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Regulation of wakefulness by neurotensin neurons in the lateral hypothalamus

Running title: Arousal Control by LH Neurotensin

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Abstract:

The lateral hypothalamic region (LH) has been identified as a key region for arousal regulation, yet the specific cell types and underlying mechanisms are not fully understood. While neurons expressing orexins (OX) are considered the primary wake-promoting population in the LH, their loss does not reduce daily wake levels, suggesting the presence of additional wake-promoting populations. In this regard, we recently discovered that a non-OX cell group in the LH, marked by the expression of neurotensin (Nts), could powerfully drive wakefulness. Activation of these Nts^{LH} neurons elicits rapid arousal from non-rapid eye movement (NREM) sleep and produces uninterrupted wakefulness for several hours in mice. However, it remains unknown if these neurons are necessary for spontaneous wakefulness and what their precise role is in the initiation and maintenance of this state. To address these questions, we first examined the activity dynamics of the Nts^{LH} population across sleep-wake behavior using fiber photometry. We find that Nts^{LH} neurons are more active during wakefulness, and their activity increases concurrently with, but does not precede, wake-onset. We then selectively destroyed the Nts^{LH} neurons using a diphtheria-toxin-based conditional ablation method, which significantly reduced wake amounts and mean duration of wake bouts and increased the EEG delta power during wakefulness. These findings demonstrate a crucial role for Nts^{LH} neurons in maintaining normal arousal levels, and their loss may be associated with chronic sleepiness in mice.

Keywords

Neurotensin, Arousal, Hypersomnia, Locomotion, Fiber Photometry, Orexins, Conditional Ablation

Highlights

1. Neurotensin-expressing neurons in the lateral hypothalamus (Nts^{LH}) are more active during wakefulness and rapid eye movement (REM) sleep than during non-REM (NREM) sleep.
2. Selective ablation of Nts^{LH} neurons results in reduced time spent in wakefulness, shorter wake bouts and increased EEG delta power during wakefulness.
3. Despite their maximal activity during REM sleep, the loss of Nts^{LH} neurons does not alter REM sleep amounts or architecture.
4. This work reveals the necessary role of Nts^{LH} neurons in normal arousal control and suggest that their loss may be associated with hypersomnia and excessive sleepiness.

Introduction

Excessive daytime sleepiness is a hallmark of central disorders of hypersomnolence and a frequent symptom in numerous sleep, neurological, and psychiatric disorders (Dubessy & Arnulf, 2023; Happe, 2003; Hawley et al., 2010; Maestri et al., 2020; Pérez-Carbonell et al., 2022; Rosenberg et al., 2024). Sleepiness and the associated fatigue adversely affect daily activities, reduce work productivity, and diminish the overall quality of life (Barnes & Watson, 2019; Léger & Stepnowsky, 2020). Furthermore, it impacts decision-making, increases the likelihood of medical errors, and raises the risk of motor vehicle accidents (Jha et al., 2001; Zhang & Chan, 2014). However, the neural mechanisms underlying arousal control and sleepiness remain poorly understood.

Since Von Economo's observations on the post-mortem brains of encephalitis lethargica patients, the lateral hypothalamic area (LH) has been recognized as a key site for regulating wakefulness (Economo, 1930). Electrolytic lesions of this region in monkeys and knife-cut lesions that isolated LH from the rest of the brain in rats induced hypersomnolence, although sleep-wake was only assessed by behavioral criteria (Nauta, 1946; Ranson, 1939). Nevertheless, several studies thereafter showed that neurotoxic lesions or pharmacological inhibitions of the LH reduced daily wake amounts and the ability to remain awake for longer periods, confirming the necessity of LH for normal levels of wakefulness (Cerri et al., 2014; de Ryck & Teitelbaum, 1978; Gerashchenko et al., 2003; Gerashchenko et al., 2001; McGinty, 1969; Shoham & Teitelbaum, 1982). However, the specific neural elements within the LH involved in this control are not completely understood and are being actively explored. Orexin-producing neurons (OX) are considered to be the primary wake-promoting population in the LH (Chemelli et al., 1999; Mochizuki & Scammell, 2003; Overeem et al., 2002; Yamanaka et al., 2003; Yamashita & Yamanaka, 2017). While the loss of OX signaling in humans and animals causes sleep-wake fragmentation and excessive sleepiness, it does not increase total sleep time (Chemelli et al., 1999; Hara et al., 2001; Mochizuki et al., 2004; Tabuchi et al., 2014; Thannickal et al., 2000), suggesting that additional wake-promoting neurons are located within the LH region. In this regard, we recently identified another peptidergic population expressing neurotensin (Nts) within the LH that can promote robust wakefulness in mice (Naganuma, Kroeger, et al., 2019). Acute optogenetic activation of Nts^{LH} neurons induced rapid arousal from NREM sleep, and their sustained activation using chemogenetics caused uninterrupted wakefulness lasting up to 5 h, indicating their ability to induce and sustain wakefulness (Naganuma, Kroeger, et al., 2019). However, it remains unknown if these neurons are necessary for spontaneous wakefulness and

their precise role in initiating and maintaining wakefulness. To address these questions, herein, we examined the population activity of Nts^{LH} neurons across sleep-wake behavior using fiber photometry. We then specifically ablated Nts^{LH} neurons using conditional genetic methods and assessed the consequent changes in sleep-wake behavior.

In addition to arousal, LH is implicated in several other functions, including physical activity and thermoregulation (Bonnavion et al., 2016; Cerri et al., 2014; Di Cristoforo et al., 2015; Gutierrez et al., 2011; Qualls-Creekmore & Münzberg, 2018; Satinoff & Shan, 1971), and our previous study shows that Nts^{LH} neurons contribute to these functions. Chemoactivation of Nts^{LH} neurons increases locomotor activity (LMA) and body temperature, and these changes can be elicited independently by optogenetic stimulations (Naganuma, Kroeger, et al., 2019). Hence, in the current study, we sought to determine the necessary role of Nts^{LH} neurons in spontaneous locomotor activity and in maintaining body temperature (Tb) levels. Therefore, we examined the effects of Nts^{LH} lesions on LMA and Tb levels at baseline conditions in addition to assessing the effects of sleep-wake behavior.

Materials and Methods

Animals

We used 8- to 12-week-old mice expressing cre recombinase (cre) in neurons producing Nts (Nts-Cre mice) for fiber photometry experiments and mice expressing both cre and green fluorescent protein (GFP) in Nts neurons (Nts-GFP mice) for conditional ablation experiments (Kempadoo et al., 2013; Leinninger et al., 2011; Naganuma, Kroeger, et al., 2019). Nts-Cre mice were originally obtained from Jackson's laboratories ($Nts^{tm1(cre)\ Mgmi}$/J mice; Jackson Laboratory, Stock No. 017525), bred, and maintained on a mixed background in our vivarium. Eutopic recombination of cre in Nts neurons in the LH has been verified previously in our lab and others (Brown et al., 2019; Goforth et al., 2014; Kempadoo et al., 2013; Naganuma, Kroeger, et al., 2019; Woodworth et al., 2017). Nts-GFP mice were generated by crossing the Nts-Cre mice with L10-green fluorescent protein reporter mice (Rosa26-loxSTOPlox-L10-GFP) (Krashes et al., 2014). The mice were maintained under a 12:12 h light-dark cycle (lights on at 0700; 150 lux) at an ambient temperature of 22±1°C with unrestricted access to chow diet and water. Care of the animals met National Institutes of Health standards, as set forth in the Guide for the Care and Use of Laboratory Animals, and all protocols were approved by the BIDMC Institutional Animal Care and Use Committee.

