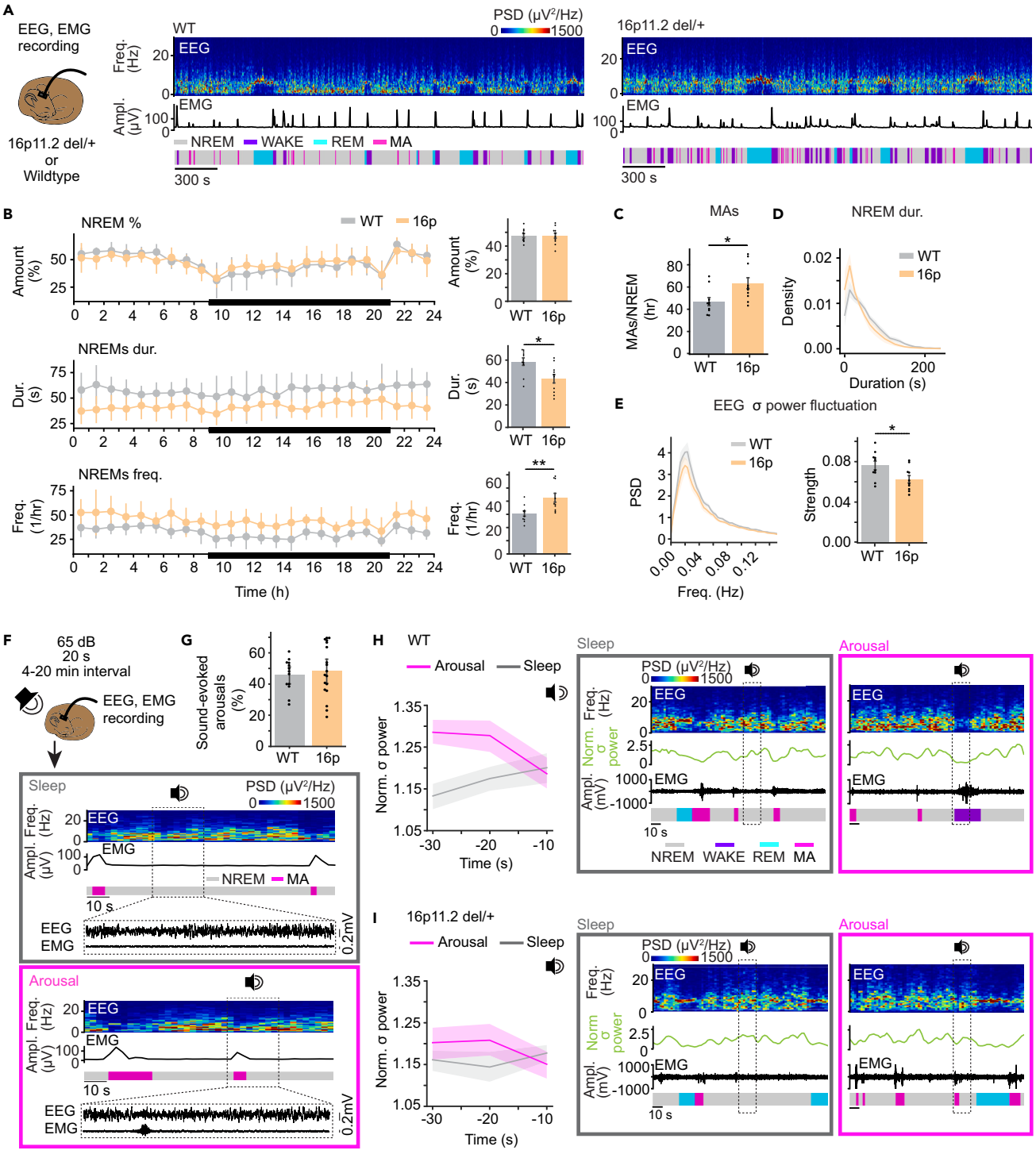

Figure 1. 16p11.2 del/+ mice exhibit fragmented sleep and impaired phase-coupling of sound-evoked arousals with infraslow σ rhythm

(A) Schematic of EEG and EMG recordings in 16p11.2 del/+ mice and WT littermates (left). Example sessions from WT and 16p11.2 del/+ mouse (middle and right). Shown are EEG power spectra, EMG amplitude, and color-coded sleep-wake states.

(B) Percentage of NREMs, duration and frequency of NREMs episodes during 24 h recordings. Black line represents the dark phase.

(C) Number of MAs during NREMs.

(D) Distribution of NREMs episodes depending on their episode duration.

(E) Power spectral density (PSD) of normalized EEG σ power during NREMs.

Figure 1. *Continued*

(F) Schematic illustrating sound-evoked arousals. During about half of the trials, mice stayed asleep (gray box) or transitioned to wake (pink box) after sound. Shown are EEG power spectra, EMG amplitude, color-coded sleep-wake states, and EEG and EMG raw traces during selected periods (dotted box, sound stimuli). Representative recordings are from a 16p11.2 del/+ mouse.

(G) Percentage of sound-evoked arousals in WT and 16p11.2 del/+ mice.

(H) Left, EEG σ power before sound-evoked arousal and sleep-through trials in WT mice. Middle and right, example sleep-through trial with rising EEG σ power and wake-up trial with falling EEG σ power in a WT mouse. Shown are EEG power spectra, EEG σ power, EMG raw traces and color-coded sleep-wake states. Dotted box, sound stimuli. Norm. σ value at −10 (s) indicates the averaged σ values from −10 to 0 s.

(I) Left, EEG σ power before sound-evoked arousal and sleep-through trials in 16p11.2 del/+ mice. Middle and right, example sleep-through and wake-up trials in a 16p11.2 del/+ mouse.

(B–E) n = 10 WT and 10 16p11.2 del/+ mice.

(F–I) 16p11.2 del/+ mice crossed with B6129SF1/J mice or GAD2-Cre mice were used.n = 18 16p11.2 del/+ and 16p11.2 del/+ x GAD2-Cre mice and 13 WT and WTx GAD2-Cre mice.

Bars and lines, averages across mice; dots, individual mice; error bars, SEM. t tests, $^{**}p < 0.01$; $^{*}p < 0.05$. See also Figure S1 and Table S1.

in the novel or familiar cage (Figure 3B; p = 0.629). The number of MAs in the novel cage was significantly higher in 16p11.2 del/+ x DBH-Cre mice compared with WT x DBH-Cre mice (Figure 3B; p = 0.001). During NREMs, EEG δ, θ, and σ power were similar between the two groups (Figure S2B). In both groups, the amount of REMs was significantly decreased in the novel cage compared with the familiar cage, while the amount was significantly lower in 16p11.2 del/+ x DBH-Cre mice compared with WT x DBH-Cre mice (Figures 3C and S2C; mixed ANOVA, genotype p = 0.031, condition p = 1.333e-13, interaction p = 0.198; pairwise t tests with Holm correction, p = 0.029). In both groups of mice, exposure to the novel cage significantly increased the amount of wake while decreasing NREMs compared with the familiar cage (Figures 3C and S2D; mixed ANOVA, condition p = 1.893e-7, 1.998e-5 for wake and NREMs). Moreover, we found that the number of LC-NE calcium transients and their overlap with MAs was significantly higher in 16p11.2 del/+ x DBH-Cre mice than in WT x DBH-Cre mice in the novel cage (Figures 3D and 3E; t tests p = 0.030 and 0.014). The number of calcium peaks was positively correlated with the number of MAs and negatively correlated with the amount of REMs (Figure 3F; Mixed linear model regression, MA freq z = 4.173, p = 0.000, group comparison z = −0.166, p = 0.868; REMs z = −2.343, p = 0.019, group comparison z = 0.554, p = 0.579). Thus, elevated activity of LC-NE neurons in 16p11.2 del/+ x DBH-Cre mice following exposure to a novel cage leads to increased MAs during NREMs and reduced REMs.

Optogenetic inhibition of LC-NE neurons and clonidine reverses sleep fragmentation in 16p11.2 del/+ mice

To test whether inhibiting LC-NE neurons reverses sleep fragmentation in 16p11.2 del/+ mice, we bilaterally injected AAVs encoding the bistable chloride channel SwiChR++ (AAV-DIO-SwiChR++-eYFP) or eYFP (AAV-DIO-eYFP) into the LC of 16p11.2 del/+ x DBH-Cre mice to optogenetically inhibit LC-NE neurons (Figures 4A, 4B, and S3A). We performed sustained optogenetic inhibition (3 s step pulses at 3 min intervals for 6 h) and compared the number of MAs as well as the percentage, duration, and frequency of each state. SwiChR++-mediated inhibition of LC-NE neurons significantly decreased the number of MAs, while increasing and decreasing the duration and frequency of NREMs episodes, respectively (Figures 4C and 4D; t tests, p = 2.938e-4, 0.377, 0.002, 6.865e-4 for MAs, NREM %, duration, and frequency). The overall time spent in NREMs, REMs, and wakefulness was not changed in both groups (Figures 4D, S3B and S3C; t tests, $p > 0.161$). Optogenetic inhibition significantly decreased the peak frequency of the infraslow rhythm (Figure S3D; 0.015 ± 0.005 Hz in eYFP and 0.006 ± 0.002 Hz in SwiChR; t test, p = 5.198e-5), which may contribute to reduced number of MAs. Thus, inhibiting LC-NE neurons in 16p11.2 del/+ x DBH-Cre mice reversed sleep fragmentation without changing the overall amount of sleep.

