Microglia modulate stable wakefulness via the thalamic reticular nucleus in mice

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Microglia are important for brain homeostasis and immunity, but their role in regulating vigilance remains unclear. We employed genetic, physiological, and metabolomic methods to examine microglial involvement in the regulation of wakefulness and sleep. Microglial depletion decreased stable nighttime wakefulness in mice by increasing transitions between wakefulness and non-rapid eye movement (NREM) sleep. Metabolomic analysis revealed that the sleep-wake behavior closely correlated with diurnal variation of the brain ceramide, which disappeared in microglia-depleted mice. Ceramide preferentially influenced microglia in the thalamic reticular nucleus (TRN), and local depletion of TRN microglia produced similar impaired wakefulness. Chemogenetic manipulations of anterior TRN neurons showed that they regulated transitions between wakefulness and NREM sleep. Their firing capacity was suppressed by both microglial depletion and added ceramide. In microglia-depleted mice, activating anterior TRN neurons or inhibiting ceramide production both restored stable wakefulness. These findings demonstrate that microglia can modulate stable wakefulness through anterior TRN neurons via ceramide signaling.

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Microglia account for 10–15% of all cells in the adult brain. They not only serve as the main defense against external/internal immune challenges in the central nervous system, but also participate in synaptic formation and elimination, neurogenesis, and learning and memory.[1–3] Accumulating evidences indicate a correlation between microglial activity and sleep/wake states: microglial morphology and activity exhibit distinct dynamics based on the time of day and brain states[4–7], and sleep dysfunction is prevalent in diseases related to abnormal microglial activity, such as Alzheimer’s disease and parasitic infections.[8,9] Although it has been shown that sleep deprivation altered microglia activation[10–12], it remains unknown, in the opposite direction, whether and how microglia regulate sleep/wake.

Wakefulness and sleep are essential components of daily life. Sleep can be broadly categorized into two states on the basis of electroencephalogram (EEG) and electromyogram (EMG) recordings: rapid eye movement (REM) sleep and nonrapid eye movement (NREM) sleep.[13,14] Many neural circuits have been implicated in the regulation of sleep/wake states, most of which are in subcortical brain regions[15,16]. The thalamic reticular nucleus (TRN), a group of GABAergic interneurons (98% of which are parvalbumin positive) that provide inhibitory input to the thalamus, is critical for regulating sleep rhythm and attention.[17–19]. The TRN has been anatomically divided into two main sections based on projection targets: the anterior TRN (aTRN), involved primarily in limbic function, and the posterior TRN which is involved with processing sensory information.[20–24] Several recent studies have shown that aTRN neurons display differential firing rates during wake, NREM sleep, and REM sleep.[17,20,25,26]. However, the cellular and molecular mechanisms underlying these differential firing rates remain unknown.

To determine whether and how microglia modulate sleep, we used a transgenic mouse line in which the diphtheria toxin (DT) receptor is conditionally expressed in microglia for the purpose of microglial depletion.[27,28] We found that microglial depletion dramatically decreased stable wakefulness due to an increase in transitions between wakefulness and NREM sleep. Through metabolic screening and immunostaining, we revealed that the diurnal dynamics of brain ceramide was highly correlated with sleep-wake behavior, and this correlation was abolished in microglia-depleted mice. Microglia in the TRN are sensitive to changes of ceramide levels, and microglial depletion or addition of ceramide decreased aTRN neuronal activity. Importantly, the chemogenetic suppression of aTRN neuronal activity promoted increased transitions between wakefulness and NREM sleep in control mice, whereas chemogenetic activation of aTRN neuronal activity in microglia-depleted mice rescued impaired stable wakefulness. Together, these findings indicate that microglia can modulate stable wakefulness through anterior TRN neurons via ceramide signaling.

Results

Microglial depletion reduced stable wakefulness by increasing transitions between wakefulness and NREM sleep. In contrast to their immune-surveillance function, the regulatory role of microglia in vigilance remains poorly understood. In this cohort of experiments, we examined the role of microglia in regulating wakefulness and sleep.

Changes in wakefulness and sleep were assessed in adult mice with or without microglia depletion. To genetically deplete microglia, as illustrated in Fig. 1a or as previously reported[27,28], we administered two doses of tamoxifen (TAM, 0.5 mg/g) over 48 h for initiation of cre-dependent iDTR transgenes recombination under CX3CR1 promoter in a group of adult CX3CR1Cre-ERT2+;R26iDTR+/ mice. Four weeks after this induction (defined as day 0), we injected DT (i.p., once per day for three consecutive days) to deplete DT receptor-expressing cells. Another group of mice with the same genetic background and tamoxifen treatment received 3 days of saline injection instead of DT to serve as controls. Microglia in the brain have much longer lifespan (>8 weeks) than other CX3CR1-expressing cells in periphery (<2 weeks)[29,30], which allows a time window to specifically deplete microglia in the brain but not affect other CX3CR1-expressing cells in the periphery. Peripheral CX3CR1-expressing cells with DT receptor expression disappeared at 4 weeks after tamoxifen treatment due to their quick renewal.[27,28,31]. Consistently, we found at 4 weeks after tamoxifen administration, DT injection led to a prominent decrease in density of CX3CR1-expressing cells in the brain (i.e., microglia), but not in kidney and spleen (Fig. 1b). To further validate depletion efficiency in microglia, DT-injected mice were sacrificed on day 0 (prior to DT injection), day 4, or day 6. We performed immunostaining with the antibody against GFP (EYFP expression was driven by CX3CR1 promoter in this mouse line[32], and another against Iba1, a microglia/macrophage-specific calcium-binding protein.[32] We then counted the number of Iba1+/GFP+ microglia in different brain regions. We found that on day 4 (one day after the last DT injection), the numbers of microglia decreased sharply to low levels in various brain regions compared to those on day 0 (Fig. 1c). Note that microglia depletion had no effect on body weight, locomotion and other cell populations in the brain, such as astrocytes and neurons (Supplementary Fig. 1a, b and d–f), and saline injections had no detectable effect on microglial density (Supplementary Fig. 1c).