Experiment 1: Nts^{LH} activity dynamics across sleep-wake cycles

Surgery and recordings

Nts-Cre mice (*n* = 7) and wild-type littermates (*n* = 3) were deeply anesthetized (100 mg/kg ketamine + 10 mg/kg xylazine; i.p.) and stereotaxically injected with a cre-dependent adeno-associated viral (AAV) vector expressing the genetically encoded calcium indicator, GCaMP6s (AAV1.Syn.Flex.GCaMP6s.WPRE.SV40; 1×10^{13} vg/mL; University of Pennsylvania Vector core, USA) unilaterally into the LH (-1.7 mm anteroposterior; -5.1 mm dorsoventral and 1.1 mm lateral; 50 nL) (Kroeger et al., 2019; Naganuma, Kroeger, et al., 2019). We then implanted these mice with an optical fiber (400 µm diameter, NA 0.39, ThorLabs, Newton, NJ) targeting 50 µm above the LH and with electrodes for recording electroencephalogram (EEG) and electromyogram (EMG) (Kroeger et al., 2018; Kroeger et al., 2019; Zhang et al., 2021). Three weeks after surgery, we transferred the mice to a sound-attenuated chamber and acclimated them to the EEG/EMG connection cables and optical patch cord for one week. We then performed fiber photometry with concurrent EEG/EMG recordings and time-lock video for 2 hours during the light period (11 am – 1 pm) in each mouse. The EEG/EMG signals were amplified (A.M. Systems, Carlsborg, WA), filtered (EEG: 40 Hz low pass; EMG: 40 Hz high pass), and digitized with time-lock video recordings (VitalRecorder, Kissei Comtec, Nagano, Japan). For fiber photometry, the implanted optical fiber was connected to an LED source, and constant, focused blue light (473 nm; intensity 0.1-0.2 mW) was directed to the LH. GCaMP6-expressing neurons absorb the blue light and return the green light (530 nm) depending upon the neuron's intracellular Ca^{2+} activity. The emitted green light (Ca^{2+} signal) was directed through a dichroic mirror and a GFP emission filter before being recorded by a sensitive photodetector (Fig 1A). The photodetector signal was digitized and synchronized along with the EEG/EMG signals and video recordings.

Data analysis

EEG and EMG recordings were divided into 5-s epochs and manually scored into wake, NREM sleep, or REM sleep as per the conventional criteria using Sleepsign for Animals (Kissei Comtec) (Kroeger et al., 2019; Lu et al., 2000; Vetrivelan et al., 2009). Fiber photometry data was analyzed as described previously (Ito et al., 2023). In brief, we first corrected the digitized Ca^{2+} fluorescence traces for any photobleaching by fitting an exponential to the fluorescent time course and subtracting prior to analyzing the data using a custom-written MATLAB (MathWorks, Natick, MA) script. Then, 1024 Hz of recorded GCaMP6s signal was down-sampled to 1 Hz using and converted to ΔF/F (%) as follows: ΔF/F=100*(F(t)-Fmin)/Fmin). F(t) is the GCaMP6s signal and

Fmin is the minimum value of the signal. To assess the signal across different animals, ΔF/F was normalized to Z-score using S.D. and an average of analysis range (60 sec before and after each transition). Finally, the mean ∆F/F for each sleep-wake state was calculated by averaging ∆F/F during all epochs scored as a particular stage.

Histology

Upon completion of these recordings, we deeply anesthetized the mice with chloralhydrate (500 mg/kg BW, i.p., Sigma Aldrich, USA) and intracardially perfused them with 10% formalin. We harvested the brains and post-fixed them in 10% formalin overnight and in 30% sucrose solution for two days. We then sectioned brains at 20 µm (brains processed for in situ hybridization; *n* = 3) or 40 µm into three series using a freezing stage microtome.

In one series of sections from each mouse, we mapped the injection site by visualizing the GCaMPs-expressing neurons (i.e., GFP+) and the location of the optical fiber tip. We included mice in the analysis if GCaMPs+ neurons covered the LH region and the optical fiber tip was within 0.2 mm above the injection site.

To verify the expression of GCaMPs in Nts^{LH} neurons, we double-labeled a second series for *Nts mRNA* by multiplex fluorescence in situ hybridization (RNAscope kit V2; Catalog#323100; Advanced Cell Diagnostics, CA) and GFP by immunohistochemistry (to label the GCaMPs-expressing neurons) as described earlier (Kroeger et al., 2018; Naganuma, Bandaru, et al., 2019) Briefly, after pretreatment in hydrogen peroxide for 20 minutes, we placed the slides in a 1x Target retrieval reagent (at >99°C) for 5 minutes and dehydrated the sections in 90% alcohol. We then air-dried the sections, treated them with protease reagent (Protease III, RNAscope; 30 minutes at 40°C), and incubated them in an RNAscope probe for Nts (Mm-Nts-C1, Catalog #420441-C1, Advanced Cell Diagnostics) at 40°C for 2 hours. We then performed 3 amplification steps at 40°C (AMP1-FL and AMP2-FL: 30 mins each; AMP3-FL: 15 minutes) and incubated slides in HRP C1 (15 minutes). Finally, we incubated the sections in TSA plus Cy3 fluorophore (Catalog # NEL741001, Perkin Elmer, Waltham, MA) for 30 mins for visualization (Channel 2 at 488nm) of Nts mRNA and HRP blocker for 15 minutes. Then, to immunolabel these sections for GFP, we incubated them in Rabbit anti-GFP (1:2000 dilution; Thermo Fisher Scientific catalog #: A11122; 1:10,000 dilution) overnight at 4°C, washed in PBS (2X2 minutes) and incubated in secondary antibody (Alexa fluro488 Goat anti-Rabbit, Catalog # A-110342, Invitrogen, USA) for 2 h at room temperature. After two rounds of washes, we dried the slides and cover-slipped them with Prolong Gold antifade reagent (Catalog # P36934, Invitrogen, Carlsbad, CA).

Finally, to validate the specificity of AAV-GCaMPs, we immunolabeled a third series for GFP and orexin (*n* = 2 mice) or melanin-concentrating hormone (MCH; *n* = 2 mice) (Kroeger et al., 2018; Naganuma, Kroeger, et al., 2019). For this, we incubated the sections in chicken anti-GFP (1:10000 dilution; Cat. No: A10262, Invitrogen) and Rabbit anti-orexin A (1:7500; Catalog # H-003-30, Phoenix Pharmaceutical Inc, Burlingame, CA) or Rabbit anti- MCH (1:7500, a generous gift from Dr. Maratos-Flier, BIDMC) overnight at 4°C. The next day, we washed the sections in PBS (2X2 minutes) and incubated them in secondary antibodies (1:1000, Alexa Fluor 488 Goat anti-Chicken, Catalog # A11039, Invitrogen and 1:1000, Alexa Fluor 555 Donkey anti-Rabbit, Catalog # A-31572, Life Technologies) for 2 h at room temperature. After 2X2 min washes, we dried the slides and cover-slipped them with Vectashield (Catalog # h-1400, Vector Laboratories, Burlingame, CA).