To test whether pharmacologically blocking noradrenergic transmission in 16p11.2 del/+ mice also reverses sleep fragmentation, we next tested the effect of clonidine, an α2 adrenergic receptor (ADRA2A) agonist in 16p11.2 del/+ mice (1 mg/kg, intraperitoneal). Injection of clonidine significantly decreased the number of MAs while increasing the duration of NREMs episodes (Figures S4A–S4C; paired t tests, p = 0.002, 0.019 for MAs and NREMs duration). The overall time spent in NREMs was also significantly increased, while wakefulness and REMs were decreased (Figures S4C–S4E; p = 0.001, 0.027, 1.230e-5 for NREMs, wake, and REMs %). These effects on the amount of NREMs, REMs, and wakefulness were different from those observed following optogenetic LC-NE inhibition. This may be caused by the fact that the pharmacological blockade of presynaptic ADRA2A using clonidine decreases overall noradrenaline release from both central and peripheral nerve terminals whereas optogenetic inhibition specifically inhibits the activity of noradrenergic cell bodies within the LC. Taken together, both optogenetic inhibition of LC-NE neurons and pharmacological blockade of noradrenergic transmission improved sleep continuity, while clonidine further increased the amount of NREMs and decreased wakefulness and REMs.

Inhibiting LC-NE neurons restores memory in 16p11.2 del/+ mice

Previous studies showed that 16p11.2 del/+ mice exhibit impaired memory.[14,15,18,34] We tested whether 16p11.2 del/+ mice exhibit impaired memory in spatial object recognition (SOR) task compared with WT mice (Figure 5A). Mice were first habituated to the box on the first two days. During the training session on the third day, mice were placed in the arena with two identical objects, and then returned to their home cage for 5 h. During the test session, mice were placed in the arena with the two familiar objects, one displaced to a new location. WT mice exhibited a significant increase in the preference for the moved object, whereas in 16p11.2 del/+ mice the memory was impaired (Figure 5B; paired t tests, p = 0.025, 0.250 in WT and 16p mice; t test, p = 0.020 for discrimination ratio). To test whether inhibiting the activity of LC-NE neurons after the training session reverses memory deficits in 16p11.2 del/+ mice, we performed SwiChR++-mediated optogenetic inhibition

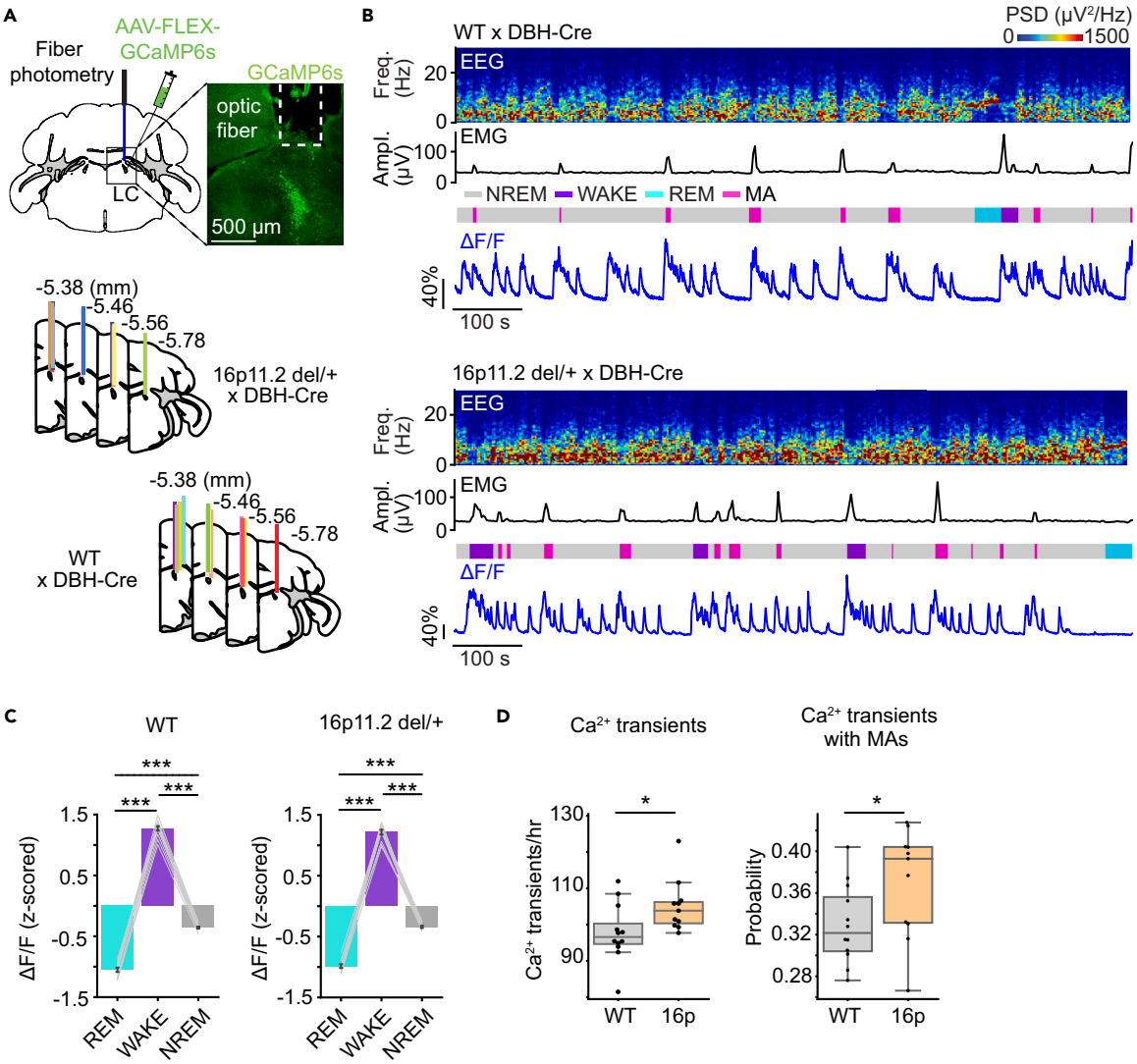

Figure 2. Increased activation of LC-NE neurons during NREMs in 16p11.2 del/+ mice

(A) Schematic of fiber photometry with simultaneous EEG and EMG recordings in LC-NE neurons. Mouse brain figure adapted from the Allen Reference Atlas - Mouse Brain (atlas.brain-map.org). Top right, fluorescence image in a 16p11.2 del/+ x DBH-Cre mouse injected with AAV-FLEX-GCaMP6s into the LC. Scale bar, 500 µm. Bottom, location of optic fiber tracts. Each colored bar represents the location of optic fibers for photometry recordings.

(B) Example fiber photometry recordings of LC-NE neurons in a WT x DBH-Cre (top) and a 16p11.2 del/+ x DBH-Cre mouse (bottom). Shown are EEG power spectra, EMG amplitude, color-coded sleep-wake states, and ΔF/F signal.

(C) *Z* scored ΔF/F activity in WT x DBH-Cre and 16p11.2 del/+ x DBH-Cre mice. Bars, averages across mice; lines, individual mice; error bars, SEM. One-way rm ANOVA followed by pairwise t tests with Bonferroni correction, ***$p < 0.001$.

(D) Left, number of calcium transients in LC-NE neurons during NREMs. Right, proportion of calcium transients coinciding with MAs. Boxplots; dots, individual mice. t tests, *$p < 0.05$.n = 12 WT x DBH-Cre and 11 16p11.2 del/+ x DBH-Cre mice. See also Table S1.

of LC-NE neurons in 16p11.2 del/+ x DBH-Cre and WT x DBH-Cre mice (Figure 5C). Immediately following the training session, optogenetic inhibition was performed for 5 h in the home cage as described previously (3 s step pulses at 3 min intervals). WT mice still showed a significant preference for the moved object after inhibition of LC-NE neurons, and the change in the preference between the training and test session was comparable for eYFP - WT x DBH-Cre and SwiChR - WT x DBH-Cre groups (Figure 5D; paired t tests, $p = 0.018$, 0.049 in eYFP and SwiChR mice; t test, $p = 0.254$ for discrimination ratio). However, in 16p11.2 del/+ x DBH-Cre mice, SwiChR++-mediated inhibition of LC-NE neurons significantly increased the preference for the moved object compared with the eYFP-expressing 16p11.2 del/+ x DBH-Cre control mice (Figure 5E; paired t tests, $p = 0.382$, 0.002 in eYFP and SwiChR mice; t test, $p = 0.017$ for discrimination ratio). Optogenetic inhibition after the training in 16p11.2 del/+ x DBH-Cre mice also decreased the amount of MAs, similar to Figure 4D (Figure S5A; t test, $p = 0.048$) without changing the amount of NREMs, REMs, and wakefulness (Figures S5B–S5D). Taken together, inhibiting LC-NE neurons reversed memory impairment in 16p11.2 del/+ mice.