Next, we analyzed vigilance states in microglia-depleted mice. Both EEG and EMG recordings were performed before and after DT injections for 6 h during the day (Zeitgeber time 3–9) and 6 h during the night (Zeitgeber time 15–21) at 4 weeks after tamoxifen administration (Fig. 1a). Wakefulness, NREM sleep, and REM sleep epochs were measured based on EEG/EMG recordings as previously described.[13,15]. We then compared sleep architecture between day 0 (baseline) and day 4, for both day and night, in the saline or DT-injected CX3CR1Cre-ERT2+;R26iDTR+ mice (MGDTR+ Veh. and MGDTR+ DT respectively; microglia were depleted in MGDTR+ DT group) and DT-injected CX3CR1Cre-ERT2+;R26iDTR+ mice (MGDTR– DT) which lack of iDTR transgene (Fig. 1a, d). We first validated that neither TAM nor DT treatment per se had a detectable effect on sleep architecture (Supplementary Fig. 2a, b). We next found that microglia-depleted mice exhibited decreased total wakefulness durations (82.1 ± 5.5% of day 0) and increased total NREM sleep durations (133.7 ± 8.1% of day 0) on the night of day 4 (Fig. 1d, Supplementary Fig. 2d, 3a–c). Note that there was no change between day 0 and day 4 in saline-injected MGDTR+ mice and DT-injected MGDTR– mice (Fig. 1d, Supplementary Fig. 2b–c, 3a–c), indicating that the change of sleep architecture is due to microglia depletion but not DT or saline injection per se. Further analysis revealed a sharp decrease in wakefulness-bout durations at night (Fig. 1e), mainly in stable wakefulness but not quick arousal (Fig. 1f). To further understand these vigilance dynamics, we analyzed the transitions between different brain states. The numbers of transitions from wake to NREM sleep (WN) and from NREM sleep to wake (NW) were significantly increased in microglia-depleted mice (Fig. 1g, Supplementary Fig. 3b). Interestingly, microglial depletion had no effect on REM sleep durations (Fig. 1d–e, Supplementary Fig. 2d, 3a–c) and transitions from NREM sleep to REM sleep (NR) and REMs-to-wake (RW) (Fig. 1g). The vigilance dynamics between three groups of mice were comparable prior to microglial depletion (Day 0)
**Fig. 1 Microglia are essential for stable wakefulness at night.** a Microglia were noninvasively depleted by injecting DT (i.p.) into CX3CR1-Cre-ERT2/+; R26GFP/ΔDT transgenic mice. EEG/EMG signals were recorded before and after injection for 6 h during the middle of day and night periods. Three groups of mice were included: CX3CR1-Cre-ERT2/+;R26GFP/ΔDT mice with tamoxifen (TAM) and DT injection (MGDTR−DT), CX3CR1-Cre-ERT2/+;R26GFP/ΔDT mice with tamoxifen injection (MGDTR+ TAM) and CX3CR1-Cre-ERT2/+;R26GFP/ΔDT mice with tamoxifen injection (MGDTR+ TAM) and DT injection (MGDTR+ DT). b Four weeks after TAM treatment, DT injection sharply decreased CX3CR1-expressing cells in the brain (p = 1.1 × 10−10), with no effect on kidney and spleen of CX3CR1-Cre-ERT2/+; R26GFP/ΔDT mice. Control, 3 mice; n = 14 images from brain; n = 20 images from kidney; n = 22 images from spleen; DT, 2 mice; n = 10 images from brain, n = 16 images from kidney, n = 18 images from spleen. Each dot represents cell density obtained from one image. Scale bar, 20 µm. c DT-depleted ~80% of microglia (cells co-labeled with GFP and Iba1) across the whole brain (day 0 vs day 4, p < 0.01 for all examined regions; day 0 vs day 6, p < 0.01 for all examined regions). Representative images show cortical microglia. Microglial density was normalized to day 0 values. CTX cortex, VLPO ventrolateral preoptic nucleus, TRN thalamic reticular nucleus, LH lateral hypothalamus, VLPAG ventrolateral periaqueductal gray, PZ parafacial zone. At least four images were collected for each brain region of one mouse, each dot represents cell density obtained from one mouse. day 0, n = 5 mice; day 4, n = 4 mice; day 6, n = 7 mice. Scale bar, 50 µm. d, e A hypnogram measured from one mouse during the day (top, left) and at night (top, right) before and after microglial depletion (W wakefulness, N NREM sleep, R REM sleep). Sleep architecture (bottom in d) total duration for each state; e: bout duration for each state) was compared before (day 0) and after (day 4) DT or vehicle injection for each animal, and the difference (day 4–day 0) was further compared between three groups of mice. Each dot represents the difference between day 0 and day 4 in one mouse. For Δ % total duration at night in d, W: p = 0.025 for MGDTR− DT vs MGDTR+ Veh; p = 0.005 for MGDTR+ Veh vs MGDTR− DT; p = 0.008 for MGDTR− DT vs MGDTR+ DT; p = 0.002 for MGDTR+ Veh vs MGDTR+ DT. For Δ bout duration in e: W: p = 0.01 for MGDTR− DT vs MGDTR+ Veh; p = 0.003 for MGDTR+ Veh vs MGDTR− DT. MGDTR+ DT, n = 8 mice; MGDTR− Veh, n = 5 mice. MGDTR+ DT, n = 5 mice. f Microglial depletion shortened wakefulness stability (bottom) but not quick arousals (top) at night. g Microglial depletion enhanced the number of wakefulness to NREM sleep (WN) and NREM sleep-to-wakefulness (NW) transitions at night, the transition from NREM sleep to REM sleep (NR) and REM sleep-to-wake were still intact (RW). WN: p = 0.005 for MGDTR− DT vs MGDTR+ DT; p = 0.008 for MGDTR+ Veh vs MGDTR− DT; NW: p = 1.9 × 10−4 for MGDTR− DT vs MGDTR+ DT; p = 6.2 × 10−4 for MGDTR+ Veh vs MGDTR− DT. MGDTR+ DT, n = 8 mice; MGDTR− Veh, n = 5 mice. MGDTR− DT, n = 5 mice. p < 0.05; two-sided un paired t-test for d, e, and g; two-sided Kolmogorov-Smirnov test for f. Data are reported as mean ± SEM. See also Supplementary Figs. 1–3.
We then determined whether ceramide is involved in microglial modulation of wakefulness stability. First, we assessed whether microglia depletion affected ceramide concentration. We collected brain tissues from subcortical regions of microglia depleted and control mice with sampling standard consistent with (Fig. 2b, c). Among the lipids, we found that ceramide, a bioactive metabolite, displayed a substantially lower concentration during the wakeful period (night-time) compared to the sleep period (daytime) (Fig. 2d, Supplementary Fig. 4d). This diurnal change of ceramide concentrations suggests a potential modulatory effect between ceramide and sleep: either sleep causes accumulation of ceramide, or high ceramide concentration promotes sleep. If it is the former case, sleep deprivation will block brain ceramide accumulation. We thus measured brain ceramide concentrations following 6 h and 12 h of sleep deprivation (Fig. 2e), suggesting that ceramide increases during the day (Sleepday) and awake mice at night (Wakenight) (Fig. 2a). A total of 155 metabolites exhibited significant concentration differences between Sleepday and Wakenight (Supplementary Fig. 4a–c, Supplementary Table 1). Among these metabolites, the majority were lipid family members, including unsaturated fatty acids, sphingolipids, and glycerophospholipids

Fig. 2 Circadian variation of ceramide correlated with sleep-wake behavior. a Subcortical brain regions were collected during the day when mice were asleep (Sleepday, n = 6 mice) and at night when mice were awake (Wakenight, n = 6 mice) for metabolomic analysis. b A heatmap of lipid species showing significant Sleepday/Wakenight expression differences. The p value for each lipid was obtained by compare the difference between Sleepday and Wakenight. LCFAs, long chain saturated fatty acids, p = 0.019; LC-MUFA, long chain monounsaturated fatty acids, p = 0.006; LC-PUFAs long chain polyunsaturated fatty acids, p = 0.016; DA dicarboxylic fatty acids, p = 0.01; LCACs long chain saturated acyl carnitines, p = 0.004; PUACs polyunsaturated acyl carnitines, p = 0.022; Carnitine, p = 0.036; ACs acylcholines, p = 0.048; HFA dihydroxy fatty acids, p = 0.008; EC endocannabinoids, p = 0.04; PLP phospholipids, p = 0.018; PC phosphatidylycerolines, p = 0.01; PE phosphatidylethanolamine, p = 0.019; PS phosphatidylserine, p = 0.024; PG phosphatidylycerol, p = 0.019; LP lysophospholipid, p = 0.026; PL plasmalogen, p = 0.034; lyPL lysoplasmalegen, p = 0.044; MG monacylglycerol, p = 0.021; DAG diacylglycerol, p = 0.034; CERs ceramides, p = 0.004; HexCERs hexosylceramides, p = 0.018; dHSMs dihydroxyphosphoeylins, p = 0.003; SMs sphingomyelins, p = 0.021; SPHs sphingosines, p = 0.003; Sterol, p = 0.042. \( * \) A pathway analysis indicated that lipid-related pathways were most affected by Sleepday/Wakenight. d A volcano plot of measured sphingolipids; differentially represented metabolites with \( >1.3 \)-fold change (Sleepday vs Wakenight) and p values \(<0.10\) are indicated in color; n.s. nonsignificant. e Sleep deprivation promoted subcortical ceramide accumulation. Subcortical region was sampled at ZT12 (timepoint of light off). p = 0.035 for CII vs SD12. CII control mice, n = 8; SD6, mice with 6 h of sleep deprivation, n = 8; SD12, mice with 12 h of sleep deprivation, n = 8. f Tight correlation between brain ceramide level and wakefulness. Wakefulness was defined with video recording for 1 h before sampling (ZT12). Pearson’s correlation, p value was calculated using two-sided hypothesis test. Eight mice. \( * p < 0.05; \) two-sided unpaired t-test for b, d, and e. Data are reported as mean ± SEM. See also Supplementary Fig. 4.
Veh. vs Car.: mice) and control mice (left; daytime, nocturnal ceramide levels induced by carmofur administration facilitated state transitions between wakefulness and NREM sleep and decreased stable two-sided unpaired after administration on day 0 (before depletion) and on day 4 (after depletion). Each dot represents the difference in % total duration for each brain state microglia-depleted mice. GW-4869 or vehicle was injected three times before microglial depletion. Night-time EEG/EMG signals were recorded for 6 h between day 0 and day 4 in one mouse. Veh. vs GW: car and microglia-depleted mice, respectively. Indeed, when we observed a restoration of wakefulness stability (Fig.3d).