Experiment 2: Conditional ablation of Nts^{LH} neurons

Surgery and recordings

Under anesthesia (100 mg/kg ketamine + 10 mg/kg xylazine; i.p.), Nts-GFP mice (*n* = 15) and WT littermates (*n* = 7) were injected with 50 nL of a cre-dependent AAV vector encoding diphtheria toxin A subunit (DTA) (AAV-EF1a-mCherry-flex-DTA; University of North Carolina Vector core, USA) bilaterally into the LH (Chen et al., 2017; Fan et al., 2023; Kaur et al., 2017). We then intraperitoneally implanted these mice with biotelemetry transmitters (TLM2-F20EET; Data Science International, St. Paul, MN) that allow simultaneous recording of EEG, EMG, Tb, and LMA as described earlier (Kroeger et al., 2018; Naganuma, Kroeger, et al., 2019; Vetrivelan et al., 2016). Briefly, the transmitters were placed in the peritoneal cavity and the EEG leads were channeled subcutaneously and inserted into the skull (1.5 mm to the right of the sagittal suture, 1 mm anterior and 3.5 mm posterior to bregma for obtaining frontoparietal EEG) with tips touching the dura while the EMG leads were similarly channeled and attached to the neck extensor muscles. Three weeks after surgery, we transferred the mice to a sound-attenuated chamber and acclimated for one week. We then performed telemetric recording of EEG, EMG, Tb, and LMA for 24 h (Dataquest ART 4.1 software; Data Science International) as described previously (Kroeger et al., 2018; Naganuma, Kroeger, et al., 2019; Vetrivelan et al., 2016).

Data analysis:

We divided EEG and EMG recordings into 12-s epochs and manually scored them into wake, NREM sleep or REM sleep. From the scored data, we calculated the time spent in each sleep-

wake state and the total number and the average duration of bouts during the light, dark, and entire 24-hour periods. As this routine analysis of bout length and numbers does not consider the total time spent in those states, we then calculated the fragmentation index for all lesioned mice – bout numbers as a function of time spent in that state normalized to control values. Finally, EEG spectral power was calculated in 0.5 Hz bins using fast Fourier transformation of each 12-s epoch. Power in the 0.5-40 Hz range of artifact-free epochs was averaged in groups across each sleep-wake state, and the mean values were plotted 0.5 Hz bins (Kroeger et al., 2018; Wen et al., 2020).

We calculated the total LMA counts and mean Tb individually during the dark- and light periods and the entire 24-hour periods. As Nts^{LH} neuron activation increases LMA and Tb independent of its wake-promoting effects, we then analyzed LMA counts per unit time of wake and mean Tb in each sleep-wake state. Finally, to quantify the state-dependent changes in Tb, we isolated 6 sleep-wake episodes consisting both NREM and REM sleep with at least 3 minutes wake before and after this episode and quantified the Tb changes during state-transitions.

Sleep-wake, Tb and LMA data from lesioned animals (Nts-GFP mice receiving AAV-DTA) were compared with control mice (DTA-injected WT mice) using repeated measures of ANOVA followed by post-hoc, student's t test.

Histology: On completion of telemetric recordings, the mice were perfused with 10% formalin, and brains were harvested and cut into three series of 40 µm sections. The AAV-DTA selectively targets and kills the cre-expressing Nts neurons (i.e., GFP+ neurons in Nts-GFP mice) in the injection site. The cre-negative neurons that are not killed by the DTA instead express mCherry, thereby marking the injection site. Therefore, in one series of sections from each mouse, we mapped the injection site by visualizing the mCherry+ neurons. To assess any non-specific cell loss, we immunolabelled a second series for MCH (1:20000, Rabbit Anti-MCH, a generous gift from Dr. Maratos-Flier, BIDMC) and orexin (1:10000; Rabbit Anti-orexin A, Cat. No: H-003-30; Phoenix Pharmaceutical Inc, Burlingame, CA) and counted MCH- or orexin-positive neurons in all mice (Naganuma, Kroeger, et al., 2019; Vetrivelan et al., 2016). All cell counting was performed by constructing a 500 µm x 500 µm counting box on the LH bilaterally on 2 sections (one every 120 µm) and the cell counts were corrected using Abercrombie's formula (Guillery, 2002).

Results

Activity dynamics of Nts^{LH} neurons

In brain sections from Nts-Cre mice injected with AAV-GCaMP6s, numerous GFP+ neurons were observed in the LH (Fig 1B), but none were found in the WT mice injected with the same AAV. Over 98% of GFP+ neurons were also positive for Nts mRNA (Fig 1C) whereas none of them were positive for MCH or orexin (Fig 1D, E), indicating the eutopic expression of AAV-GCaMPs in Nts neurons in the LH.

Consistent with the specific cre-dependent expression of GCaMPs, we observed robust fluctuations in Ca^{2+} fluorescence ($\Delta F/F$) across sleep-wake states in Nts-Cre mice but not in WT mice. The mean $\Delta F/F$ in the Nts-Cre mice was lowest during NREM sleep, higher during wakefulness, and maximal during REM sleep (Fig 2A, B). We observed several peaks in Nts^{LH} $\Delta F/F$ during wake or REM sleep, but they were rare during NREM sleep, indicating that Nts^{LH} neurons may be relatively silent during NREM sleep. Analysis of state transitions revealed that Nts^{LH} activity ($\Delta F/F$) began increasing a few seconds prior to REM sleep onset and started declining a few seconds prior to the end of REM episodes (or wake-onset) (Fig 2E, F). Such a close temporal association between Nts^{LH} activity and REM sleep points out a crucial role for Nts^{LH} in regulating this stage of sleep or its characteristic features including cortical and hippocampal activation. On the other hand, Nts $\Delta F/F$ did not increase prior to wake onset, but rather increased at the same time as NREM-wake transitions (Fig 2D) and persisted at higher levels throughout the wake episodes, suggesting that Nts^{LH} neurons may be necessary for wake maintenance but not for eliciting spontaneous arousals from sleep. However, the Nts^{LH} activity started declining a few seconds before the NREM sleep onset (Fig 2C), suggesting that their lower activity may contribute to the initiation of NREM sleep.

Selective deletion of Nts^{LH} neurons

In the AAV-DTA injected Nts-GFP mice, no GFP+ neurons were observed within the boundary of the injection site in the LH marked by mCherry+ neurons (Fig 3A). In contrast, MCH and orexin neurons, which are intermingled with Nts^{LH} neurons, remained unaffected in the AAV-DTA-injected mice (445±37 MCH neurons vs. 415±43 in sham controls; 387±16 OX neurons vs 393±22 in sham controls; n = 5 each; 2 sections/mice), further confirming the selectivity of lesions. We then analyzed the lesion sites in each case and found 7 out of 15 mice had a near-complete loss of Nts^{LH} neurons, with the AAV injections covering at least 75% of the LH region bilaterally and minimal spread in the surrounding regions (hereafter 'Nts^{LH}-lesioned mice'). The other 8 mice were categorized as partial lesions.

Sleep-wake changes

Consistent with our prior data indicating potent wake-promoting effects of Nts^{LH} activation (Naganuma, Kroeger, et al., 2019), Nts^{LH} lesioned mice displayed an average 16% decrease in wakefulness over a 24-hour period, with a concomitant increase in NREM sleep. These changes were particularly prominent during the dark period, with NREM sleep levels 30% higher than controls (Fig 3B-D). NREM sleep levels were also higher during the light period, but the difference was less pronounced. In contrast, REM sleep levels during the light and dark periods in the Nts^{LH}-lesioned mice did not differ from controls (Fig 3B-D). For a comparison, orexin knockout mice displayed no changes in total amounts of sleep but slightly higher REM sleep during the dark period (Mochizuki et al., 2004). Similarly, chronic deletion of several other wake-promoting neuronal populations had little effect on spontaneous sleep-wake amounts (Blanco-Centurion et al., 2007; Gerashchenko et al., 2004; Gompf et al., 2010; Lu, Sherman, et al., 2006; Webster & Jones, 1988).

We then analyzed the changes in the sleep-wake bouts and found that the mean duration of wake bouts decreased, and the number of NREM bouts increased in the Nts^{LH}-lesioned mice (Table 1). However, this routine analysis does not consider differences in sleep-wake amounts, so we also calculated the fragmentation index for each state. The fragmentation index of wake, but not that of NREM or REM sleep, was higher in the lesioned mice (Fig 3E), indicating that Nts^{LH} loss selectively disrupts wakefulness.