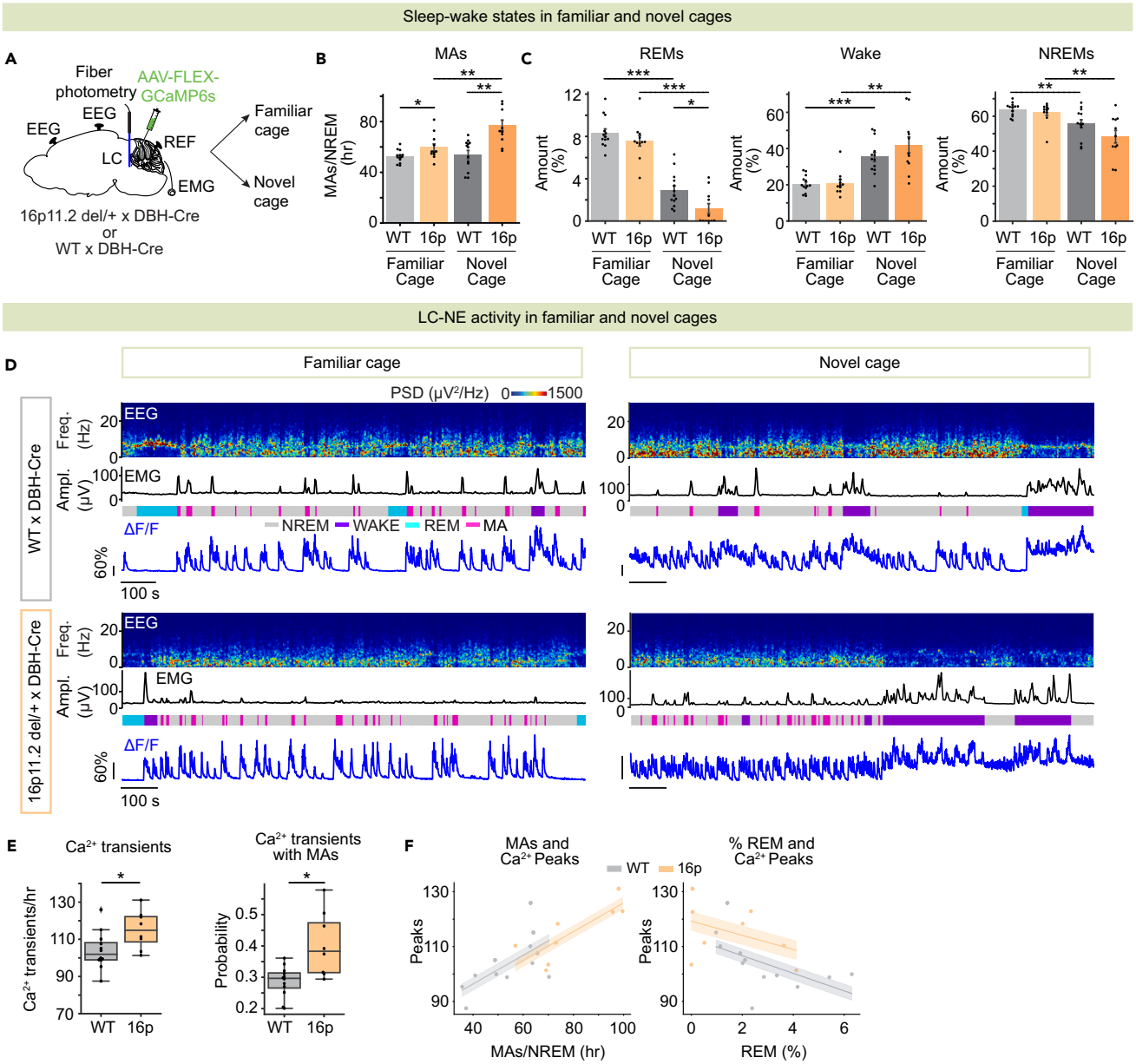

Figure 3. Elevated activity of LC-NE neurons in novel environment leads to fragmented NREMs and reduced REMs in 16p11.2 del/+ mice

(A) Schematic of fiber photometry with simultaneous EEG and EMG recordings in LC-NE neurons during exposure to a familiar or novel cage.

(B) Number of MAs during NREMs in 16p11.2 del/+ x DBH-Cre and WT x DBH-Cre mice in the novel and familiar cages for 3 h recordings.

(C) Percentage of time spent in REMs, wakefulness, and NREMs.

(D) Example fiber photometry recordings of LC-NE neurons in a WT x DBH-Cre (top) and a 16p11.2 del/+ x DBH-Cre mouse (bottom) in familiar (left) and novel (right) cages. Shown are EEG power spectra, EMG amplitude, color-coded sleep-wake states, and ΔF/F signal.

(E) Left, number of calcium transients in LC-NE neurons during NREMs in a novel cage. Right, proportion of calcium transients coinciding with MAs. Boxplots; dots, individual mice.

(F) Correlation of the number of calcium transients with the number of MAs (left) or the amount of REMs (right). Shadings, SEM.

(B and C) Bars, averages across mice; dots, individual mice; error bars, SEM. Mixed ANOVA followed by pairwise t tests with Holm correction. ***$p < 0.001$; **$p < 0.01$; *$p < 0.05$ $n = 11$ 16p11.2 del/+ x DBH-Cre and 13 WT x DBH-Cre mice.

(E and F) t tests, *$p < 0.05$. $n = 8$ 16p11.2 del/+ x DBH-Cre and 12 WT x DBH-Cre mice. See also Figure S2 and Table S1.

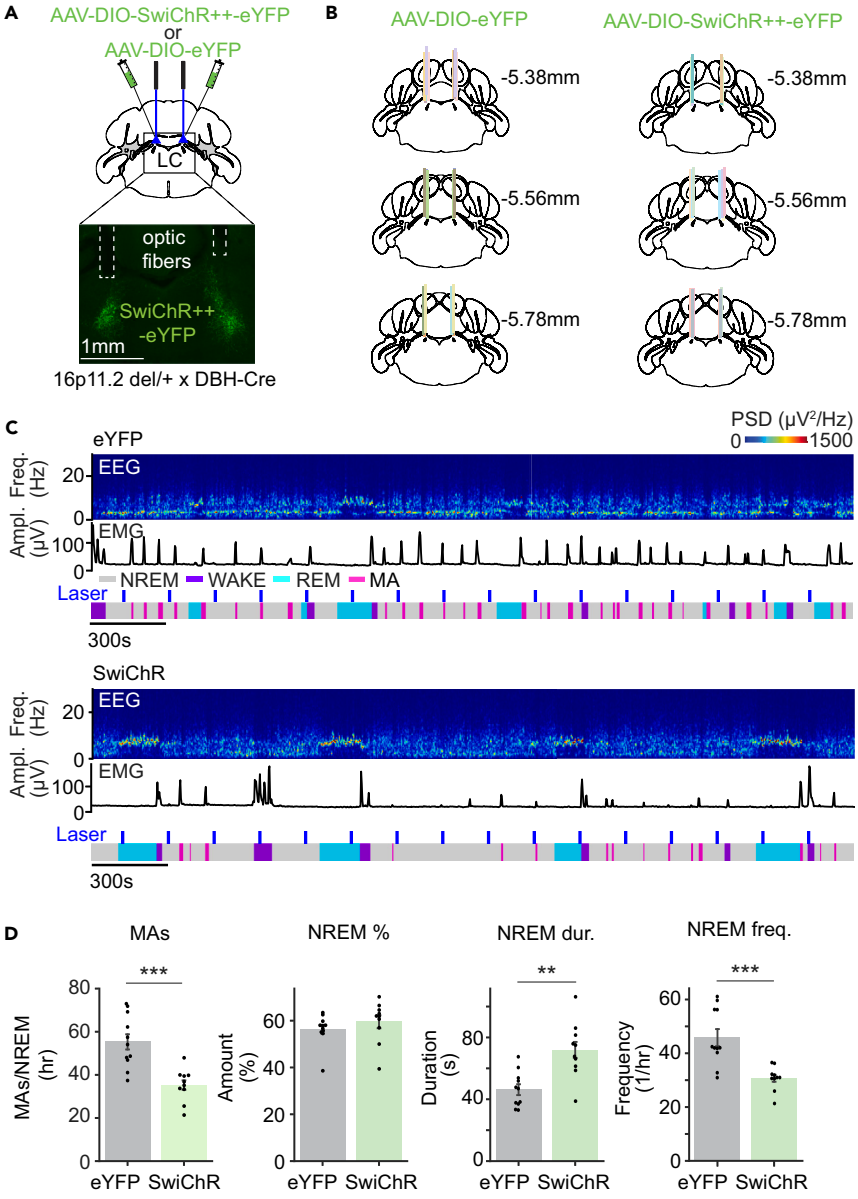

Figure 4. Optogenetic inhibition of LC-NE neurons reverses sleep fragmentation in 16p11.2 del/+ mice

(A) Schematic of SwiChR++-mediated inhibition experiments and a fluorescence image of LC in a 16p11.2 del/+ x DBH-Cre mouse bilaterally injected with AAV-DIO-SwiChR++-eYFP into the LC.

(B) Location of fiber tracts. Each colored bar represents the location of optic fibers.

(C) Example sessions from eYFP- (top) or SwiChR++- (bottom) expressing 16p11.2 del/+ x DBH-Cre mouse with laser stimulation. Shown are EEG power spectra, EMG traces, and color-coded sleep-wake states.

(D) Number of MAs during NREMs, percentage of NREMs, duration and frequency of NREMs episodes in eYFP- or SwiChR++- expressing 16p11.2 del/+ x DBH-Cre mice during the 6 h laser recordings. Bars, averages across mice; dots, individual mice; error bars, SEM. t tests, ***p <0.001, ** p <0.01. n = 11 eYFP-16p11.2 del/+ x DBH-Cre and 10 SwiChR-16p11.2 del/+ x DBH-Cre mice. See also Figures S3, S4 and Table S1.