This led us to investigate whether manipulation of the nighttime ceramide concentration would mimic, or mitigate, the difference in % total duration for each brain state between day 0 and day 4 in one mouse. Veh. vs GW; p = 0.032 for W; p = 0.026 for N. GW, GW-4869 (n = 5 mice); Veh., vehicle (n = 5 mice). t test for the right panels in a, two-sided paired t-test for b, and left panel in d, two-sided paired t-test for left panel in c, two-sided Kolmogorov-Smirnov test for the right panels in c and d. Data are reported as mean ± SEM. See also Supplementary Fig. 4.

TRN microglia exhibited preferential sensitivity to brain ceramide levels, and local depletion of TRN microglia decreased stable wakefulness at night. We next examined the possible neural circuit involved in the microglia-ceramide modulation of nighttime wakefulness. Microglial morphology is tightly linked to their activity6,7,38. Activated microglia display shorter but thicker processes, larger cell bodies, and higher Iba1 expression1,39. Via morphological analysis of microglia in brain regions involved in sleep/wakefulness regulation15,16, with the sampling standard described in Fig. 2a, we identified multiple subcortical regions showing diurnal morphological changes between Wake_{night} and Sleep_{day} in mice, such as median preoptic area (MnPO), lateral preoptic area (LPO), thalamic reticular nucleus (TRN), suprachiasmatic nucleus (SCN) and dorsomedial hypothalamic nucleus (DM) (Fig. 4a). To explore whether the diurnal morphological difference of microglia in these brain regions is affected by changes in ceramide concentration, we elevated brain ceramide levels by directly applying exogenous C2-ceramide into mouse brains with i.c.v. injections and assessed morphological microglial morphology in these brain regions with Iba1 staining at 3 h after administration (Fig. 4b). We first found that the Iba1 expression was specifically higher in TRN, a brain region that surrounding the thalamus and implicated in regulating sleep rhythm24,40-42 (Fig. 4b, upper panel). We next revealed that TRN microglia retracted their processes in response to ceramide application (3 h after injection), whereas other examined regions showed no significant morphological change (Fig. 4b, lower panel). We further validated this result in alkaline ceramidase-3 knockout (Acer3^{−/−}) mice, that have higher brain ceramide levels than wild-type mice43. Consistently, microglia in Acer3^{−/−} mice.
had higher Iba1 expression and shorter processes in the TRN region compared to those in wild-type mice (Fig. 4c, Supplementary Fig. 5a). To further validate the relationship between TRN microglia activity and brain ceramide level, we measure subcortical ceramide concentration and TRN microglial morphology from the same naïve mice and analyzed their correlation (Fig. 4d). We found that higher ceramide level was tightly correlated with shorter process length of TRN microglia (Fig. 4d). And TRN microglia dramatically retract their process in response to 12-hours of sleep deprivation (Fig. 4e), which is consistent with increased brain ceramide following the treatment (Fig. 2e). Taken together, these data suggest that TRN may be an essential region for microglia-mediated modulation of wakefulness stability.

To test this hypothesis, we locally depleted TRN microglia using focal clodronate liposome injections as shown in Fig. 4f and performed the same experiment and analyses as described in Fig. 1. Consistent with our findings for global microglia depletion, mice with microglia depleted locally in the anterior TRN (aTRN) displayed decreased wake durations at night and increased transitions between wakefulness and NREM sleep (Fig. 4f, Supplementary Fig. 5b).

We further hypothesized that microglia in the aTRN modulate wakefulness stability through influencing the neuronal activity via ceramide. To test this hypothesis, we first recorded aTRN neuronal activity with tetrode recordings in mice during day and night times. We found that aTRN neurons displayed higher averaged firing rates at night than at day in normal mice (Fig. 4g).

To test whether microglia depletion affects aTRN intrinsic excitability (such as firing capacity), we performed whole-cell patch recordings onto individual aTRN neurons in acute-prepared brain slices, by injecting various levels of currents and quantifying the elicited action potentials. We found that aTRN neuronal firing capacity was suppressed in microglia-depleted mice as compared to normal mice (Fig. 4h). In microglia-depleted mice, we have shown that night-time ceramide concentration was relatively elevated (Fig. 3b). Consistently, extracellular application of ceramide suppressed aTRN neuronal firing capacity from normal mice (Fig. 4i). Together these results indicated an inhibitory role of ceramide in modulating aTRN neuronal activity.

These data suggest that microglia may modulate stable wakefulness through ceramide signaling to aTRN neurons.

**aTRN neuronal activity regulated transitions between wake and NREM sleep.** To determine how aTRN neurons potentially modulate wakefulness stability, we first analyzed the night-time activity of aTRN neurons. We performed tetrode recordings in...
Fig. 4 High sensitivity of TRN microglia to brain ceramide. a Diurnal differences in the total lengths of microglial processes in sleep/wakefulness-related brain regions (Sleepday, 3 mice; Wake night, 3 mice). A sagittal brain cartoon showing the regions examined, with red dots representing regions with significant Sleepday/Wake night differences in microglial morphology. The white dashed line on the representative microglia indicates the measured processes. Scale bar, 10 μm. The p value for each brain region was obtained by comparing the difference of microglial process length between Sleepday and Wake night, each dot represents log₂ of ratio (Sleepday/mean value of Wake night). MnPO, median preoptic area, p = 0.015 for Sleepday (n = 65) vs Wake night (n = 49); LPO lateral preoptic area, p = 5.4 × 10⁻⁶ for Sleepday (n = 84) vs Wake night (n = 92); VLPO ventrolateral preoptic area (Sleepday, n = 53; Wake night, n = 55); TRN thalamic reticular nucleus, p = 1.5 × 10⁻⁵ for Sleepday (n = 64) vs Wake night (n = 70); SCN suprachiasmatic nucleus, p = 0.006 for Sleepday (n = 48) vs Wake night (n = 69); LH lateral hypothalamus (Sleepday, n = 59; Wake night, n = 70); DM dorsomedial hypothalamus, p = 0.393 for Sleepday (n = 83) vs Wake night (n = 88); VPAG ventral periaqueductal grey matter (Sleepday, n = 64; Wake night, n = 59); LDTg laterodorsal tegmental nucleus (Sleepday, n = 89; Wake night, n = 53); PZ parafacial zone (Sleepday, n = 16; Wake night, n = 25); LPi lateral paragigantocellular nucleus (Sleepday, n = 37; Wake night, n = 48). b Exogenous application of C2-ceramide specifically altered iba fluorescence and microglial morphology by a retraction of processes in the TRN region (Veh. Vehicle, 6 mice; MnPO, n = 38 cells; LPO, n = 25 cells; TRN, n = 85 cells; DM, n = 32 cells; SCN, n = 45 cells. C2-Cer, C2-ceramide, 4 mice; MnPO, n = 25 cells; LPO, n = 55 cells; TRN, n = 57 cells; DM, n = 31 cells; SCN, n = 51 cells). p = 2.5 × 10⁻⁵ in TRN region for Veh. vs C2-Cer. Each dot represents one microglia. Brain tissue was collected 3 h after i.c.v. injection. Representative images with iba staining were taken from TRN-containing brain sections, with the TRN outlined with white dashed lines. The dashed square within the cartoon inset indicates the brain regions from which images were taken. Scale bar, 200 μm. c Total process lengths of TRN microglia in wild-type (WT) and Acer3 knockout (Acer3⁻/⁻) mice (WT, n = 30 cells from 2 mice; Acer3⁻/⁻, n = 31 cells from 3 mice; p = 8.1 × 10⁻¹⁰ for WT vs Acer3⁻/⁻). Each dot represents one microglia. Scale bar, 10 μm. d Correlation between brain ceramide level and microglial morphology in naïve mice. Subcortical ceramide level and total process length of TRN microglia were analyzed in the same mouse. Total process length of at least 20 TRN microglia was measured for each mouse, and the averaged value was used for further analysis. Pearson’s correlation, p value was calculated using two-sided hypothesis test. n = 8 mice. e TRN microglia retracted processes following sleep deprivation. Representative image with iba staining shown TRN microglia in control and sleep-deprived mice. Scale bar, 20 μm. f CTL control mice, 86 cells from 4 mice; SD6, mice with 6 h of sleep deprivation, 91 cells from 4 mice; SD12, mice with 12 h of sleep deprivation, 81 cells from 4 mice. Each dot represents one microglia. f Local injection of clonodone liposomes into the anterior thalamic reticular nucleus (aTRN) effectively depleted microglia (top) and facilitated NREM sleep specifically at night (bottom). Scale bar, 100 μm. Clonodone vs Control at night: p = 0.003 for W; p = 0.003 for N. Clonodone liposomes, n = 6 mice; control liposomes, n = 6 mice. Each open circle represents the difference, before and after local administration, in one mouse, and these differences were compared between clonodone-treated and control mice. g aTRN neuronal firing rate (n = 14 mice with 248 neurons been collected) during daytime and night-time. Each line represents change in mean firing rate of aTRN neurons between day and night in one mouse. p = 0.012 for day vs night. h Compared to control mice (11 neurons from 3 mice), action potentials induced by higher current injections were reduced in aTRN neurons from microglia-depleted mice (17 neurons from two mice: p < 0.05 for current injection high than 120 pA). i Application of C2-ceramide (10 μM) reduced aTRN neuronal excitability (9 neurons from 3 mice; p < 0.05 for current injection higher than 200 pA). *p < 0.05; two-sided unpaired t-test for a-c, e, f, and h. Two-sided paired t-test for g and i. Data are reported as mean ± SEM. See also Supplementary Fig. 5.