Analysis of EEG spectra revealed that delta power (2-4 Hz) in wakefulness during both dark and light periods was higher in the Nts^{LH}-lesioned mice, but theta (6-9 Hz) or higher frequency components (10-40 Hz) did not differ from controls (Fig 4). We also observed a strong trend towards higher delta power during REM sleep, but EEG spectra during NREM sleep did not show any significant differences. Taken together with the higher NREM sleep bouts, these data suggest that Nts^{LH} neuron loss may be associated with EEG slowdown and sleepiness.

Finally, in the mice with partial Nts^{LH} loss, sleep-wake behavior or EEG spectra did not differ significantly from controls, although a trend for changes in a similar direction was observed. suggesting a functional redundancy of Nts^{LH} neurons.

Changes in locomotor activity and body temperature

Nts^{LH}-lesioned mice displayed substantially lower LMA counts, which were more pronounced during the dark period (Fig 5A). As these mice had lower wake levels than controls, we analyzed LMA counts as a function of time spent in wakefulness. The LMA per hour of

wakefulness was still lower in the lesioned mice, indicating that Nts^{LH} neurons are necessary for maintaining normal levels of spontaneous activity. These data are consistent with the robust increase in LMA after the chemoactivation of Nts^{LH} neurons in our previous study. In contrast to LMA, mean Tb in the Nts^{LH}-lesioned mice either during the entire 24-h period or specifically during the light and dark periods did not differ significantly from control mice (Fig 5B). We then examined the Tb changes during individual sleep-wake states. Both control and Nts-lesioned mice exhibited higher Tb during wakefulness when compared to NREM or REM sleep as expected, but there was no significant difference between control and Nts-lesioned mice (Fig 5C, D). These data suggest that Nts^{LH} neurons may not be necessary for maintaining Tb under baseline conditions, but considering the strong hyperthermia induced by Nts^{LH} activation (Naganuma, Kroeger, et al., 2019), it is likely these neurons are necessary for thermoregulation, especially in a cold environment.

Discussion

To investigate the necessary role of Nts^{LH} in arousal control, we examined their activity dynamics across sleep-wake cycles and the impact of their loss on spontaneous sleep-wake behavior in mice in this study. We find that Nts^{LH} neurons are more active during wakefulness and REM sleep than during NREM sleep. Consistently, we find that selective ablation of Nts^{LH} neurons reduces wake amounts, the average duration of wake bouts, and locomotor activity. Nts^{LH} loss also increases delta power during wake, and NREM sleep amounts. However, despite their high activity during REM sleep, Nts^{LH} loss does not alter REM sleep amounts or architecture. Overall, these data demonstrate the necessary role of Nts^{LH} neurons in maintaining normal arousal and activity levels.

The LH has been identified as a key region for arousal regulation, yet the intricacies regarding specific cell types and underlying mechanisms remain somewhat elusive. Our recent work has shed light on a population of neurons expressing Nts within the LH, and characterized for their wake-promoting effects. These neurons facilitate rapid arousal from NREM sleep and sustain arousal for extended periods, when activated using optogenetic and chemogenetic methods (Naganuma, Kroeger, et al., 2019). As their chemoinhibition did not reduce spontaneous wakefulness but altered the arousal effects of novel environment or fasting, we had concluded that Nts^{LH} neurons primarily optimize arousal levels in response to external and internal stressors (Naganuma, Kroeger, et al., 2019). However, such chemoinhibition lasting for a few hours is insufficient to examine their necessary role in the induction and maintenance of spontaneous

wakefulness over a 24-hour cycle. Hence, in this study, we selectively ablated Nts^{LH} neurons, which significantly decreased wake levels over a 24-hour period and particularly during the dark period. To our knowledge, this is the first report where selective ablation of a neurochemically distinct cell group within the LH increases time spent in sleep, similar to non-specific neurotoxic lesions. For example, neither the loss of orexin neurons nor other LH subpopulations altered spontaneous sleep amounts (Hara et al., 2001; Heiss et al., 2024; Mochizuki et al., 2004; Tabuchi et al., 2014). A vast majority (>90%) of Nts^{LH} neurons contain GABA (Brown et al., 2019; Mickelsen et al., 2019) and recent literature has pointed out the importance of $GABA^{LH}$ neurons in arousal regulation (Gazea et al., 2021; Herrera et al., 2016; Venner et al., 2016; Venner et al., 2019). However, the LH houses at least 15 distinct subsets of GABA neurons (Mickelsen et al., 2019) and both wake-active and sleep-active GABA neurons have been identified in this region (Alam et al., 2002; Hassani et al., 2010; Koyama et al., 2003). Consistently, the DTA-mediated lesions of $GABA^{LH}$ neurons that did not differentiate these subpopulations had no impact on sleep-wake amounts (Heiss et al., 2024). The significant sleep increase after selective deletion of Nts^{LH} population in this study, in conjunction with our previous data demonstrating the potent wake-promoting effects of Nts^{LH} neuron activation, identifies Nts as a marker for the wake-promoting $GABA^{LH}$ population. Further, considering the lack of sleep-wake changes after lesions in other established arousal populations (Blanco-Centurion et al., 2007; Gerashchenko et al., 2004; Gompf et al., 2010; Lu, Sherman, et al., 2006; Webster & Jones, 1988), the increase in sleep after Nts^{LH} lesions, assumes immense significance and assigns Nts^{LH} neurons a critical spot in the wide-spread arousal network. Importantly, the 30% increase in NREM sleep during the dark period after Nts^{LH} lesions is comparable to or even greater than that resulting from lesions of dopaminergic neurons in the ventral periaqueductal gray (~20%) or glutamatergic neurons in the parabrachial nucleus (PB; ~ 17-38%), two other established arousal populations (Kaur et al., 2013; Lu, Jhou, et al., 2006).

Reduced wake levels in the Nts^{LH} lesioned mice were mainly due to a decrease in the average duration of wake bouts, suggesting that Nts^{LH} neurons may primarily contribute to the maintenance of wakefulness rather than initiating spontaneous arousals from sleep. This is consistent with the activity dynamics of Nts^{LH} neurons. While the NREM sleep-wake transitions are associated with a rapid increase in Nts^{LH} activity, this increase coincides with but does not precede wake onset defined by EEG and EMG features. Thus, Nts^{LH} neurons are likely to become active during the wake onset, and the higher activity persists throughout the wake period, contributing to the maintenance of this state. Nevertheless, the ability of Nts^{LH} neurons to induce

arousals, especially from NREM sleep, is undeniable as their activation consistently induced short latency arousals in our previous study (Naganuma, Kroeger, et al., 2019). While the duration of wake bouts reduced, the number of wake bouts and consequentially fragmentation index increased after Nts^{LH} lesions. In contrast, the fragmentation index for NREM and REM sleep remained unchanged. Such selective wake fragmentation is different from the fragmentation of both sleep and wake bouts observed in animals with orexin system dysfunction (Chemelli et al., 1999; Diniz Behn et al., 2010; Mochizuki et al., 2004). While orexin loss destabilizes all states, Nts^{LH} loss appears to specifically impair wake maintenance.

Nts^{LH} activity reduces a few seconds prior to sleep onset, suggesting that Nts^{LH} neurons, like other wake-promoting neurons, must be inhibited to permit sleep entry and that decline in Nts^{LH} activity contributes to sleep preparedness and sleep initiation, which is also consistent with the increased number of NREM sleep bouts in the Nts^{LH}-lesioned mice. Further, the lesioned mice also exhibit high delta power during wake, indicating a higher homeostatic sleep pressure and sleepiness in these mice. This idea is further supported by the previous observations that Nts^{LH} neuronal inhibition decreased the latency to sleep and increased the time spent in sleep in a novel environment (Naganuma, Kroeger, et al., 2019).