Changes of LC-NE presynaptic input distributions in 16p11.2 del/+ mice

Differences in the distribution of presynaptic inputs may contribute to the different activity dynamics of LC-NE neurons in 16p11.2 del/+ and WT mice. To compare the organization of presynaptic inputs between 16p11.2 del/+ and WT mice, we performed mono-synaptically restricted rabies virus tracing from LC-NE neurons. AAVs with Cre-dependent expression of the TVA receptor fused with mCherry (TC66T) and rabies glycoprotein (RG) were injected into the LC of 16p11.2 del/+ x DBH-Cre and WT x DBH-Cre mice (Figure 6A). 12 days later, a modified rabies virus expressing GFP (RV*d*G-GFP) was injected into the LC. The majority of starter cells expressing both TC66T and GFP were

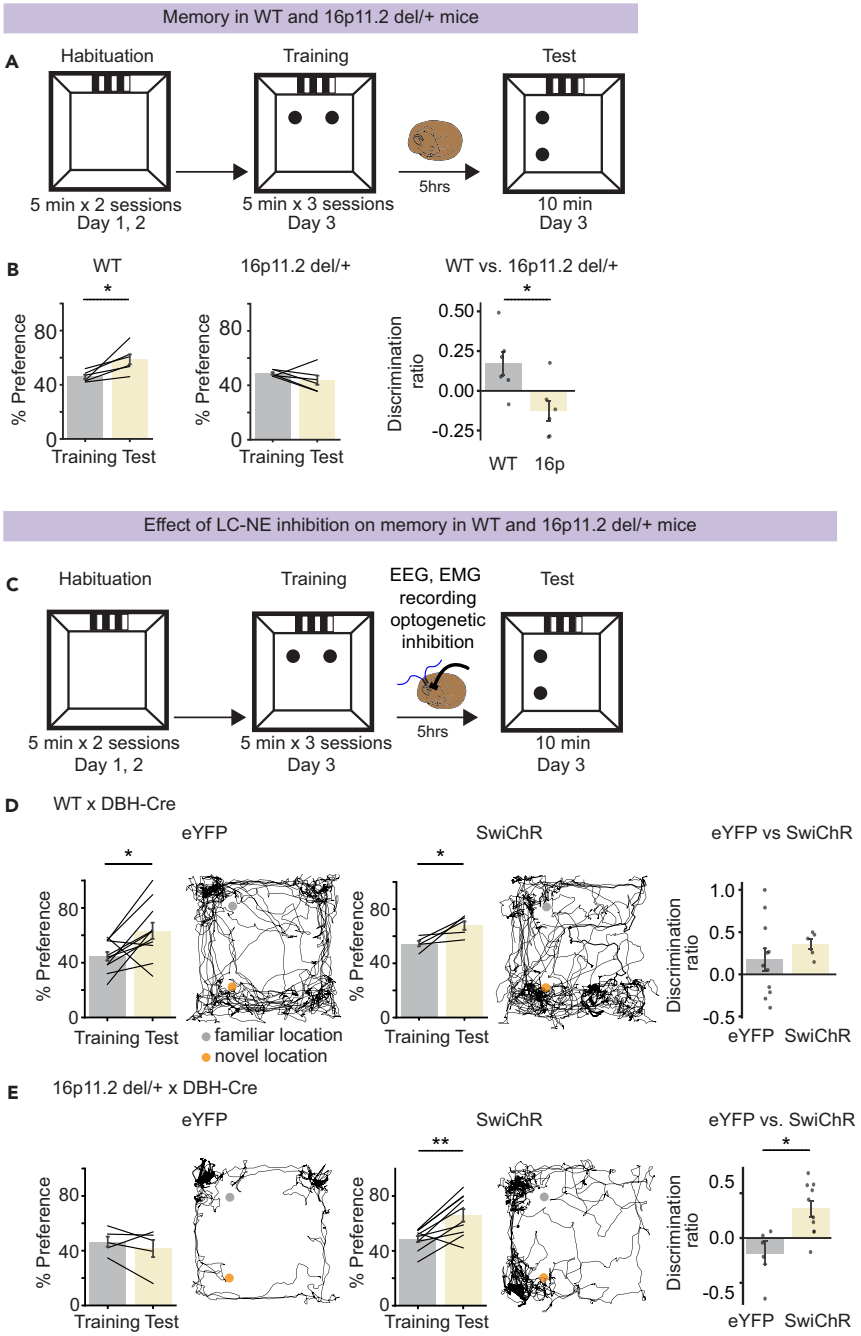

Figure 5. Optogenetic inhibition of LC-NE neurons restores memory in 16p11.2 del/+ mice

(A) Schematic of SOR task in WT and 16p11.2 del/+ mice.

(B) Preference (%) for a moved object during training and testing sessions in WT and 16p11.2 del/+ mice (left and middle). Preference (%) is calculated as $\frac{(T\ novel\ location)}{(T\ familiar\ location+T\ novel\ location)} \times 100$. Discrimination ratio (right) is calculated as $\frac{(T\ novel\ location - T\ familiar\ location)}{(T\ familiar\ location+T\ novel\ location)}$. $n = 6$ WT and 6 16p11.2 del/+ mice.

(C) Schematic of SOR task combined with SwiChR++-mediated LC-NE inhibition.

(D) Effect of LC-NE inhibition on SOR memory in WT x DBH-Cre mice. $n = 11$ eYFP- WT x DBH-Cre and 5 SwiChR - WT x DBH-Cre mice.

(E) Effect of LC-NE inhibition on SOR memory in 16p11.2 del/+ x DBH-Cre mice. $n = 5$ eYFP-16p11.2 del/+ x DBH-Cre and 10 SwiChR-16p11.2 del/+ x DBH-Cre mice.

Bars, averages across mice; dots and lines, individual mice; error bars, SEM. Unpaired and paired t tests, $^{**}p < 0.01$; $^{*}p < 0.05$.See also Figure S5 and Table S1.

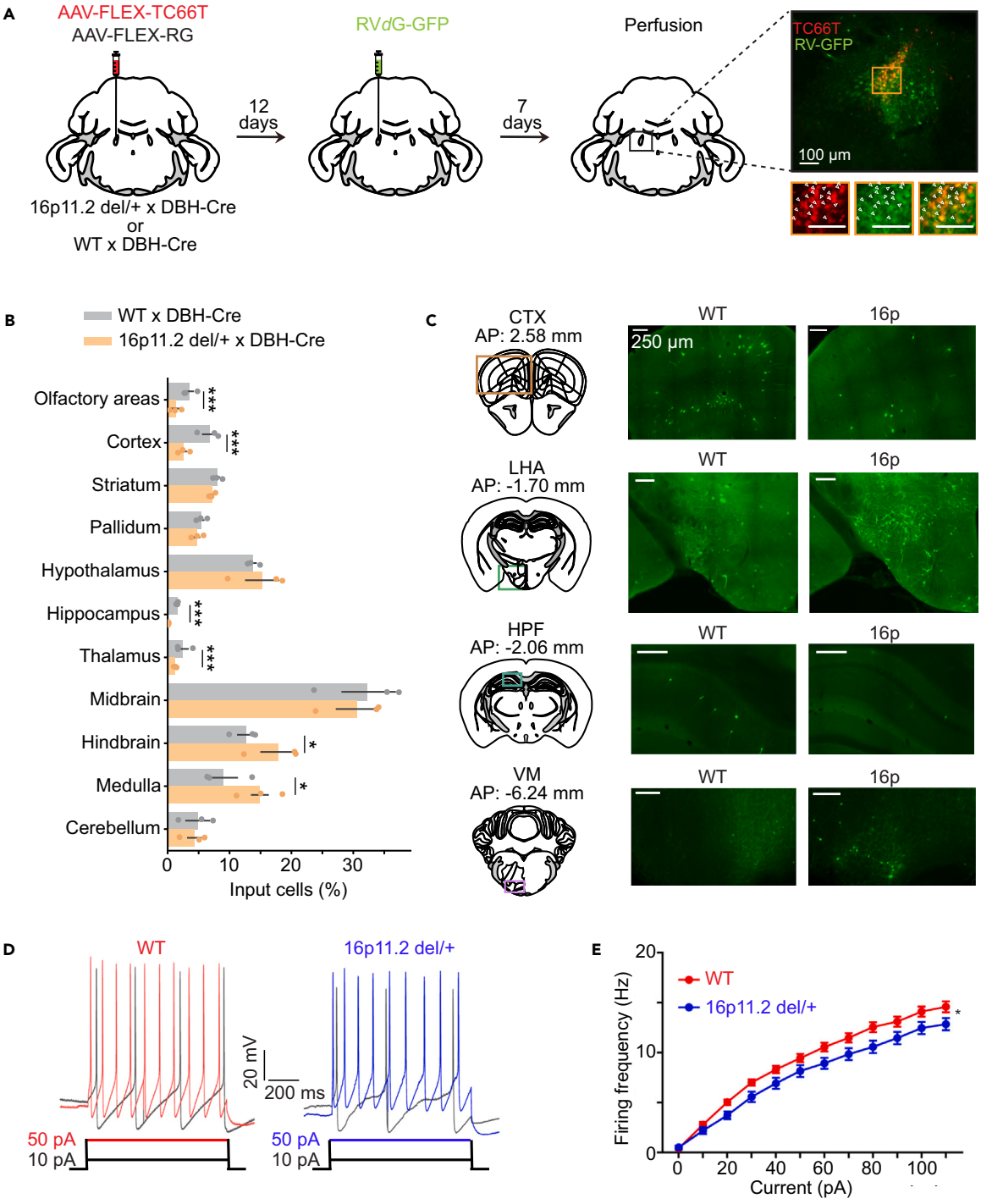

Figure 6. Presynaptic input distributions and electrophysiological properties of LC-NE neurons in 16p11.2 del/+ mice

(A) Schematic illustration of rabies-mediated tracing of monosynaptic inputs to LC-NE neurons. AAVs expressing Cre-dependent mutant EnvA receptor fused with mCherry (TC66T) and Cre-dependent rabies glycoprotein (GP) were injected into the LC of 16p11.2 del/+ x DBH-Cre and WT x DBH-Cre mice. 12 days later, we injected EnvA-pseudotyped, G-deleted, and GFP expressing rabies virus (RV*d*G-GFP). Starter cells co-express TC66T and GFP (right). Scale bar, 100 μm.

