

# Serotonergic Integration of Circadian Clock and Ultradian Sleep–Wake Cycles

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In mammals, the suprachiasmatic nucleus (SCN) of the hypothalamus generates a 24 h rhythm of sleep and arousal. While neuronal spiking activity in the SCN provides a functional circadian oscillator that propagates throughout the brain, the ultradian sleep–wake state is regulated by the basal forebrain/preoptic area (BF/POA). How this SCN circadian oscillation is integrated into the shorter sleep–wake cycles remains unclear. We examined the temporal patterns of neuronal activity in these key brain regions in freely behaving rats. Neuronal activity in various brain regions presented diurnal rhythmicity and/or sleep–wake state dependence. We identified a diurnal rhythm in the BF/POA that was selectively degraded when diurnal arousal patterns were disrupted by acute brain serotonin depletion despite robust circadian spiking activity in the SCN. Local blockade of serotonergic transmission in the BF/POA was sufficient to disrupt the diurnal sleep–wake rhythm of mice. These results suggest that the serotonergic system enables the BF/POA to couple the SCN circadian signal to ultradian sleep–wake cycles, thereby providing a potential link between circadian rhythms and psychiatric disorders.

## Introduction

The suprachiasmatic nucleus (SCN) of the hypothalamus regulates a broad range of biological and behavioral rhythms, including hormonal secretion, metabolism, neuronal firing, locomotor activity, and sleep–wake cycles (Mistlberger, 2005; Maywood et al., 2006; Saper et al., 2010). Autonomous neuronal spiking occurs with ~24 h periodicity in the SCN and serves as an essential signal for circadian activity (Inouye and Kawamura, 1979; Schwartz et al., 1987; Ralph et al., 1990; Colwell, 2011), but the mechanism by which the rest of the brain synchronizes with the SCN clock remains unclear.

To sustain the circadian sleep rhythm, the ultradian sleep–wake cycle must be linked to circadian SCN activity. Sleep-active or wake-active neurons are found in the basal forebrain and preoptic area (BF/POA), both of which are located near the SCN, and

the stimulation or lesion of these structures alters the sleep–wake architecture (Jones, 2005; Huang et al., 2007; Takahashi et al., 2009). Despite great advances in elucidating the genetic and molecular circadian mechanisms functioning within SCN cells (Takahashi et al., 2008), precisely how this SCN activity controls the circadian pattern of shorter sleep–wake cycles remains unclear, partly because of the many brain regions and pathways that are involved in the regulation of behavioral state (Deurveilher and Semba, 2008; Saper et al., 2010).

Serotonergic (5-hydroxytryptamine, 5-HT) neurons are located in the dorsal and median raphe nuclei of the brainstem. These neurons innervate the entire brain, including the SCN (Morin and Allen, 2006) and BF/POA (Deurveilher and Semba, 2008), and they affect neuronal excitability via various types of 5-HT receptors. Consequently, serotonin neurons have been implicated in the regulation of multiple brain regions and physiological processes, including sleep and arousal (Frank et al., 2002; Popa et al., 2005; Monti and Jantos, 2008). We recently observed that acute depletion of brain 5-HT leads to a disruption of diurnal sleep–wake cycles and locomotor activity in rats (Nakamaru-Ogiso et al., 2012). Here, we examine the physiological processes underlying circadian organization *in vivo* before and during 5-HT depletion.

To investigate the functional link between circadian rhythms and ultradian sleep–wake cycles, we continuously recorded extracellular neuronal firing (multi-unit activity, MUA) (Nishihara et al., 1994; Yamazaki et al., 1998; Nakamura et al., 2011) in several key brain regions involved in sleep–wake regulation in behaving rats or mice. Our results indicate that the SCN circadian signal is integrated in the BF/POA via the serotonergic system.