We find Nts^{LH} neurons are about three times more active during REM sleep than during wake and the REM-associated increase in Nts^{LH} activity precedes rather than follows REM transitions. Similarly, Nts^{LH} activity decreases a few seconds prior to the end of REM bouts. Despite this strong temporal correlation, REM sleep levels were unaffected by Nts^{LH} lesions. It is entirely possible that subpopulations of Nts^{LH} neurons may be specifically active during REM sleep and/or Wake. Further studies investigating the activity pattern of Nts^{LH} neurons at the cellular level using miniscope calcium imaging methods may be necessary to address this issue.

Another important observation in this study is the association between Nts^{LH} activity and LMA and the drastic reduction of LMA after Nts^{LH} lesions. Taken together with the robust increase in LMA induced by chemoactivation, these data indicate a key role for Nts^{LH} neurons in locomotor control. The role of LH in this locomotor control has been established, especially in the context of motivation and reward, and recent studies indicate that Nts^{LH} neurons could participate in these behaviors (Bonnavion et al., 2016; Kempadoo et al., 2013; Torruella-Suárez & McElligott, 2020; Tyree & de Lecea, 2017). Although we did not specifically study motivational arousal, attenuation of novelty-induced increases in locomotor activity was observed after chemoinhibition of Nts^{LH} neurons (Naganuma, Kroeger, et al., 2019), which are in line with this idea. However, based on

rapid electrocortical and behavioral arousal from NREM sleep induced by brief photostimulations of Nts^{LH} neurons (Naganuma, Kroeger, et al., 2019) and the higher sleepiness (an increase in wake delta power and higher NREM bouts) after Nts^{LH} lesions, we hypothesize that the reward effects of Nts^{LH} neuron manipulations are due to general arousal-promoting and motor effects.

The major consequences of Nts^{LH} lesions, impaired wake maintenance and higher sleepiness are primary symptoms of narcolepsy and hypersomnia disorders (Arnulf et al., 2023; Barateau et al., 2022; Rosenberg et al., 2024; Scammell, 2015), suggesting that Nts^{LH} loss may contribute to these disorders. It is well-established that the loss of orexin neurons causes narcolepsy, and one study found that Nts^{LH} ablations led to reductions in orexin expression and loss of orexin neurons in the LH (Brown et al., 2018). However, we observed neither orexin neuron loss nor cataplexy in the Nts^{LH}-lesioned mice, suggesting that ectopic expression of AAV-DTA or its long-term effects could have caused orexin loss in that previous study (Brown et al., 2018). Thus, the current findings do not support the idea of non-specific loss of orexin neurons contributing to the sleep effects in the Nts^{LH} lesioned mice. On the other hand, it is possible that loss of Nts^{LH} neurons may worsen the narcolepsy induced by loss of orexins. Our prior anterograde tracing data indicate that the Nts^{LH} neurons may project to similar sleep-wake regulatory regions as orexin neurons (Naganuma, Kroeger, et al., 2019; Peyron et al., 1998). Thus, it is likely that Nts^{LH} and orexin neurons may independently regulate cortical and behavioral arousal via parallel pathways. Irrespective of the involvement of orexin systems, higher sleep amounts and wake fragmentation induced by Nts^{LH} lesions indicate that dysfunction of these neurons may underlie central disorders of hypersomnia. In addition to orexin neurons, Nts^{LH} neurons heavily project to the key wake-promoting regions, including the ventral tegmental area (VTA), locus coeruleus (LC), and parabrachial region (PB), and the sleep-promoting lateral preoptic area (LPOA) (Naganuma, Kroeger, et al., 2019). While Nts^{LH} neurons do not appear to directly project to the cortex, they innervate the basal forebrain, which is crucial for cortical activation during wakefulness (Fuller et al., 2011; Naganuma, Kroeger, et al., 2019). As Nts^{LH} neurons contain both excitatory (Nts) and inhibitory (GABA) neurotransmitters (Brown et al., 2019; Mickelsen et al., 2019), they could activate the above arousal regions and/or inhibit the LPOA to promote cortical and behavioral arousal. Future studies investigating the specific neurocircuits and mechanisms through which the Nts^{LH} neurons impact arousal levels will be greatly helpful in the understanding of etiology of disorders of hypersomnia.

In this study, we primarily focused on spontaneous sleep-wake behavior and did not attempt to investigate the role of Nts^{LH} neurons in arousal maintenance in challenging conditions.

However, we addressed this question in our previous study and demonstrated that Nts^{LH} neurons are necessary to optimize sleep-wake behavior in response to external and internal stressors. Similarly, we did not investigate sleep homeostasis in the Nts^{LH}-lesioned mice. However, higher delta during wake and more frequent NREM sleep transitions were observed in these mice. Further, in our previous study, we showed that Nts^{LH} inhibitions in a novel environment reduced the latency to sleep and increased sleep amounts. These data indicate that reduced activity of Nts^{LH} neurons may be associated with higher homeostatic sleep pressure.

Conclusions

In summary, our data demonstrate that Nts^{LH} neurons are an integral part of the arousal network, and they are necessary for maintaining normal levels of active wakefulness. Their loss leads to sleepiness, hypersomnolence, and hypoactivity, especially during their active phase, which are primary symptoms of several hypersomnia disorders and chronic fatigue syndrome, suggesting that Nts^{LH} neuron dysfunction may contribute to the development of these disorders. Future research should examine specific Nts^{LH} projections that may impact wake and physical activity levels. Defining these circuits will lead to a better understanding of the arousal mechanisms and should ultimately identify novel targets for the treatment of disorders of hypersomnia.