(B) Proportion of RV-GFP labeled inputs to LC-NE neurons across brain regions defined by the Allen Reference Atlas - Mouse Brain (atlas.brain-map.org). Proportion of input cells (%) was calculated by dividing the number of RV-GFP labeled neurons found in a specific brain region by the total number of input neurons. Bars, average across mice; dots, individual mice; error bars, SEM $n = 3$ mice. Bootstrap, ***$p < 0.001$; *$p < 0.05$.

Figure 6. *Continued*

(C) GFP-labeled input neurons in the cortex (CTX), lateral hypothalamic area (LHA), hippocampus (HPF) and ventral medulla (VM, the shown image contains the magnocellular reticular nucleus and paragigantocellular reticular nucleus within the medulla) of 16p11.2 del/+ x DBH-Cre and WT x DBH-Cre mice. Scale bar, 250 μm.

(D) Example firing patterns of LC-NE neurons in response to current injections (10 and 50 pA). Baseline membrane potential = −60 mV.

(E) Firing frequencies of LC-NE neurons in WT (29 cells from 4 animals) and 16p11.2 del/+ (26 cells from 4 animals) mice plotted against the injected currents. Two-way ANOVA (cell type and current step as two factors). *$p < 0.05$. Data are shown as mean ± SEM. See also Figure S6 and Table S1.

located in the LC (Figure 6A). The proportion of input neurons in the hypothalamus was similar in both groups (Figure 6B; bootstrap, $p = 0.48$). However, the proportion in the lateral hypothalamic area (LHA) was higher in 16p11.2 del/+ mice compared with WT mice (Figures 6C and S6; $p = 0.012$). We have recently shown that LHA glutamatergic neurons are wake and MA promoting.[35] While the cell type of these presynaptic inputs in the LHA remains to be determined, we predict that the increased number of inputs from the LHA to the LC in 16p11.2 del/+ mice may contribute to increased frequency of MAs. The proportion of input neurons in the medulla of 16p11.2 del/+ mice was higher compared with WT mice (Figure 6B; $p = 0.042$). In particular, we found more presynaptic cells in the ventral medulla (VM) of 16p11.2 del/+ mice (Figures 6C and S6; $p < 0.001$). The VM sends excitatory inputs to the LC.[36] Activation of glutamatergic neurons in the VM strongly promotes wakefulness,[37] and these inputs to the LC may lead to increased arousal in 16p11.2 del/+ mice. A small proportion of input neurons was found in the cortex and hippocampus of WT mice, and their number was further reduced in 16p11.2 del/+ mice (Figures 6B and 6C; $p < 0.001$).

To examine whether the increased activity of LC-NE neurons is due to the changes in their intrinsic excitability, we performed whole-cell patch clamp recordings of LC-NE neurons in 16p11.2 del/+ x DBH-Cre and WT x DBH-Cre mice. We injected AAV-DIO-tdTomato into the LC of these mice. Three weeks later, we performed patch clamp recordings of tdTomato expressing LC-NE cells. We found that LC-NE neurons in 16p11.2 del/+ x DBH-Cre mice display slightly lower firing frequencies upon current injection than WT x DBH-Cre mice (Figures 6D and 6E; two-way ANOVA, $p = 0.022$ for cell type).

Consequently, changes in the presynaptic inputs and not an increase in their intrinsic excitability likely underlie the increased activation of LC-NE neurons during NREMs.

DISCUSSION

We have identified a neural mechanism underlying sleep fragmentation and memory deficits in the 16p11.2 deletion mouse model of ASD. We found that 16p11.2 del/+ mice exhibit frequent MAs, a weakened infraslow σ rhythm and impaired infraslow phase coupling of sound-evoked arousals (Figure 1). The frequent MAs in 16p11.2 del/+ mice are reflected in an increased number of LC-NE calcium transients during NREMs (Figure 2). Exposure to a novel environment further disrupted their sleep quality (Figure 3). In contrast, optogenetic inhibition of LC-NE neurons and pharmacological blockade of noradrenergic transmission using clonidine reversed sleep fragmentation (Figure 4). In addition, inhibition of LC-NE neurons restored memory in 16p11.2 del/+ mice (Figure 5). Rabies virus-mediated screening revealed an altered connectivity of LC-NE neurons with presynaptic partners in 16p11.2 del/+ mice that may contribute to the observed sleep disturbances (Figure 6). In sum, our findings elucidate a mechanism by which heightened LC-NE neuron activity underlies sleep disturbances and memory impairment in the 16p11.2 del mouse model of ASD.

Previous studies on LC have demonstrated its critical role in regulating the overall macro- and micro-architecture of sleep. Lesioning LC led to either no changes in the overall amount of sleep or an increase in NREMs.[38–42] Opto- and chemogenetic inhibition of the LC-NE neurons increased NREMs during the dark phase.[43,44] Our previous and current study demonstrates that chemogenetic and optogenetic inhibition of LC-NE neurons decreases the number of MAs to consolidate sleep (Figure 4).[20] In addition, optogenetic inhibition of LC-NE neurons led to an increase in transitions to REMs.[19,21,24] In contrast, pharmacological blockade of noradrenergic transmission by activating α2 adrenergic receptors using clonidine decreases REMs, while the low dose of the drug elevated REMs in humans.[45] This may be due to the fact that clonidine affects both central and peripheral noradrenergic tone.[46] In addition, LC-NE neurons release other neuromodulators such as dopamine.[47] Thus, the effect of inhibiting LC-NE neurons on sleep could be mediated by these co-released neuromodulators and therefore differ from the effect of pharmacologically activating α2 adrenergic receptors.

In addition to the LC-NE system, previous studies also found disturbances in the serotonergic system, causing hyperactivity, reduced sociability and difficulties in coping with acute stress in 16p11.2 del/+ mice.[48,49] Serotonergic neurons in the dorsal raphe nucleus are also activated with the infraslow σ rhythm during NREMs.[50] For future studies, it would be interesting to test whether the activity of serotonergic neurons in 16p11.2 del/+ mice is also disturbed during sleep, possibly contributing to sleep disturbances and whether restoring sleep can alleviate hyperactivity and improve sociability. Besides NE and serotonin, dysfunctional dopaminergic signaling has also been shown to cause sleep disturbances. A homozygous InsG3680 mutation in the *Shank3* gene caused a reduction of NREMs.[51] Opto- and chemogenetic inhibition of ventral tegmental area dopaminergic neurons during the critical developmental period increased NREMs and restored social novelty preference in adulthood. For future studies, it would be interesting to test whether these deficits in dopaminergic signaling are specific to Shank3 mutant mice or whether different ASD mouse models including 16p11.2 del/+ mice share dysfunctions in these neuromodulatory systems.

16p11.2 del/+ mice model a deletion on human chromosome 16p11.2 that contains 27 genes, and they are located within chromosome 7 in mice.[52] In particular, the ERK1 gene (MAPK3) and the major vault protein (MVP) gene may contribute to changes in neural activity. The extracellular signal-regulated kinase/mitogen-activated protein kinase (ERK/MAPK) is an essential component in intracellular signaling pathways

regulating neural development and synaptic plasticity.[53,54] MVP acts as an ERK scaffold that could regulate the ERK signaling pathway.[55,56] 16p11.2 del/+ mice exhibit dysregulated ERK activity in the developing cortex and hippocampus.[14] LC neurons express large numbers of GABA receptors to exert tonic inhibition by GABA.[57] These GABA receptors can mediate the whole-cell current through the activation of ERK in LC neurons, and ERK dependent autoregulation of GABA receptors could influence the activity of LC neurons.[57] Aberrant activity in the ERK pathway is associated with ASD.[58] Therefore, disrupted ERK signaling pathways in 16p11.2 del/+ mice may contribute to the heightened activity of LC-NE neurons.