the aTRN of normal mice together with EEG/EMG recordings, as shown in Fig. 5a and Supplementary Fig. 6a, and analyzed neuronal firing rates during different vigilance states. The tetrode data showed that 81% of aTRN neurons displayed higher firing rates during wakefulness than during NREM sleep (Fig. 5b). We then analyzed any changes in neuronal firing during the transition periods between these two states. We found that a large cohort of aTRN neurons dramatically decreased their firing rates from wake to NREM sleep, and increased their firing rates from NREM sleep to wake (Fig. 5c, d), indicating close synchronization to the onsets of these transitions. For 56–58% of these neurons, their firing rate changes within a 40-s window around transition onset were represented well by a logistic function (Fig. 5c, d; Supplementary Fig. 6b). We found that 75% of well-fitted neurons exhibited firing rate decreases that preceded transitions from wake to NREM sleep (Fig. 5c, right panel), and that most neurons exhibited increased firing rates closely around the point of transition from NREM sleep to wake (Fig. 5d, right panel).

We then explored whether alterations of aTRN neuronal activity are sufficient to impact transitions from wake to NREM sleep. We employed a chemogenetic method to silence or activate aTRN neurons (Fig. 5e, f, Supplementary Fig. 8). To facilitate viral labeling specificity, we injected adeno-associated virus (AAV) carrying cre-dependent transgenes into GAD2Cre mice in which all GABAergic neurons expressed Cre recombinase. Briefly, we bilaterally injected AAV-Hsyn-DIO-DOR-mCherry into the aTRN to express KORD (DIO-KORD), a kappa opioid receptor-based chemogenetic receptor (Fig. 5e, Supplementary Fig. 8b) that can be activated by salvinorin B (SalB). We verified that SalB suppressed the activity of KORD-expressing aTRN neurons (Supplementary Fig. 6c). To exclude the potential effect of SalB injection per se, we prepared a group of GAD2Cre control mice with local expression of AAV-hSyn-DIO-mCherry in aTRN (DIO-mCherry). In mice expressing KORD receptors in aTRN neurons, SalB administration (compared to vehicle alone) dramatically increased the number of transitions between wake and NREM sleep, whereas administration of SalB in mice without KORD expression showed no change of transition (compared to vehicle injection) (Fig. 5e, Supplementary Fig. 7a). Consistently, suppression of aTRN neurons increased the bout numbers of wake and NREM sleep and decreased the bout durations of wake (Supplementary Fig. 7a,b). We should note that postmortem validations confirmed that viral expression was mainly in the aTRN (Supplementary Fig. 8b). We next chemogenetically activated aTRN neurons with AAV carrying hM3Dq (AAV-Hsyn-DIO-hM3Dq-mCherry) (Fig. 5f, Supplementary Fig. 8c), which can be activated by clozapine-N-oxide (CNO)46. When we compared the sleep architecture of these mice between CNO-administration sessions and vehicle administration sessions, we found that the transitions between wake and NREM sleep were decreased (Fig. 5f, Supplementary Fig. 7c), the bout numbers of wake and NREM sleep decreased and bout durations of wake increased (Supplementary Fig. 7c,d). There was no such change in control mice without hM3Dq expression that received the same CNO/vehicle injections (Fig. 5f, Supplementary Fig. 7c,d). Although there is a slight increase in bout durations of NREM sleep upon CNO injection in both mCherry-expressing and hM3Dq-expressing mice, no significant difference was observed between two mouse groups (Supplementary Fig. 7c,d). These results indicated that aTRN neuronal activity regulated the transitions between wake and NREM sleep, which in turn influence the stability of wakefulness. Combined with the finding
To examine whether impaired wakefulness after microglial activity under both physiological and pathological conditions,

Microglial depletion decreased aTRN neuronal activity during wakefulness. Microglia have been shown to regulate neuronal activity under different vigilance states before and after microglial depletion (Fig. 6a). In microglia-depleted mice, aTRN neuronal firing rates during wake were significantly lower on day 4 compared to day 0 (from 10.2 ± 1.71 spikes/s to 6.9 ± 1.7 spikes/s, p = 0.036) (Fig. 6b), whereas during NREM sleep, firing rates exhibited little change from day 0 to day 4 (from 6.3 ± 1.3 spikes/s to 4.8 ± 1.5 spikes/s, p = 0.093). As a control, we performed the
same set of experiments in mice with the same genetic background and saline administration instead of DT, and found no changes between day 0 and day 4 during either wake or NREM sleep (Fig. 6b). Importantly, firing rate differences between wake and NREM sleep decreased after microglia depletion (Fig. 6c–f), suggesting that the closer activity levels of aTRN neurons during these two states may have accounted for the easier transitions between them. We should note that there were no detectable changes in the proportion of neurons that exhibited logistic firing rate or lag time changes during these state transitions in microglia-depleted mice (Supplementary Fig. 9a,b). The same set of analyses were performed in control mice without microglia depletion, and no changes were observed (Supplementary Fig. 9c–d).