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Figure legends

Figure 1

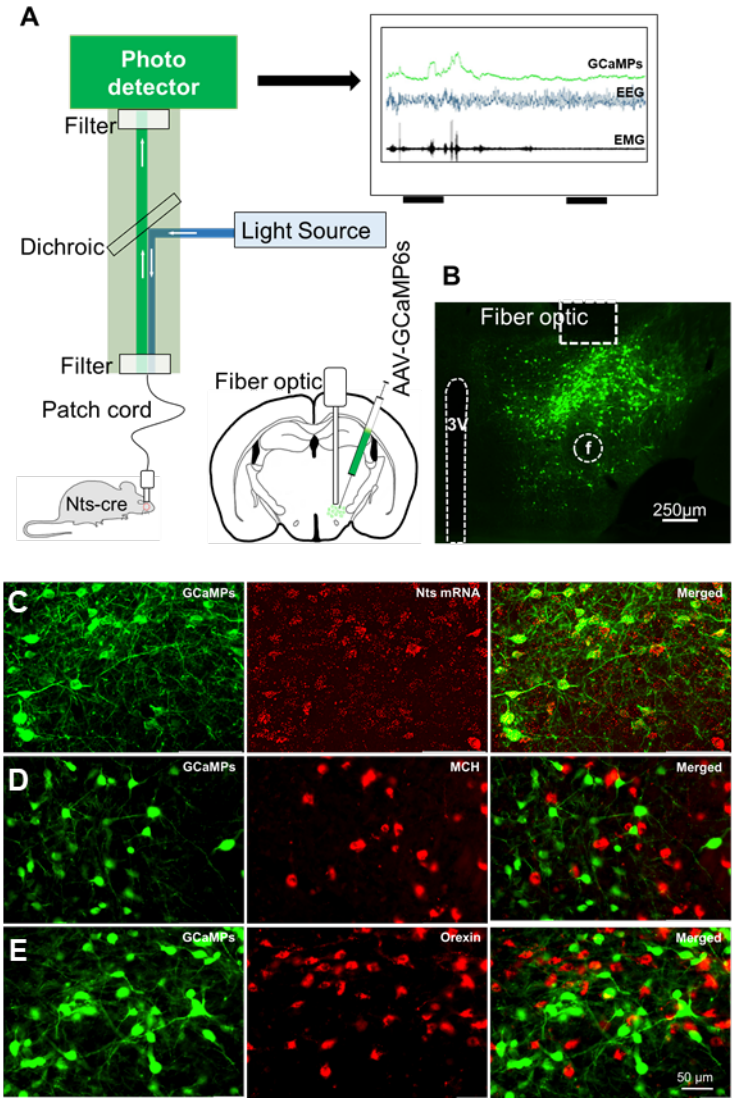

Figure 1. Imaging Nts^{LH} neuron activity across sleep-wake states using fiber photometry. (A) Schematic representation of concurrent fiber photometry and EEG/EMG recordings from a Nts-Cre mouse injected with AAV-GCaMP6s into the LH. **(B)** Representative brain section showing the GCaMPs+ cells in the LH; dashed white line indicates the optic fiber tract. **(C-E)** Eutopic expression of GCaMP6s in Nts neurons; >98% of GCaMPs+ neurons (green cells) in the LH were positive for Nts mRNA (Red cells in C), but none were positive for MCH (Red cells in D)

or orexin (Red cells in E). GFP, green fluorescent protein; LH, lateral hypothalamus; MCH, melanin-concentrating hormone; Nts, neurotensin; OX, orexin.

Figure 2

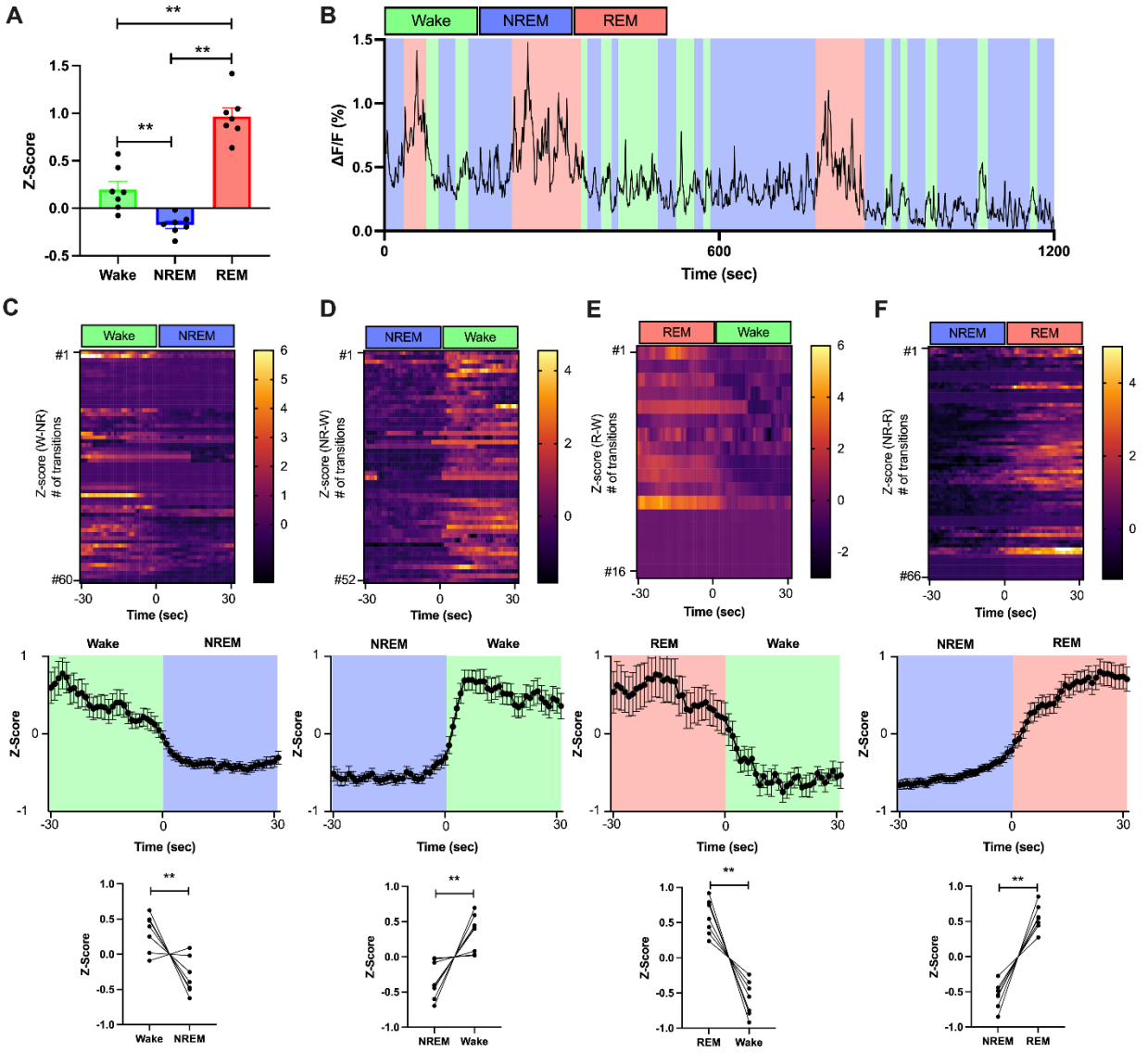

Figure 2. **Nts^{LH} neuron activity across sleep-wake states measured by fiber photometry.** (A) Summary of Nts^{LH} neuron activity across sleep-wake states, expressed as Z-scores. *P < 0.05, **P < 0.01: One-way ANOVA followed by Tukey's multiple comparisons test (n = 7: F = 59.01, P < 0.0001, wake vs NREM: P = 0.0074, wake vs REM: P < 0.0001, NREM vs REM: P < 0.0001). (B) Representative Nts^{LH} neuronal activity across sleep-wake states. (C-F) Nts^{LH} neuron activity (Z-scores 30-sec before and 30-sec immediately after) during sleep-wake transitions; 60 wake-NREM, 52 NREM-wake, 16 REM-wake and 66 NREM-REM transitions were analyzed (Top panel); mean Z-score in 1 sec intervals (C-F middle panel) or entire 30 sec periods (C-F, bottom

panel) **$P < 0.01$: Paired t test (n = 6-7, wake to NREM: $P = 0.0010$, NREM to wake: $P = 0.0083$, REM to wake: $P = 0.0002$, NREM to REM: $P < 0.0001$). Values are the mean ± SEM.