While we found that the LC-NE neurons in 16p11.2 del/+ mice are more frequently activated during NREMs, their intrinsic excitability in whole-cell patch clamp recordings was slightly lower compared with WT mice (Figure 6). Our rabies virus tracing data suggest that changes in the distribution of presynaptic inputs may contribute to different activity patterns during sleep in 16p11.2 del/+ mice. In particular, 16p11.2 del/+ mice receive an increased number of presynaptic inputs from the LH. It remains to be tested whether these presynaptic inputs are positive for VGLUT2, or hypocretin/orexin as neurons labeled for these markers were shown to be crucial for regulating wakefulness and MAs.[35,59] A recent study showed that hypocretin/orexin neurons become activated during NREMs in synchrony with an infraslow σ rhythm, similar to LC-NE neurons.[60] Hypocretin knockout mice exhibit an increased number of LC-NE activity peaks during NREMs, suggesting that the hypocretin system mediates LC-NE activity levels during sleep. Therefore, dysregulated hypocretin/orexin or VGLUT2 system in the LH may contribute to hyper-activation of LC-NE neurons during sleep in 16p11.2 del/+ mice. In addition, the LH contains a variety of molecularly distinct populations that could be potentially involved in regulating various types of behaviors.[61] Leptin receptor expressing neurons, a population that do not overlap with Hcrt neurons, promote place preference and decrease plasma corticosterone levels when optogenetically stimulated.[62] In addition, somatostatin expressing neurons promote repetitive locomotor behaviors.[61] Therefore, investigating the identity of presynaptic inputs in the LH of 16p11.2 del/+ mice and their roles in regulating sleep-wake states and LC-NE activity will elucidate the circuit mechanisms underlying hyperactivated LC neurons during sleep and associated ASD-related behaviors. Another major input region to the LC is the ventrolateral medulla,[36,63] and we found that the number of presynaptic inputs was significantly increased in the VM of 16p11.2 del/+ mice (Figure S6). Glutamatergic neurons in the VM are wake-promoting, and send excitatory inputs to the LC.[36,37] It remains to be investigated whether glutamatergic neurons in the VM also become activated during NREMs to regulate MAs, and whether an increased number of inputs from these neurons contribute to frequent LC activation and thereby fragmented sleep in 16p11.2 del/+ mice. Taken together, differential activity patterns from these presynaptic inputs may contribute to regulate the activity dynamics of LC-NE neurons.

Consistent with previous reports,[14,15,18,34] we observed an impairment in SOR memory in 16p11.2 del/+ mice. Inhibiting LC-NE activity during subsequent sleep to reduce MAs and consolidate sleep, improved memory. A previous study found reduced activation of LC-NE neurons in 16p11.2 del/+ mice during motor learning, and activating LC-NE neurons during rotating disk tasks improved motor learning.[64] Thus, while increased LC-NE activity during motor learning is beneficial, our study, consistent with previous studies,[21,23] implies that reducing heightened LC activity during subsequent sleep improves learning, likely as a result of improved sleep quality.

Given that the rate of sound-evoked arousals in 16p11.2 del/+ mice is similar to that in WT mice (Figure 1G), their sleep disturbances are likely the result of internal processes causing the heightened frequency of MAs, rather than caused by external stimuli. It is generally assumed that fragmented sleep in individuals with ASD is due to an increased sensitivity to the external stimuli such as sound.[65] While we observed highly fragmented NREMs in 16p11.2 del/+ mice, our results contradict this assumption. The likelihood that WT and 16p11.2 del/+ mice wake up to sound is similar, but the phase coupling of the awakenings with the infraslow σ rhythm is impaired. The infraslow σ rhythm is thought to partition sleep into a consolidated phase, ideal for sleep-dependent processes such as memory consolidation, followed by a fragile sleep phase, where the organism is sensitive to external stimuli and can be easily awakened.[30] Disruption in the infraslow σ rhythm may consequently interfere with internal memory processes, resulting in poor memory consolidation. In humans, an enhanced infraslow σ rhythm, in particular within the fast spindle frequency band, was shown to be associated with better overnight memory consolidation.[30] Our rabies virus tracing experiments indicate that the number of presynaptic input neurons from the hippocampus to the LC is decreased in 16p11.2 del/+ mice which may contribute to their memory impairment. LC projections to the hippocampus are crucial for learning and memory,[47,66–68] and the reduced number of LC-projecting neurons in the hippocampus may impair a feedback mechanism, possibly contributing to the memory deficits in 16p11.2 del/+ mice.

In children with ASD, exposure to a novel environment worsens sleep efficiency,[32,33] and similarly the exposure to a novel cage exacerbated sleep quality in 16p11.2 del/+ mice, reflected in reduced REMs and a tendency toward less NREMs compared with WT mice (Figures 3B and 3C). 16p11.2 del/+ mice took longer time to habituate to their sleep environment, and the degree of habituation may therefore significantly impact the amount and quality of sleep in mouse models of ASD.[16,17] Our findings further imply that blocking noradrenergic transmission can not only improve sleep in ASD patients,[69] but help them to more quickly habituate to new sleep environments.

Taken together, our results demonstrate that heightened activity of LC-NE neurons contribute to fragment sleep and impaired memory in the 16p11.2 del mouse model of ASD. Elucidating the circuit mechanisms underlying various aspects of sleep disturbances will provide valuable insights for the development of targeted therapeutic interventions to specifically improve distinct features of sleep and related behaviors in ASD.

Limitations of the study

Our study focuses on the population activity of LC-NE neurons, which limits our ability to determine whether sleep disturbances in 16p11.2 del/+ mice are driven by changes in LC-NE activity at the single-cell level. Future studies utilizing optrode recordings or

miniscope imaging will be useful for identifying single-cell activity changes in LC-NE neurons. In addition, although our study identifies differences in the distribution of presynaptic inputs to LC-NE neurons in 16p11.2 del/+ mice compared to WT mice, future studies are needed to identify the specific presynaptic inputs that regulate LC-NE neurons, contributing to fragmented sleep in 16p11.2 del/+ mice.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Shinjae Chung (shinjaec@pennmedicine.upenn.edu).

Materials availability

This study did not generate new unique reagents.

Data and code availability

All datasets will be shared by the lead contact upon request after publication. All data have been deposited at Zenodo (https://zenodo.org) and are available as of the date of publication. All original code is deposited in: https://github.com/tortugar/Lab. DOI for datasets is listed in the key resources table. Any additional information required to reanalyze the data reported in this paper is available from the lead contact upon request.

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AUTHOR CONTRIBUTIONS

Conceptualization, A.C. and S.C.; methodology, A.C., B.K., A.W., and J.P.B.; software, B.K., H.A., and F.W.; investigation, A.C., B.K., E.L., Y.W., J.S., H.S., X.J., I.A., and J.H.; resources, S.T. and K.B.; writing, review and editing, A.C., M.M., F.W., and S.C.; visualization, A.C., B.K., E.L., A.W., and Y.W.; supervision, S.C.; funding acquisition, S.C.

DECLARATION OF INTERESTS

The authors declare no competing financial interests.

STAR★METHODS

Detailed methods are provided in the online version of this paper and include the following:

SUPPLEMENTAL INFORMATION

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STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
anti-GFP antibody	Aves lab	Cat# GFP-1020; RRID:AB_10000240
anti-GFP antibody	Abcam	Cat# ab300643
Alexa Fluor 488-AffiniPure donkey anti-chicken IgY (IgG) (H + L)	Jackson ImmunoResearch Laboratories	Cat# 703545155; RRID:AB_2340375
anti-tyrosine hydroxylase antibody	Abcam	Cat# ab112; RRID:AB_297840
Alexa Fluor 488-AffiniPure donkey anti-chicken IgY (IgG) (H + L)	Jackson ImmunoResearch Laboratories	Cat# 703545155; RRID:AB_2340375
Bacterial and virus strains		
AAV1-Syn-Flex-GCaMP6S-WPRE-SV40	Penn vector core/Addgene	100845-AAV1; RRID: Addgene_100845
AAV2-EF1α-DIO-eYFP	University of North Carolina vector core	27056; RRID: Addgene_27056
AAV2-EF1α-DIO-SwiChR++-eYFP	University of North Carolina vector core	55631; RRID: Addgene_55631
AAV-DIO-tdTomato	Addgene	28306-AAV2; RRID:Addgene_28306
AAV-CAG-FLEX-TC66T	UC Irvine/Kevin Beier	Cat# 48331; RRID: Addgene_48331
AAV-CAG-FLEX-RG	UC Irvine/Kevin Beier	Cat# 48333; RRID: Addgene_48333
RVdG	UC Irvine/Kevin Beier	N/A
Chemicals, peptides, and recombinant proteins		
Hoechst solution	Thermo Scientific	Cat# 33342
Fluoromount-G mounting medium	Southern Biotechnic	Cat# 0100-01
Deposited data		
Source data	This paper	https://doi.org/10.5281/zenodo.13901368
code	This paper	https://github.com/tortugar/Lab
Experimental models: Organisms/strains		
16p11.2 del/+ mice	Jackson lab	Cat# 013128; RRID:IMSR_JAX:013128
B6129SF1/J	Jackson lab	Cat# 101043; RRID:IMSR_JAX:101043
GAD2-Cre	Jackson lab	Cat# 010802; RRID:IMSR_JAX:010802
DBH-Cre mice	Penn/Steven Thomas (Antila et al.[20], Tsetsenis et al; [70])	N/A
Software and algorithms		
Python Programming Language	https://www.anaconda.com/	RRID: SCR_008394
RHD USB Interface Board Software	https://intantech.com/downloads.html?tabSelect=Software&yPos=0	RRID: SCR_019278
Synapse	Tucker-DavisTechnologies, https://www.tdt.com/component/synapse-software/	RRID: SCR_006495
SciPy	https://www.scipy.org/	RRID: SCR_008058
Pingouin	https://pingouin-stats.org/build/html/index.html	RRID: SCR_022261
Other		
Nanoject II	Drummond Scientific	Cat# 3-000-204
Raspberry Pi 3 Model B	RaspberryPi, https://www.raspberrypi.org/	N/A
RHD 2000 USB Interface Board	Intan Technologies	Cat# C3100
RHD2132 amplifier	IntanTechnologies, https://intantech.com/products_RHD2000.html	Cat# C3334