Chemogenetic activation of aTRN neurons restored the microglial depletion-induced decrease in wakefulness stability. Our findings that aTRN neuronal activity regulated transitions between wake and NREM sleep, and that microglial depletion increased transitions between wake and NREM sleep and decreased aTRN neuronal activity, support the hypothesis that microglia modulate the transitions through influencing aTRN neuronal activity. Thus, we may be able to restore normalcy of state transitions in microglia-depleted mice by increasing aTRN neuronal activity. We used chemogenetic activation of aTRN neurons in microglia-depleted mice (Fig. 6g). Briefly, 1 week after tamoxifen administration in CX3CR1Cre-ERT2/+;R26iDTR/+ mice (this induced DT receptor expression in microglia), we injected AAV-hSyn-hM3Dq-mCherry into the aTRN. Another group of CX3CR1Cre-ERT2/+;R26iDTR/+ mice with tamoxifen induction and AAV-CAG-GFP injections served as controls. Three weeks after viral injection, we used CNO (i.p.) to activate hM3Dq-expressing aTRN neurons following microglial depletion. We performed EEG/EMG recordings to monitor the sleep architecture in these mice within a time window of 3 h after vehicle/CNO injection (Fig. 6g). We found that chemogenetic activation of aTRN neurons in microglia-depleted mice indeed restored the

![Diagram](image-url)

A diagram showing the experimental setup and results of the chemogenetic activation of aTRN neurons. The diagram includes graphs illustrating changes in firing rate (FR) and percentage of normal transitions between states before and after microglia depletion and chemogenetic activation.
transition numbers between wake and NREM sleep, percentage of total time for wake, and wakefulness stability (Fig. 6h–j, Supplementary Fig. 9e).

In summary, our findings indicated that microglia could modulate wakefulness stability through ceramide-mediated interactions between microglia and neurons in the aTRN (Fig. 6k).

Discussion

The mechanisms involved in regulating vigilance state of brain expand in multiple systematic layers, from fine-tuned neural circuits controlling to circadian rhythm regulation, from internal metabolic dynamics to external environment stimuli16,51–53. Those mechanisms function in a wide-range timescale, with neurons synchronize their activity with brain state transitions in milliseconds54 whereas microglia survey the brain and sense the behavioral alterations in minutes6,38.

In this study, we depleted microglia using the CX3CR1CreERT2/+; R26iDTR/+ mice. The Cre expression in CX3CR1-expressing cells is induced by tamoxifen treatment, which in turn turns on DT receptor expression in CX3CR1-expressing cells27,28. In addition to microglia, some other peripheral and circulating immune cells also express CX3CR129,30,37. Thus, the wakefulness change observed in our study may include contribution of cells other than microglia. However, we believe it is less likely for the following evidences. First, we confirmed that in the brain parenchyma, the CX3CR1-expressing cells are microglia (Fig. 1c and Supplementary Fig. 1c), consistent with previous reports27,28,31,38,30,38. Second, taking the advantage that peripheral CR3CR1-expressing cells have much higher turnover rates than microglia (<2 weeks versus >8 weeks), we perform the DT injection 4 weeks after the tamoxifen treatment to specifically deplete microglia, with no detectable effect on peripheral CX3CR1-expressing cells (Fig. 1b). Third, DT receptor mediated microglia depletion do not compromise the blood-brain barrier28, exclude the possibility of circulating immune cells infiltrating the brain. We also confirm that within the time window of our analyses, there is no change in densities of neuron and astrocyte (Supplementary Fig. 1 d–f).

It would be ideal to quantify the correlation of metabolites and microglia morphology directly with wake/sleep instead of day/night in mice since mouse sleep/wake cycle is less homogenous than the human13. However, it is technically challenging to monitor metabolite concentrations and/or microglia processes in free-moving mice with EEG recordings to identify wake/sleep epochs. Currently ceramide sensor has poor specificity and limited availability59. Two-photon imaging that can monitor the microglia processes required head-fixed configuration of animals60, whereas single-photon imaging that can be coupled on freely moving animals is not sensitive enough to identify the thin processes of microglia. Therefore, in this study we addressed this question in an alternative strategy. First, the brain samples we collected for metabolite and microglia analyses (Figs. 2–4) were (1) all from sleeping mice at day or awake mice at night, and (2) at time points mice having 65% sleeping time or 25% sleeping time, respectively (Supplementary Fig. 4d). Thus, although mouse sleep/wake cycle is not exactly synchronized with day/night, the samples were collected from mice with big differences in sleep and wake periods. Second, we monitored the mouse behaviors through infrared video recording for 1 h before sampling. We found the percentage of wake periods in the last 1 h before sample collection is highly correlated with the brain ceramide concentration (Fig. 2f), and the brain ceramide concentration is highly correlated with the total length of TRN microglia processes (Fig. 4d). Together, we believe our analyses reveal the sleep/wake related correlations in metabolite concentration and microglia processes.

Previous studies associated microglia with sleep in two lines of evidence. First, sleep deprivation activates microglia9,10,11,60,61 and microglia morphology and activity change in different brain vigilance states, such as being awake or under anesthesia6,7. Second, sleep is impaired in diseases accompanied with microglial activation, such as Alzheimer’s disease and parasitic infections8,9. Our current study provides the evidence for microglial function in modulating stable wakefulness. The finding that microglial depletion decreases the duration of night-time wakefulness due to an increase in transitions from wake to NREM sleep (Fig. 1) indicates that microglia are important for maintaining the stability of wakefulness. It should be noted that we observed these changes only during night-time (Fig. 1d, Supplementary Fig. 3a–c). One reason for this difference between day and night observations is that mice sleep mostly during the day, and the few stable bouts of wake during the day may limit our ability to detect changes. However, it is also possible that sleep (and/or transitions between wakefulness and NREM sleep) is regulated differently.
between day and night. Further studies, especially those using tools and paradigms for in vivo monitoring and manipulation of microglial activity, are needed to examine these possibilities. Microglial quickly repopulated after depletion, during which astroglisis may observed28,62–64. However these alterations do not occur right after microglia depletion27,28, which is the time frame of our study. As repopulated microglia can promote brain recovery after injury65, benefit neurological disorder treatments66, and improve cognitive function in aging mice67,68, further studies are also needed to understand whether microglial repopulation affects stable wakefulness.

In the current study we showed that aTRN regulates the transitions between wake and NREM sleep. The sharp changes in firing rates of aTRN neurons precede transition onsets from wake to NREM sleep, but not from NREM sleep to wake, suggesting that aTRN neuronal activity may only drive transitions from wake to NREM sleep. Previous studies showed that optogenetic silencing of TRN led to a rapid arousal from NREM sleep69,70, which is consistent with our findings that chemogenetic silencing of aTRN increases the number of transitions from NREM sleep to wake (Fig. 5e, Supplementary Fig. 7a–b). However, it is unclear whether previous findings match our findings of increased transitions from wake to NREM sleep. It is probable that our study focuses on different portions of the TRN compared to previous studies: limbic TRN vs sensory TRN. The TRN is a heterogeneous brain region with high molecular, cellular, anatomical, and functional diversity19,24. From anterior to posterior, the TRN can be divided into different regions based on target projections, such as the limbic TRN and sensory TRN19. TRN neurons also exhibit distinct firing patterns from anterior to posterior; the limbic TRN (aTRN) is composed mainly of wakeful/REM sleep-active neurons, whereas activity in the sensory TRN (posterior TRN) is either state-independent or sleep-active17,20,25,26. Most of the neurons that we recorded from in this study exhibit high firing rates when mice were awake and low firing rates when mice were in NREM sleep (Fig. 5a–d), and postmortem analyses confirmed recording locations in the aTRN (Supplementary Fig. 6a). There is a growing body of evidence suggesting that anterior part of the TRN is implicated in selective attention, fear conditioning, and flight behavior20,22,23,71, for which stable wakefulness is a prerequisite. Sensory TRN exhibits strong repetitive burst discharges24,72, which is essential for generating spindle oscillations and in turn promotes sleep onset11,73–75. A recent study further dissected the contributions of subpopulations of sensory TRN neurons to spindle activity and NREM sleep-bout duration24. Combining previous reports and our current findings, it is possibly that limbic TRN activity is essential for wakefulness stability and sensory TRN activity is crucial for NREM sleep stability. Whether and how these two regions of the TRN interact in wakefulness and sleep regulation requires further study.