Figure 3

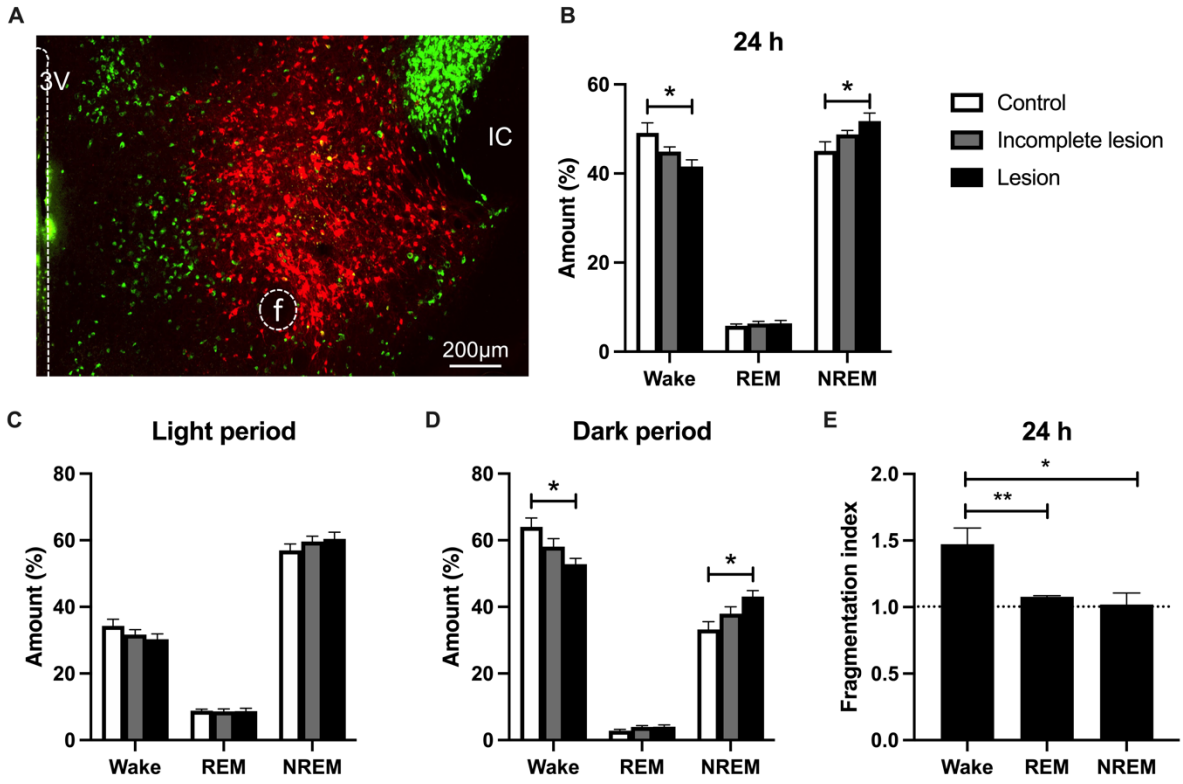

Figure 3. Changes sleep-wake following Nts^{LH} neuron lesions. (A) Representative brain section from Nts-GFP mouse injected with AAV-DTA into the lateral hypothalamus. Nts+ neurons were visualized by native GFP fluorescence (Green cells). AAV-DTA selectively kills Cre+ Nts neurons but expresses mCherry in the Cre-negative neurons (Red cells), thereby marking the injection site. Note the absence of GFP+ neurons within the boundaries of injection site. Percentages of wake, REM and NREM sleep during light (C) dark (D) or the entire 24-hr period (B) in mice with complete (>75% bilateral cell loss; black bars) or incomplete Nts^{LH} lesions (gray bars) and sham controls (white bars). (B)(D) *P < 0.05: One-way ANOVA followed by Tukey's multiple comparisons test (for B, wake: n =7-8: F = 4.88, P = 0.019, control vs complete lesion: P = 0.015, NREM: n =7-8: F = 4.12, P = 0.032, control vs complete lesion: P = 0.025) (for D, wake: n =7-8: F = 5.41, P = 0.013, control vs complete lesion: P = 0.010, NREM: n =7-8: F = 5.33, P = 0.014, control vs. complete lesion: P = 0.010). Fragmentation index of sleep-wake stages (number of bouts as a function of time spent in that stage normalized to control mice) are shown E, indicating selective fragmentation of wake in mice with complete Nts^{LH} lesions. *P < 0.05, **P < 0.01: One-way ANOVA followed by Tukey's multiple comparisons test (n = 7-8: F = 8.29, P = 0.0031, wake vs NREM: P = 0.0052, wake vs REM: P = 0.011). Values are the mean ± SEM.

Figure 4

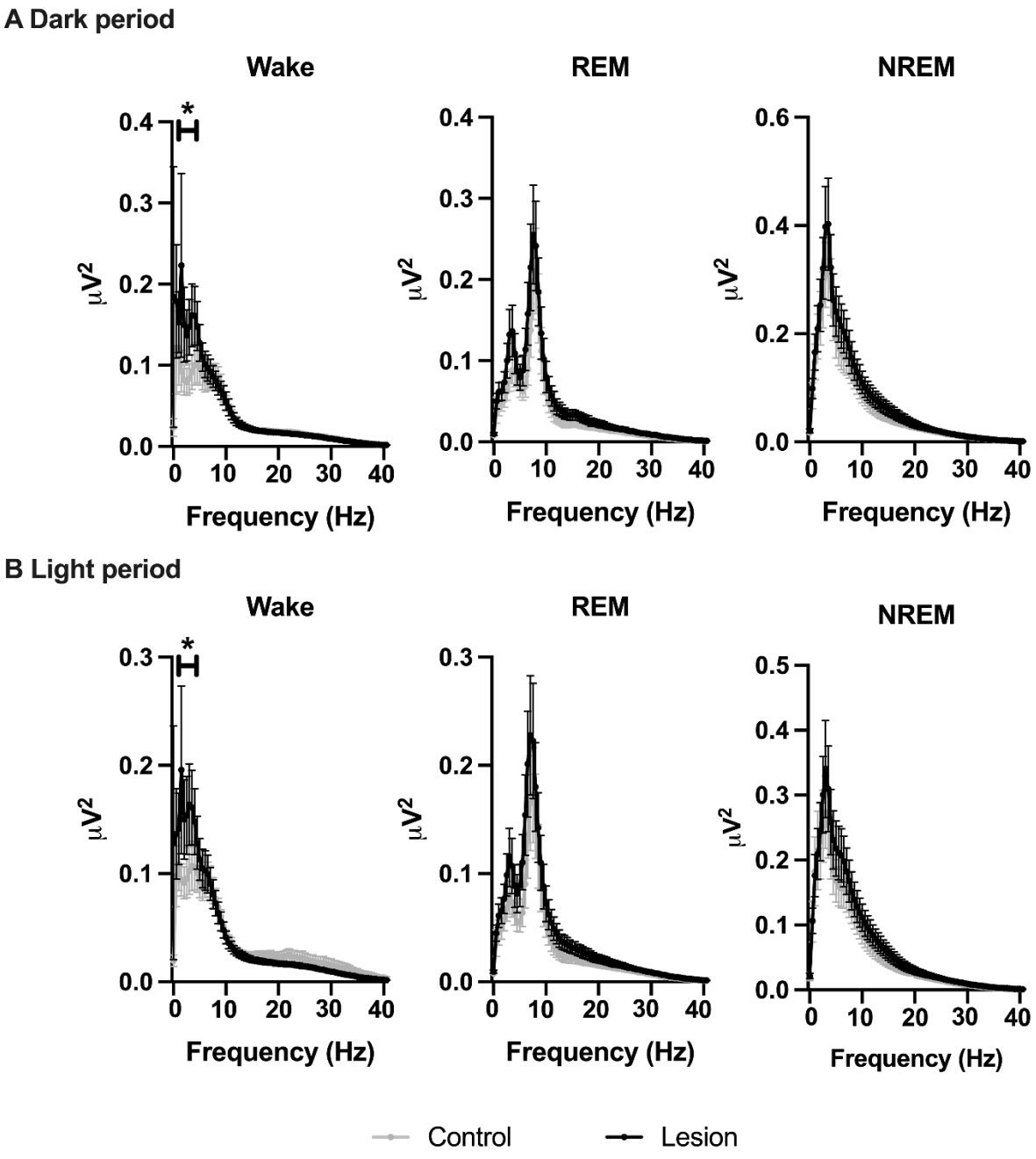

Figure 4. Changes in EEG spectra following Nts^{LH} neuron lesions. EEG spectral profiles during wakefulness (Left), REM sleep (Center), and NREM sleep (Right)) of the Nts-LH lesioned mice (>75% bilateral cell loss; black lines) and sham controls (light gray lines) during the light and dark periods. The average EEG spectra were normalized to total EEG power from 1–40 Hz in

0.5-Hz bins. Values are the mean ± SEM. EEG power from 2-4 Hz, *P < 0.05: unpaired *t-test* (for A, n = 6-7, P = 0.049, for B, n = 6-7, P = 0.048).