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REAGENT or RESOURCE	SOURCE	IDENTIFIER
RZ5P amplifier	Tucker- DavisTechnologies, https://www.tdt.com/component/rz5p/	Cat# RZ5P; RRID: SCR_024878
473 nm lasers	Laserglow	Cat# LRS-0473- PFM-00050-05
Allen Mouse Brain Atlas	https://mouse.brain-map.org/static/atlas	RRID: SCR_002978

EXPERIMENTAL MODEL AND STUDY PARTICIPANT DETAILS

Animals

16p11.2 del/+ mice,[52] B6129SF1/J and GAD2-Cre mice were obtained from Jackson lab (#013128, 101043, 010802; Jackson lab). DBH-Cre mice[20,70] were obtained from Dr. Steve Thomas, Penn. 16p11.2 del/+ mice were crossed with B6129SF1/J mice to generate 16p11.2 del/+ and 16p11.2 +/+ (WT) mice. To target LC-NE neurons for photometry recordings, optogenetic inhibition, RV tracing and patch clamp recordings, 16p11.2 del/+ mice were crossed with DBH-Cre mice to generate 16p11.2 del/+ x DBH-Cre and 16p11.2 +/+ (WT) x DBH-Cre mice. For sound stimulation experiments, 16p11.2 del/+ crossed with GAD2-Cre mice as well as 16p11.2 del/+ mice crossed with B6129SF1/J mice were used. All mice were housed on a 12 h light/12 h dark cycle (lights on 07:00 and off 19:00) with free access to food and water and were 6–16 weeks old at the time of surgery. A previous study reported male-specific sleep deficits in the 16p11.2 del/+ mice[16] and therefore male mice were used for all the experiments. All procedures were approved by Institutional Animal Care and Use Committees of the University of Pennsylvania and were done in accordance with the federal regulations and guidelines on animal experimentation (National Institutes of Health Offices of Laboratory Animal Welfare Policy).

METHOD DETAILS

Surgery

To implant electroencephalogram (EEG) and electromyogram (EMG) recording electrodes, mice were anesthetized with 1.5–2% isoflurane and placed on a stereotaxic frame. Two stainless steel screws were inserted into the skull 1.5 mm from midline and 1.5 mm anterior to the bregma, and 2.5 mm from midline and 2.5 mm posterior to the bregma. The reference screw was inserted on top of the cerebellum. Two EMG electrodes were inserted into the neck musculature. Insulated leads from the EEG and EMG electrodes were soldered to a 2 × 3 pin header, which was secured to the skull using dental cement.

For LC photometry recordings, AAV1-Syn-Flex-GCaMP6S-WPRE-SV40 (0.3 μL, #100845-AAV1, Penn vector core/Addgene) was injected into LC (AP -5.3 mm, ML +/−1.0 mm, DV -2.7–3.3 mm from the cortical surface) using Nanoject II (Drummond Scientific) via a micropipette followed by an optic fiber (400 μm in diameter) implantation on top of the injection site. For optogenetic inhibition experiments, AAV2-EF1α-DIO-eYFP or AAV2-EF1α-DIO-SwiChR++-eYFP (0.3 μL, #27056, 55631, University of North Carolina vector core) was bilaterally injected into the LC followed by bilateral implantation of optic fibers (200 μm in diameter). Dental cement was applied to cover the exposed skull completely and to secure the optic fiber and the EEG/EMG implant. After surgery, mice were allowed to recover for at least 2–3 weeks before experiments.

For patch clamp recordings, AAV-DIO-tdTomato (0.3 μl, #28306-AAV2, Addgene) was injected into LC. 3 weeks later, patch clamp recordings were performed.

For rabies tracing experiments, 0.4 μL of a mixture containing AAV-CAG-FLEX-TC66T and AAV-CAG-FLEX-RG was injected into the LC. After 12 days, 0.4 μL of EnvA-pseudotyped, rabies-glycoprotein-deleted, and GFP-expressing rabies viral particles (RV*dG*) were injected into the LC. After 7 days, mice were perfused. Viruses for rabies tracing experiments were obtained from the University of California, Irvine.

Histology

Mice were deeply anesthetized and transcardially perfused with phosphate-buffered saline (PBS) followed by 4% paraformaldehyde (PFA) in PBS. Brains were fixed overnight in 4% PFA and then transferred to 30% sucrose in PBS solution for at least one night. Brains were embedded and mounted with Tissue-Tek OCT compound (Sakura Finetek) and frozen. 50–60 μm sections were cut using a cryostat (Thermo Scientific HM525 NX) and mounted onto glass slides. Brain sections were washed in PBS, permeabilized using PBST (0.3% Triton X-100 in PBS) for 30 min and then incubated in blocking solution (5% normal donkey serum [NDS] in PBST) for 1 h. Slices were then incubated in primary antibodies (1:1000, anti-GFP antibody, #GFP-1020, Aves lab or #ab300643, abcam; 1:1000, anti-tyrosine hydroxylase antibody, #ab112, abcam) overnight at 4 °C. 18–48 h later, slices were washed with PBS and incubated with the corresponding secondary antibody (1:500, Alexa Fluor 488-AffiniPure donkey anti-chicken IgY (IgG) (H + L), #703545155, Jackson ImmunoResearch Laboratories; 1:500, Alexa Fluor 594-AffiniPure donkey anti-rabbit IgY (IgG) (H + L), #711585152, Jackson ImmunoResearch Laboratories). Brain sections were washed with PBS followed by counterstaining with Hoechst solution (#33342, Thermo Scientific) and mounted onto glass slides. Slides were cover-slipped with Fluoromount-G (Southern Biotechnic) and imaged using a fluorescence microscope (Microscope, Leica DM6B; Camera, Leica DFC7000GT; LED, Leica CTR6 LED).

For rabies tracing, 60 μm sections were cut, and every other section was collected. Presynaptic GFP cells were counted throughout all the sections that were collected, and starter cells were counted from sections containing LC. The number of starter cells was similar in both groups (159.33 ± 13.74 and 166.33 ± 47.10 cells in WT x DBH-Cre and 16p11.2 del/+ x DBH-Cre mice respectively [mean ± s.e.m.]).

Mice with no virus expression, or misplaced optic fiber were excluded from the dataset.

Sleep recordings

Sleep recordings were carried out in the animal's home cage or in a cage to which the mouse had been habituated. EEG and EMG electrodes were connected to flexible recording cables via a mini-connector. EEG and EMG signals were recorded using an RHD2132 amplifier (Intan Technologies, sampling rate 1 kHz) connected to the RHD USB Interface Board (Intan Technologies). For fiber photometry, we used a Tucker-Davis Technologies RZ5P amplifier (sampling rate 1.5 kHz). EEG and EMG signals were referenced to a ground screw placed on top of the cerebellum. To determine the sleep-wake state of the animal, we first computed the EEG and EMG spectrogram for sliding, half-overlapping 5 s windows, resulting in 2.5 s time resolution. To estimate within each 5 s window the power spectral density (PSD), we performed Welch's method with Hanning window using sliding, half-overlapping 2 s intervals. Next, we computed the time-dependent δ (0.5–4 Hz), θ (5–12 Hz), σ (12–20 Hz) and high γ (100–150 Hz) power by integrating the EEG power in the corresponding ranges within the EEG spectrogram. We also calculated the ratio of the θ and δ power (θ/δ) and the EMG power in the range 50–500 Hz. For each power band, we used its temporal mean to separate it into a low and high part (except for the EMG and θ/δ ratio, where we used the mean plus one standard deviation as threshold). REMs was defined by a high θ/δ ratio, low EMG, and low δ power. A state was set as NREMs if the δ power was high, the θ/δ ratio was low, and EMG power was low. In addition, states with low EMG power, low δ, but high σ power were scored as NREMs. Wake encompassed states with low δ power and high EMG power and each state with high γ power (if not otherwise classified as REMs). Our automatic algorithm has been published[20,35,37,71–73] and has 90.256% accuracy compared with the manual scoring by expert annotators. We manually verified the automatic classification using a graphical user interface visualizing the raw EEG and EMG signals, EEG spectrograms, EMG amplitudes, and the hypnogram to correct for errors, by visiting each single 2.5 s epoch in the hypnograms. The software for automatic sleep-wake state classification and manual scoring was programmed in Python (available at https://github.com/tortugar/Lab/tree/master/PySleep).

Sound-evoked arousal

Mice were exposed to acoustic stimuli (15000 Hz, 65 dB, 20 s) randomly every 4–20 min for 6–7 h of recordings. The duration of noise and the sound level was based on the arousal success rate that mice woke up or slept through half of trials (arousal success rate was ∼50%). For analysis, episodes with NREMs duration >120 s were used for analysis. All sound trials were divided into sleep-through and arousal trials and EEG σ power was measured before the onset of sound.