The aTRN neurons send projections to intermediodorsal, anterodorsal, and dorsal midline thalamus71,76. Those connections provide the structure basis for function of aTRN in in selective attention, fear conditioning, and flight behavior20,22,23,71, for which stable wakefulness is a prerequisite. Based on the coordinates in this study, the recording and manipulations covered most of the aTRN (Supplementary Fig. 6a, 8). It is not clear whether all aTRN neurons or a subpopulation of aTRN neurons are essential for the phenotypes we observed. Furthermore, the aTRN mainly contains limbic and motor sector of TRN area19,24, receiving projections from relevant thalamic nuclei (e.g., anterodorsal and laterodorsal thalamus) and cortical areas (e.g., prelimbic cortex and cingulate cortex)22,76. The specific inputs and outputs of the aTRN that controlling wakefulness stability and the transition between wakefulness and NREM sleep, are of great interest for future studies.

As the two main cell populations in the brain, there is a reciprocal interaction between microglia and neuron49,50,77. In this study, we show that elevation of ceramide following microglial depletion decreased intrinsic excitabilities of aTRN neurons (Figs. 4h, i, 6a–l) and manipulations of ceramide productions either mimicked or blocked these microglia depletion effects (Figs. 3c, d, 6e–j), suggesting that microglia can modulate neuronal activity through ceramide. Ceramide locates in the branching point of sphingolipids metabolism77, and most of brain cells may be able to produce ceramides. Ceramide was previously found to affect microglia activity levels and neuronal activity79–81. It remains unclear whether the ceramide critical for aTRN neuronal activity is produced from microglial cells or other cell types (e.g., astrocyte). Furthermore, whether and how ceramide directly interacts with neurons are largely unknown. Sphingosine-1-phosphate (S1P) is a main catabolite of ceramide82. A recent work from our group revealed that sphingosine-1-phosphate receptor 2 (S1PR2) is specifically located in interneuron, with remarkable expression in interneuron enriched TRN region83. Manipulations of S1P signaling via S1PR2 tune inhibition level in the neural network83. Therefore, it is possible that microglia modulate aTRN neurons via ceramide-S1p-S1PR2 signaling pathway. Although we have shown that within the study time window the cell density of astrocytes was not changed (Supplementary Fig. 1d & e), it did not rule out the possibility that astrocytes and other brain cells are also involved in the modulatory process. The molecular and cellular mechanisms underlying how microglia regulate subcortical ceramide concentrations, and how ceramide modulates neuronal excitability require further studies.

Our study does not rule out the potential involvement of other signaling molecules, such as adenosine. Adenosine-related metabolites are essential modulators of microglial morphology and activity84–86. A recent study reported that adenosine produced by microglia is crucial for the suppressive role of microglia on neuronal activity49. Adenosine maintains a low level during NREM sleep, and accumulates during wake and within REMs52,87. Manipulation of either brain adenosine level or its receptors, especially in the basal forebrain, produced a prominent effect on sleep/wake behavior52,87,88. Thus, it is highly possible that adenosine may also involve the microglia modulation of stable wakefulness, by functioning in either parallel or joint pathways with ceramide signaling. Previous studies provided limited evidence about the relationship between ceramide and adenosine. Ceramide acts as a pro-inflammation factor89 whereas adenosine is an anti-inflammation factor90. Ceramide may affect adenosine production by blocking mitochondrial ATP release91. Adenosine suppresses tumor necrosis factor-induced nuclear factor-κB (NF-κB) activation but has less effect on ceramide-induced NF-κB activation92. Adenosine executes a strong suppression on excitatory synaptic transmission, but not on inhibitory synaptic transmission93. Future studies may reveal whether and how adenosine (or other signaling molecules) participates in this microglia-mediated wakefulness modulation.

Brain ceramide levels vary across different physiological and pathological conditions. Long chain ceramides are dramatically increased in aged brains43,94. Similarly, ceramide levels have been associated with neurodegenerative disease severity, such as in Alzheimer’s disease94–96. With aging (in both rodents and humans), and in patients with neurodegenerative diseases, there are deficits in wakefulness stability97–100. The present findings that microglia regulate wakefulness stability through ceramide may shed light on potential mechanisms underlying the impaired wakefulness with aging and in other pathological conditions.
Methods

Animals. Male and female adult (2–5 months old) wild-type C57BL/6J, CX3CR1<CreERT2>+/−, CX3CR1<CreERT2>+/−; R26R<ΔDTR>+/−, GAD2<Cre> mice, and Acer3<−/−> mice were used for the experiments. Mice were entrained to a 12 h/12 h light/dark cycle with 64–79 F and 30–70% humidity, food and water available ad libitum. All procedures were approved by the Stony Brook Animal Care and Use Committee and carried out in accordance with the National Institute of Health standards.

Surgery. Adult mice were anesthetized with isoflurane (3% for induction followed by 1% for maintenance) and placed in a stereotaxic apparatus. Both eyes were covered with a layer of ointment during surgery. After shaving and sterilizing the skin, the scalp was incised to expose the skull. Connective tissue was gently removed with a small gomet and a thin layer of Self Etch was applied. For EEG/EMG electrode implantations, four holes were drilled into the skull at the following coordinates: 1st EEG channel: AP:+2 mm, ML:+1.5 mm; 2nd EEG channel: AP:+4 mm, ML:+1.5 mm, reference channel (AP:+2 mm, ML:−1.5 mm), and ground channel site (AP:−4 mm, ML:−1.5 mm). A 2-EEG/1-EMG headmount (8201, Pinnacle Technology Inc.) was aligned to the craniotomy sites and fixed to the skull with a small drop of dental cement. Four EEG screws (0.10 in EEG screws for the anterior craniotomy sites and 0.12 in EEG screws for the posterior craniotomy sites) were inserted into the corresponding channels of the headmount and screwed to the skull. Two EMG leads were inserted on each side of the neck muscles to record postural tone. All implants were secured to the skull with dental cement.

For tetrode/EEG/EMG implantations, three holes were drilled into the skull at the following coordinates: EEG channel: AP:−4 mm, ML:−1.5 mm; reference channel, AP:+2 mm, ML:+1.5 mm; ground channel, AP:−6 mm, ML:0 mm. Screw electrodes were PTTE-coated (0.005 in, WPI) and screwed into each craniotomy site, and two EMG wires were attached on each side of the neck muscles. To record aTRN neuronal activity simultaneously, one additional craniotomy was created over the aTRN (AP:−0.58 mm, ML:+1.25 mm). The tetrodes were assembled as described previously101. Briefly, each tetrode consisted of four polyimide-coated nichrome wires (12.7 μm, Coas Coast, FL, USA) twisted together, placed in a polyimide tube (ID, 0.0049 in; OD, 0.0064 in, Cole-Parmer) and gold-plated to an impedance of 0.3–0.5 Ω at 1 kHz. One end of the tetrad assembly was glued to a microdrive to ensure vertical movement of the entire apparatus. Seven customized tetrodes along with two EEG wires, two EMG wires, one reference wire, and one ground wire were anchored to an experimental interface board (EIB-32 narrow, Neuralynx), with gold EIB pins (Neuralynx). The tips of the tetrodes were brushed with Dil (V22885, Vybrant) for postmortem identification of the recording site (Supplementary Fig. 6a). The dura above the aTRN was removed, the tip of the tetrode was placed over the target area, and then it was slowly lowered to 2.8 mm below the cortical surface. Three percent agarose was applied to the exposed tetrode wire and brain tissue to protect the area from the dental cement. The implants were secured to the skull with dental cement.

For cannula/EEG/EMG implantations, two holes were drilled over the bilateral aTRN (AP:−0.58 mm, ML:+1.25 mm). Custom-designed guide cannula (3.5 mm, C135GMN, Plastics One) were slowly lowered 2.8 mm below the cortical surface and secured with a small drop of dental cement. Four additional holes were drilled into the skull at the following coordinates: 1st EEG channel: AP:−2 mm, ML:+1.5 mm; 2nd EEG channel: AP:+4 mm, ML:+1.5 mm, reference channel (AP:+2 mm, ML:−2 mm), and ground channel site (AP:−4 mm, ML:−1.5 mm). Screw electrodes were soldered with PTTE-coated silver wire (0.005 in, WPI) and screwed into each craniotomy site. Silver wires were soldered to the corresponding channels of the 2-EEG/1-EMG headmount. All implants were re-secured with dental cement. Dummy cannula (3.5 mm, C315DCMN, Plastics One) were mounted to the implanted guide cannulae after the dental cement had dried.