Figure 5

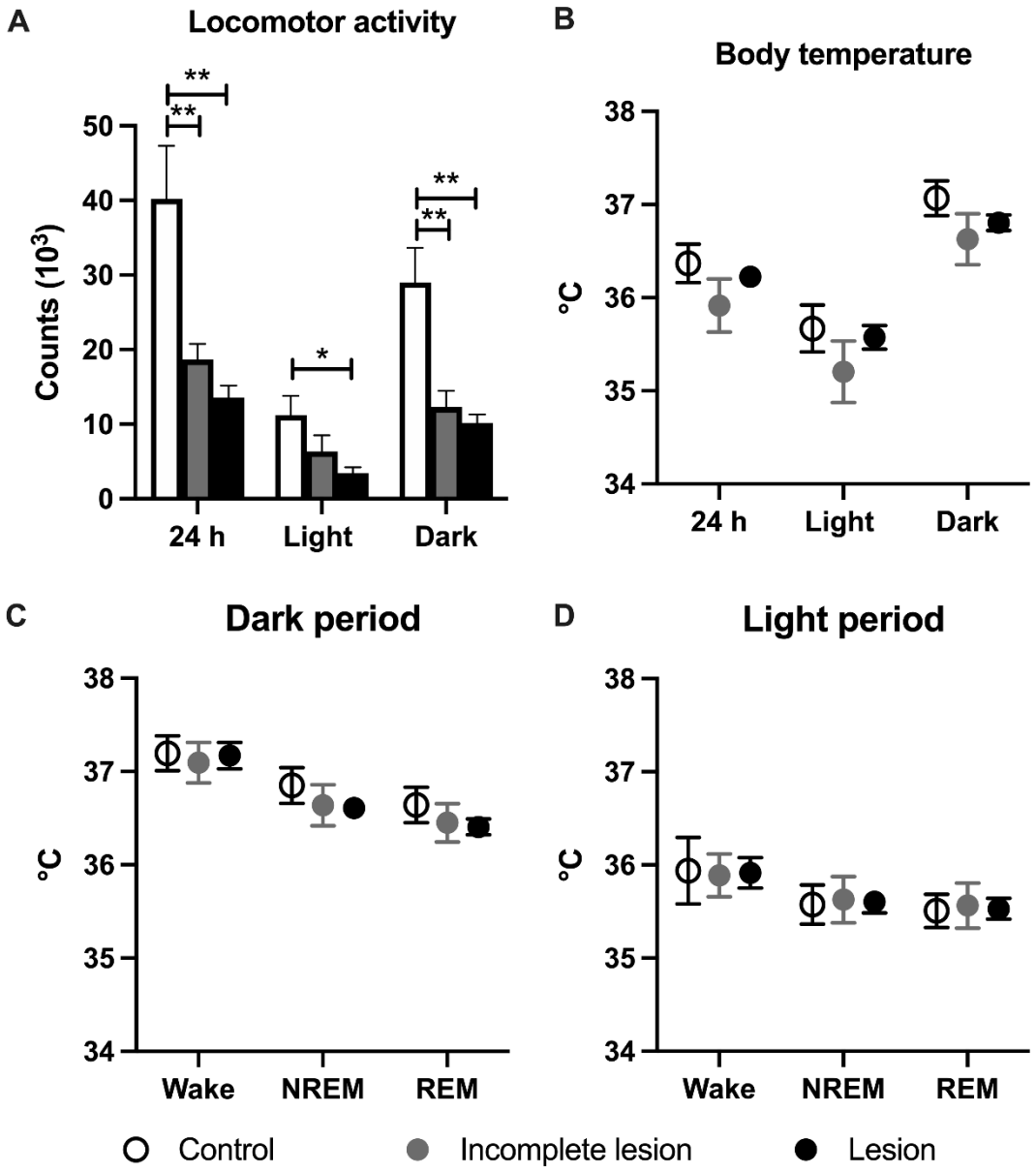

Figure 5. Changes in locomotor activity and body temperature following Nts^{LH} neuron lesions. Total locomotor activity counts (A) or mean body temperature (B) during light, dark or the entire 24-hr periods in mice with complete (>75% bilateral cell loss; black bars) or incomplete Nts^{LH} lesions (gray bars) and sham controls (white bars). C and D) mean Tb in each sleep-wake state during the light and dark periods. Values are the mean ± SEM. *P < 0.05, *P < 0.01: One-way ANOVA followed by Tukey's multiple comparisons test (for 24h; n = 7: F = 10.53, P = 0.0009,

control vs incomplete lesion: $P = 0.0070$, control vs lesion: $P = 0.0011$, for light period; $n = 7$: $F = 3.93$, $P = 0.039$, control vs. incomplete lesion: $P = 0.032$, for dark period; $n = 7$: $F = 11.73$, $P = 0.0005$, control vs. incomplete lesion: $P = 0.0028$, control vs. lesion: $P = 0.0009$).

Table 1: Changes in sleep-wake architecture following Nts^{LH} neuron lesions

		Number of bouts			Mean bout duration (s)		
		Control	Incomplete	Lesion	Control	Incomplete	Lesion
Wake	24 h	261.3 ± 17.1	320.9 ± 23.0	325.6 ± 22.8	166.3 ± 13.1	134.1 ± 14.6	114.9 ± 12.4†*
	Light	137.4 ± 12.7	169.4 ± 16.2	176.4 ± 6.1	101.1 ± 9.1	91.4 ± 11.8	74.9 ± 4.5
	Dark	124.0 ± 8.0	146.1 ± 12.2	149.3 ± 17.4	211.0 ± 16.2	176.8 ± 29.9	144.3 ± 9.9
REM	24 h	79.5 ± 17.1	91.5 ± 11.9	95.1 ± 10.0	64.0 ± 2.7	67.2 ± 6.1	58.1 ± 0.6
	Light	55.4 ± 8.3	56.9 ± 8.9	67.1 ± 7.4	63.8 ± 2.8	76.9 ± 11.1	56.4 ± 1.2
	Dark	24.1 ± 3.8	39.0 ± 9.7	28.0 ± 3.7	61.0 ± 4.4	57.5 ± 3.4	62.6 ± 1.8
NREM	24 h	268.8 ± 16.6	320.8 ± 22.9	326.0 ± 22.7	147.8 ± 9.9	133.7 ± 9.3	141.7 ± 11.7
	Light	143.8 ± 12.7	182.5 ± 12.2	176.7 ± 6.1†	161.5 ± 8.7	146.4 ± 15.5	149.3 ± 8.3
	Dark	125.8 ± 7.6	146.0 ± 12.2	150.0 ± 17.3	131.1 ± 16.2	121.0 ± 5.3	120.9 ± 11.4

Bout number and average duration of individual sleep-wake states during light and dark periods and the entire 24-h day in mice with complete (>75% bilateral cell loss) or incomplete loss of Nts^{LH} neurons and sham controls. All data are mean ± SEM. †P<0.05: One-way ANOVA, *P<0.05: post-hoc test by Tukey's multiple comparison (for mean duration of wake in 24 h; $F = 3.67$, $P = 0.044$, control vs lesion: $P = 0.038$, for the number of bouts of NREM in the light period; $F = 3.65$, $P = 0.045$).

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