Optogenetic manipulation

Light pulses (3 s step pulses at 3 min intervals, 2–4 mW) were generated by a blue laser (473 nm, Laserglow) and sent through the optic fiber (200 μm diameter, ThorLabs) that connects to the ferrule on the mouse head. TTL pulses to trigger the laser were controlled using a raspberry pi, which was controlled by a custom user interface programmed in Python. Optogenetic manipulations were conducted during the light period for 6 h in Figure 4 and 5 h in Figure 5.

Given SwiChR++'s channel closure rate of about 115 s[74], we applied 3 s step pulses every 3 min. A similar protocol has been previously used.[74] Moreover, using the similar protocol, we showed that SwiChR++-mediated inhibition of dorsomedial medulla GABAergic neurons and preoptic area GABAergic neurons suppressed REMs while inhibiting POA VGLUT2 neurons decreased MAs.[35,73,75]

Fiber photometry

For calcium imaging, a first LED (Doric lenses) generated the excitation wavelength of 465 nm and a second LED emitted 405 nm light, which served as control for bleaching and motion artifacts. The 465 and 405 nm signals were modulated at two different frequencies (210 and 330 Hz). Both lights were passed through dichroic mirrors before entering a patch cable attached to the optic fiber. Fluorescence signals emitted by GCaMP6s were collected by the optic fiber and passed via the patch cable through a dichroic mirror and GFP emission filter (Doric lenses) before entering a photoreceiver (Newport Co.). Photoreceiver signals were relayed to an RZ5P amplifier (Tucker-Davis Technologies, TDT) and demodulated into two signals using TDT's Synapse software, corresponding to the 465 and 405 nm excitation wavelengths. To analyze the calcium activity, we used custom-written Python scripts. First, both signals were low-pass filtered at 2 Hz using a 4th order digital Butterworth filter. Next, using linear regression, we fitted the 405 nm to the 465 nm signal. Finally, the linear fit was subtracted from the 465 nm signal (to correct for photo-bleaching or motion artifacts) and the difference was divided by the linear fit yielding the ΔF/F signal. To determine the sleep-wake state, EEG and EMG signals were simultaneously recorded with calcium signals using the RZ5P amplifier. Photometry recordings were performed for 3 h between ZT5-10.

To detect calcium transients occuring on the infraslow timescale, we first filtered the calcium signal with a zero-lag, 4th order digital Butterworth filter with cutoff frequency 1/15 Hz as previously described.[20] Next, we detected prominent peaks in the signal using the function find_peaks provided by the python library scipy (https://scipy.org/). As parameter for the peak prominence, we used 0.05 * distance between the 1st and 99th percentile of the distribution of the ΔF/F signal.

Analysis of infraslow σ power oscillations

To calculate the power spectral density of the EEG σ power, we first calculated for each recording the EEG power spectrogram by computing the FFT for consecutive sliding, half-overlapping 5 s windows. Next, we normalized the spectrogram by dividing each frequency component by its mean power and calculated the normalized σ power by averaging across the spectral density values in the σ range (10.5–16 Hz). As the infraslow rhythm is most pronounced in consolidated NREMs bouts,[30] we only considered NREMs bouts that lasted at least 120 s, possibly interrupted by MAs (wake periods ≦ 20s). We then calculated the power spectral density using Welch's method with Hanning window for each consolidated NREMs bout and averaged for each animal across the resulting densities. To quantify the strength of the infraslow rhythm, we computed the areas under the PSD in the ranges 0.01–0.04 Hz and 0.08–0.12 Hz, respectively, and subtracted the second value from the first value.

Sleep spindle detection

Spindles were detected using a previously described algorithm using the frontal EEG.[28] The spectrogram was computed for consecutive 600 ms windows with 500 ms overlap, resulting in a 100 ms temporal resolution. The spindle detection algorithm used two criteria to determine for each 100 ms time bin whether it was part of a spindle or not: The first criterion was that the height of the maximum peak in the σ frequency range (10–16.67 Hz) exceeds a threshold, which corresponded to the 96th percentile of all maximum peaks in the σ frequency range of the sleep recording. We determined the optimal percentile value by maximizing the performance of the algorithm on a manually annotated control dataset. Second, the power value of the peak in the σ range (10–16.67 Hz) had to be greater than half of the peak value in the range 0–10 Hz. The optimal value for this ratio (σ peak ratio) was again determined on the control dataset. Next, the algorithm merged spindle events that were temporally close to each other. First, spindle events in adjacent bins were considered as part of the same spindle. Second, we fused together sequences of spindle events that were interrupted by gaps of less than 300 ms. The optimal value for the gap was again determined on the control dataset. Finally, we discarded spindles with duration ≦ 200 ms. Of all the potential spindles, we only considered those as spindles where for at least half of the time bins the peak frequency lied in the range of 10–16.7 Hz. The parameters of the spindle detection algorithm (σ percentile threshold, σ peak ratio, and minimum fusing distance) were optimized using a manually annotated dataset.

Patch clamp recordings

Mice were deeply anesthetized with ketamine/xylazine (200/20 mg/kg body weight) and decapitated. Brains were harvested and placed immediately in ice-cold cutting solution (92 mM *N*-methyl-D-glucamine, 2.5 mM KCl, 1.2 mM NaH_2PO_4, 30 mM $NaHCO_3$, 20 mM HEPES, 25 mM glucose, 5 mM sodium L-ascorbate, 2 mM thiourea, 3 mM sodium pyruvate, 10 mM $MgSO_4$ and 0.5 mM $CaCl_2$) and continuously bubbled with 95% O_2 and 5% CO_2. 200 μm thick coronal sections were cut with a vibratome (Leica VT 1200S) and placed in artificial cerebrospinal fluid (ACSF: 126 mM NaCl, 2.5 mM KCl, 1.2 mM MgSO4, 2.4 mM CaCl2, 25 mM NaHCO3, 1.4 mM NaH2PO4, 11 mM glucose and 0.6 mM sodium L-ascorbate) and continuously bubbled with 95% O_2 and 5% CO_2. Slices were incubated at 31°C for 30 min and then at room temperature for 30 min. Brain slices were transferred into a recording chamber and perfused with oxygenated ACSF. TdTomato+ cells in the LC were located under a 40X water-immersion objective (Olympus BX61WI). Recording pipettes were pulled from borosilicate glass (Flaming-Brown puller, Sutter Instruments, P-97, tip resistance of 5–10 MΩ) and filled with pipette solution consisting of 120 mM potassium gluconate, 10 mM NaCl, 1 mM $CaCl_2$, 10 mM EGTA, 10 mM HEPES, 5 mM Mg-ATP, 0.5 mM Na-GTP and 10 mM phosphocreatine. Whole-cell patch clamp recordings were controlled via an EPC-10 amplifier and Pulse v8.74 (HEKA Electronik). Firing patterns upon current injection were recorded under current clamp mode.

Spatial object recognition task

During the habituation session (ZT 3–4, days 1 and 2), mice were habituated to the training context (13″x13″ open field arena) for two 5-min sessions. A visual cue (a rectangle containing alternating black and white stripes) is attached to one wall of the open field to help the mice orient themselves within the open field. During the training session (ZT 3–5, day 3), mice were placed in the arena with two identical objects (two small glass bottles) for three 5-min training sessions. Immediately following the training session, mice were returned to their home cage for 5 h. In Figure 5C, optogenetic manipulation was performed for 5 h in their home cage (ZT 3.5–10: the first mouse received optogenetic stimulation between ZT 3.5–8.5, and the last mouse received stimulation between ZT 5–10) before the test session. During the test session (ZT 8.5–10.5, day 3), mice were placed in the arena with the two familiar objects, one displaced to a new location, for one 10 min test session. Exploration time to the object was quantified as the time the mouse was within 2 cm of the object with its nose oriented toward the object.[76] The representative raw traces for exploration were plotted using DeepLabCut.[77]

$$\text{Preference (\%) is calculated as } \frac{(T\ novel\ location)}{(T\ familiar\ location + T\ novel\ location)} \times 100$$

$$\text{Discrimination ratio is calculated as } \frac{(T\ novel\ location - T\ familiar\ location)}{(T\ familiar\ location + T\ novel\ location)}$$

Mice that explored less than 2 s during test or at least two training sessions were excluded.

QUANTIFICATION AND STATISTICAL ANALYSIS

Statistical analyses were performed using the python packages scipy.stats (scipy.org) and pingouin (https://pingouin-stats.org).[78] We did not predetermine sample sizes, but cohorts were similarly sized as in other relevant sleep studies.[19,51] All statistical tests were two-sided. Data were compared using t tests, bootstrap or ANOVA followed by multiple comparisons tests. For unpaired two sample t tests, Welch T test was used when the sample sizes are unequal as recommended.[79] For RM and mixed ANOVA, Mauchly's test was applied to check the sphericity of the data. In case sphericity was violated, *p* values were corrected using the Greenhouse-Geisser correction. To account for multiple comparisons, *p* values were Bonferroni or Holm corrected. For all tests, a (corrected) *p* value <0.05 was considered significant. Boxplots were used to illustrate the distribution of data points. The upper and lower edges of the box correspond to the quartiles (25th and 75th percentile) of the dataset and the horizontal line in the box depicts the median, while the whiskers indicate the remaining distribution, except for outliers, i.e., points smaller than the 25th percentile - 1.5 * the interquartile range (IQR) or larger than the 75th percentile +1.5 IQR. Outliers are depicted as diamonds. Statistical results and parameters (exact value of n and what n represents) are presented in the Table S1, figure legends and results.