For virus injections, two holes were drilled into the skull bilaterally over the aTRN (AP:−0.58 mm, ML:+1.25 mm). A homemade glass micropipette (10 μm, ML:+1.25 mm) was aligned to the craniotomy sites and preloaded with the virus (300 nl) which was injected via glass pipettes connected to a lever on the heads of tethered mice, a lever was used with one end tethered to the preamplifier using a cotton thread, and the other end secured with a similarly weighted metal screw. Signals were filtered (0.5–100 Hz for EEG/EMG signal, 600–6000 Hz for spike activity) and sampled at 40,000 Hz using the Neuralyx 32-channel hybrid system and Cheetah data acquisition software (Neuralynx). Single units were isolated offline using the MATLAB-based function MCluster (The Mathworks, Natick, MA, USA). All isolated units with stable firing during days of DTx were retained for analysis. For local depletions, mice were recorded 6 h after clodronate/control liposome injections. Differences before and after injections were calculated for each animal and compared between groups. For GAD2<−/−> mice with hM3Dq/control virus expression and CX3CR1<CreERT2>+/−; R26R<ΔDTR>+/− mice with hMD5q/control virus expression, 3 h recordings were performed at night following CNO or vehicle (saline) i.p. injections. For mice with DIO-KORD/control virus expression, 2 h recordings were performed at night following SalB or vehicle (DMSO) i.c. injections. For mice with carmofur injections, signals were collected at night for 4 h following injections. For the GW-4869-mediated rescue experiments, mice were recorded at night for 6 h following GW-4869/vehicle injections. The start time of the recording was fixed for each animal. Differences between vehicle and drug injections were compared for each animal.

For mice with EEG/EMG/tetrode implantations, we simultaneously recorded EEG, EMG, and aTRN neuronal activity across spontaneous sleep-wakeful states in freely moving mice for 2 h at night. Mice were anesthetized with iso flurane (1% for maintenance) and placed in a stereotaxic apparatus. Both eyes were covered with a layer of ointment to reduce stress levels and minimize their exploration in a novel environment. All cage maintenance work was performed after each daily recording session was completed. Mice were able to move around their cages during their cage Electrophysiology experiments. For recordings that included repeated i.p. injections (SalB, CNO, and carmofur), the interval between injections was strictly controlled to ≥3 days to reduce the potential effect of injection-induced stress on sleep architecture.

Mice with the 2-EEG/1-EMG headmount implants were tethered to a preamplifier (8202-SL, Pinnacle Technology, Inc.) and a commutator (8204, Pinnacle Technology, Inc.) for free-moving recordings. Signals were collected with a 100x gain, 0.5 Hz high-pass filter and 400 Hz sampling rate using Sirenia Acquisition software (Pinnacle Technology, Inc.) and analyzed with Synereia sleep software (Pinnacle Technology, Inc.). Simultaneous video recording was performed to monitor animal behavior. Brain state scoring was first processed using the cluster scoring feature of Sirenia Sleep, and then manually reinspected epoch-by-epoch based on the frequency and amplitude signatures of the signals, and corresponding behaviors on video. States were assigned based on consecutive, nonoverlapping, 4-s windows as either wakeful, NREM sleep or REM sleep using scoring criteria described previously13,18. Briefly, low-amplitude desynchronized EEG and high EMG activity were defined as wakeful; synchronized EEG with high-amplitude low-frequency (0.5–4 Hz) EEG activity and low EMG activity were defined as NREM sleep; and prominent theta frequency (6–9 Hz) EEG and low EMG activity were defined as REM sleep (Proprio) (% for duration) for day and night, bout duration, and bout number/4 for each brain state were calculated. A state transition was defined as a change in brain state lasting for more than 12 s. Due to innate properties of mouse sleep19, only state transitions from wakefulness to NREM sleep, from NREM sleep to REM sleep, from NREM sleep to wakefulness, and from REM sleep to wakefulness were calculated. As wakefulness is highly variable within a wide range in mice92, we defined wakeful states as either quick arousals (short wakeful bouts lasting more than 12 s, followed by a transition back to sleep) or stable wakefulness (long wakeful bouts for essential behavior, such as food intake and exploration) based on the duration of each wakeful episode; wakeful bouts less than 12 s were defined as quasi arousals, and wakeful bouts longer than 2 min were defined as stable wakefulness.

For animals assigned to the microglial depletion experiments (both global and local depletions), recordings were performed during the middle of the day (Zeitgeber time [ZT] 3–9 and night (ZT 15–21). For global depletions, mice were recorded before and after 3 days of DTx treatment to ensure that DTx-mediated neuronal activity of identified units was aligned with the sleep architecture for each recording. The averaged neural activity for different brain states was calculated separately as the total number of spikes during a certain state divided by the total recording time spent in the state. Neuronal activity during the transition period was defined as spikes that occurred within 20 s before and after the onset of state transitions. State transitions that satisfied the following criteria were included in further analyses of neuronal activity during the transition period: (1) the prior and subsequent state lasted ≥20 s; and (2) the analyzed transition behavior occurred within 5 min of the transition period. Neuronal activity was defined as the averaged firing rate of a neuron during periods of the same transition behavior across a single recording. Neuronal transition activity was fitted by the
following logistic function:

\[
y = \min + \frac{\max - \min}{1 + 10^{(-x-a)}} + \beta
\]

where \( y \) is the neuronal activity, \( x \) is the time relative to the onset of the state transition, \( \max \) and \( \min \) represent maximal and minimal values of the fitted curve, \( a \) is a slope, and \( \beta \) is the lag time.

For each neuron, the quality of the fit was assessed using the following equation:

\[
R^2 = 1 - \frac{\sum_{i} (y_i - \hat{y}_i)^2}{\sum_{i} (y_i - \bar{y})^2}
\]

where \( f_i \) represents the fitted neuronal activity, \( y_i \) represents the raw neuronal activity, and \( \beta_i \) is the averaged activity of this unit. Units with \( R^2 > 0.3 \) were retained for the calculation of the lag time.

Tetrodes were lowered by 50 μm per day to find the optimal position from which to maximally identify cells inside the aTRN. Once the optimal position was found, the tetrodes were kept in this position until all the recordings for that animal were completed. The start time of the recording was fixed for each animal.

Drug applications. We used a CX3CR1CreER/T2/+;R26DTR/+ transgenic mouse for global microglial depletion.27,28 Mice were administered tamoxifen (T5648, Sigma) to initiate DT receptor expression (500 mg/kg, gavage twice, with 2-day intervals) at 4 weeks before (3 times, with 3-day intervals) and after microglial depletion; 2.5% D-glucose, 2 Thiourea, 5 Na-ascorbate, 3 Na-pyruvate, 0.5 CaCl2, and 10 MgSO4 (250 μm thickness) with a vibratome (HM650V, Thermo Scientific, Waltham, MA, USA). Data analysis was conducted using Clampﬁlter and PatchMaster software (HEKA Electronics, Lambrecht (Pfalz), Germany). The NMDG ACSF contained (in mM): 93 NMDG, 2.5 KCl, 1.2 NaH2PO4, 30 NaHCO3, 20 HEPES, 25 D-glucose, 2 Thiourea, 5 Na-asorbate, 3 Na-pyruvate, 0.5 CaCl2, and 10 MgSO4 with a pH of 7.3–7.4. The HEPELS ACSF contained (in mM): 92 NaCl, 2.5 KCl, 1.2 NaH2PO4, 30 NaHCO3, 20 HEPES, 25 D-glucose, 2 Thiourea, 5 Na-asorbate, 3 Na-pyruvate, 2 CaCl2, and 2 MgSO4. The recording ACSF contained (in mM): 129 NaCl, 3 KCl, 2.2 KH2PO4, 1.3 MgSO4, 20 NaHCO3, 2.4 CaCl2, 3 HEPS, and 10 D-glucose.

Lipidomics. CX3CR1CreER/T2/+;R26DTR/+ transgenic animals were administered tamoxifen and injected with DT 4 weeks later. Mice with the same genetic background that received DT injections, but not tamoxifen, served as controls. One day after the final DT injection, mice were deeply anesthetized with urethane when the mice were either awake (during the night) or asleep (during the day) and perfused transcardially for 30 min with 20 mM Tris-HCl (pH 7.5). The brains were cut into coronal slices (250 μm thickness). The slices were then dried at 37°C for 48 h. The slices were then homogenized in 20 mM Tris-HCl buffer (pH 7.5). The resulting supernatant was divided into two parts for lipidomic profiling and protein level measurements, respectively. Lipidomic measurements were conducted by the Lipidomics Shared Resource Core at Stony Brook University. Briefly, brain samples were homogenized in 20 mM Tris-HCL (pH 7.5) with 1% protease/phosphatase inhibitors and centrifuged. The resulting supernatant was divided into two parts for lipidomic profiling and protein level measurements, respectively. Lipidomic measurements were conducted by the Lipidomics Shared Resource Core at Stony Brook University. Briefly, brain samples were homogenized in 20 mM Tris-HCL (pH 7.5) with 1% protease/phosphatase inhibitors and centrifuged. The resulting supernatant was divided into two parts for lipidomic profiling and protein level measurements, respectively. Lipidomic measurements were conducted by the Lipidomics Shared Resource Core at Stony Brook University. Briefly, brain samples were homogenized in 20 mM Tris-HCL (pH 7.5) with 1% protease/phosphatase inhibitors and centrifuged. The resulting supernatant was divided into two parts for lipidomic profiling and protein level measurements, respectively.

Histology. Mice were deeply anesthetized with urethane and transcardially perfused with PBS followed by 4% paraformaldehyde. Brains were removed and postfixed in the same fixative at 4 °C overnight, then cryoprotected with 30% sucrose in PBS for 4 days. Coronal sections (50 μm) were obtained using a vibratome (HM650, Thermo Scientific). Coronal sections of the aTRn were kept in HEPELS ACSF. Whole-cell recordings were performed in 32 °C, oxygenated, recording ACSF under current-clamp conditions. The NMDG ACSF contained (in mM): 93 NMDG, 2.5 KCl, 1.2 NaH2PO4, 30 NaHCO3, 23 N-hydroxethylpiperazine-N-2-ethanesulfonic acid (HEPES), 25 D-glucose, 2 Thiourea, 5 Na-asorbate, 3 Na-pyruvate, 0.5 CaCl2, and 10 MgSO4 with a pH of 7.3–7.4. The HEPELS ACSF contained (in mM): 92 NaCl, 2.5 KCl, 1.2 NaH2PO4, 30 NaHCO3, 20 HEPES, 25 D-glucose, 2 Thiourea, 5 Na-asorbate, 3 Na-pyruvate, 2 CaCl2, and 2 MgSO4. The recording ACSF contained (in mM): 129 NaCl, 3 KCl, 2.2 KH2PO4, 1.3 MgSO4, 20 NaHCO3, 2.4 CaCl2, 3 HEPS, and 10 D-glucose.

Sleep deprivation. Wild-type mice in both genders were randomly assigned to three groups: mice in control group sleep freely without any interruption; mice with 6 h of sleep deprivation, which was started at ZT 6 (6 h after light on); mice with 12 h of sleep deprivation, which was started at ZT 0 (timepoint of light on). Sleep deprivation was performed in a procedural schedule comprising novel cages, move toys and nesting materials, and gentle handling. All mice were sampled at ZT21 (timepoint of light off).

Metabolomics profiling. The controlling regions for sleep and wakefulness are located primarily in subcortical brain regions,13,14. Therefore, tissues that contained the basal forebrain, thalamus, hypothalamus, midbrain, and brain stem for metabolomics profiling were collected from wild-type mice at ZT 5–7 (day) when mice were asleep (Sleep), and at ZT 17–19 (night) when mice were awake (Wake-night). Mice were deeply anesthetized with urethane and perfused with precooled PBS.

Tissues were dissected on ice and quickly frozen on dry ice and then transferred to −80 °C for storage. Metabolite profiling was performed by Metabolon, Inc. (Morrisville, NC, USA). Briefly, powdered tissues were precipitated with methanol and washed twice with chloroform. Extracts were evaporated under vigorous shaking for 2 min (Glen Mills GenoGrinder 2000) followed by centrifugation. The sample extracts were placed briefly on a TurboVap (Zymark) to remove the organic solvent and stored overnight under nitrogen before preparation for analysis. Measurements were performed via a Waters ACQUITY ultra-performance liquid chromatography and a Thermo Scientific Xevo TQ-Si Mass Spectrometer. Anti-cytochrome oxidase activity/mass resolution accurate mass spectrometer that was interfaced with a heated electro-spray ionization (HESI-II) source and Orbitrap mass analyzer operated at 35,000 mass resolution. Compounds were identified by comparison to library entries of purified standards or recurring metabolite entities. Peak area-under-the-curve analyses. Differential metabolites were defined when a \( p < 0.1 \) (by unpaired t-test) and a >1.3-fold change were observed between day and night values. Pathway analyses for these differential metabolites were performed using the web-based tool MetaboAnalyst 4.0 (https://www.metaboanalyst.ca). To generate the heatmap in Fig. 2b, metabolites belonging to the same species were summed together and z-scored between mice under different conditions.
this transgenic line, microglia were identified as cells that were positive for both Iba1 and GFP. Consistent with a previous study, we also noticed a complete overlap between Iba1- and GFP-positive cells across all examined brain regions. Cx3cr1-expressing GFP-positive cells were also observed and counted in the kidney and spleen. In general, 4–6 images were collected from each examined region for one animal. For microglial morphology analyses, images containing the cell bodies of measured microglia were stacked together, and then Neuron (a plugin for Fiji, an ImageJ, National Institutes of Health, Bethesda, MD, USA) was used to trace all the processes of each microglia. The total process length of each microglia was quantified and compared for the different treatments. For a comparison of differences in microglia morphologies between Sleep-deprived and Wake-rested mice were perfused at ZT 5–7 (day) and ZT 17–19 (night). Animal states were maintained (i.e., asleep during the day and awake at night) before anesthesia. For testing the correlation between ceramide level and animal behavior, the total distance traveled was calculated during the testing period for each animal.

Measurement of locomotor activity. Four weeks after tamoxifen administration, Cx3cr1CreERT2;ROSA26tdTomato mice were exposed to gentle handling for at least 3 days (5 min/day) before the onset of experiments. Mice were randomly divided into two groups to receive either vehicle or DT injections. We placed a ceiling-mounted video camera above the recording area, and each mouse was introduced to this area and allowed to freely explore the area for 5 min of testing. Using an automated tracking system (Ethovision XT, Noldus), we were able to track the animal traces and count the number of times that each animal moved during each test period. The total distance traveled was calculated during the testing period for each animal. Locomotor activity was compared in the same mice before and after DT/vehicle injections.

Reporting summary. Further information on research design is available in the Nature Research Reporting Summary linked to this article.

Data availability
The data that support the findings of this study are available in Source Data and Supplementary Tables. Other data are available upon reasonable request. Mouse brain atlas is available on Allen Institute (https://mouse.brain-map.org/experiment/thumbnails/100004576?image_type=atlas). Source data are provided with this paper.

Code availability
The custom codes used for data analysis in this study are provided together with the source data.

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Author contributions
H.L., S.G., and Q.X. designed the experiments. H.L. performed most of the experiments and data analysis. X.W. performed the in vitro electrophysiological recordings. Lu C. performed part of the metabolomic analysis. Liang C. performed part of the tetrode recordings. H.L., S.T., S.G., and Q.X. wrote the manuscript.

Competing interests
The authors declare no competing interests.

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