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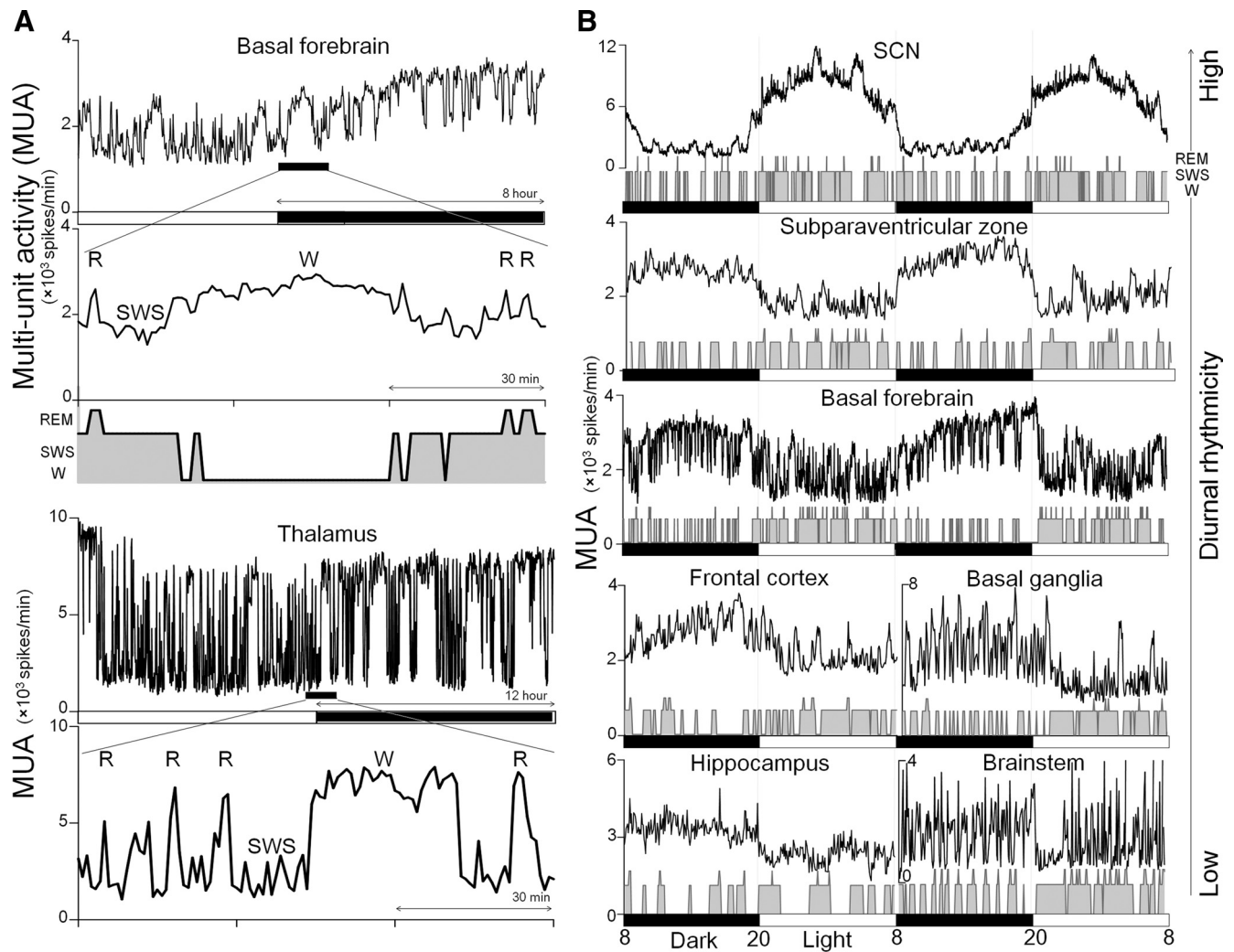
The authors declare no competing financial interests.

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**Figure 1.** Diurnal and behavioral state-dependent neuronal activities in the brain. **A**, Marked state dependence of neuronal activity (multi-unit activity or MUA) in the rat basal forebrain (medial septum/diagonal band of Broca; top panel, 14 h recording; middle panel, 1.5 h expanded recording denoted by horizontal bar in the top panel; bottom panel, hypnogram) and thalamus (centrolateral thalamic nucleus; top panel, 24 h recording; bottom panel, 1.5 h expanded recording). Note the elevated neuronal activities associated with waking (W) or rapid eye movement sleep (REM). SWS indicates slow-wave sleep. **B**, Daytime-dominant MUA rhythm in the SCN (top) and night-dominant MUA rhythm in the subparaventricular zone, basal forebrain (medial septum/diagonal band of Broca), prefrontal cortex, basal ganglia (caudate putamen), hippocampus CA1 region, and brainstem (pontine reticular nucleus pars oralis) of a freely behaving rat; 12:12 h light:dark cycle: light period (white bar), 8 A.M.–8 P.M.; dark period (black bar), 8 P.M.–8 A.M.. Hypnograms are shown for each recording site. Note the large diurnal amplitude in the SCN and prominent ultradian sleep–wake activity in the thalamus and brainstem.

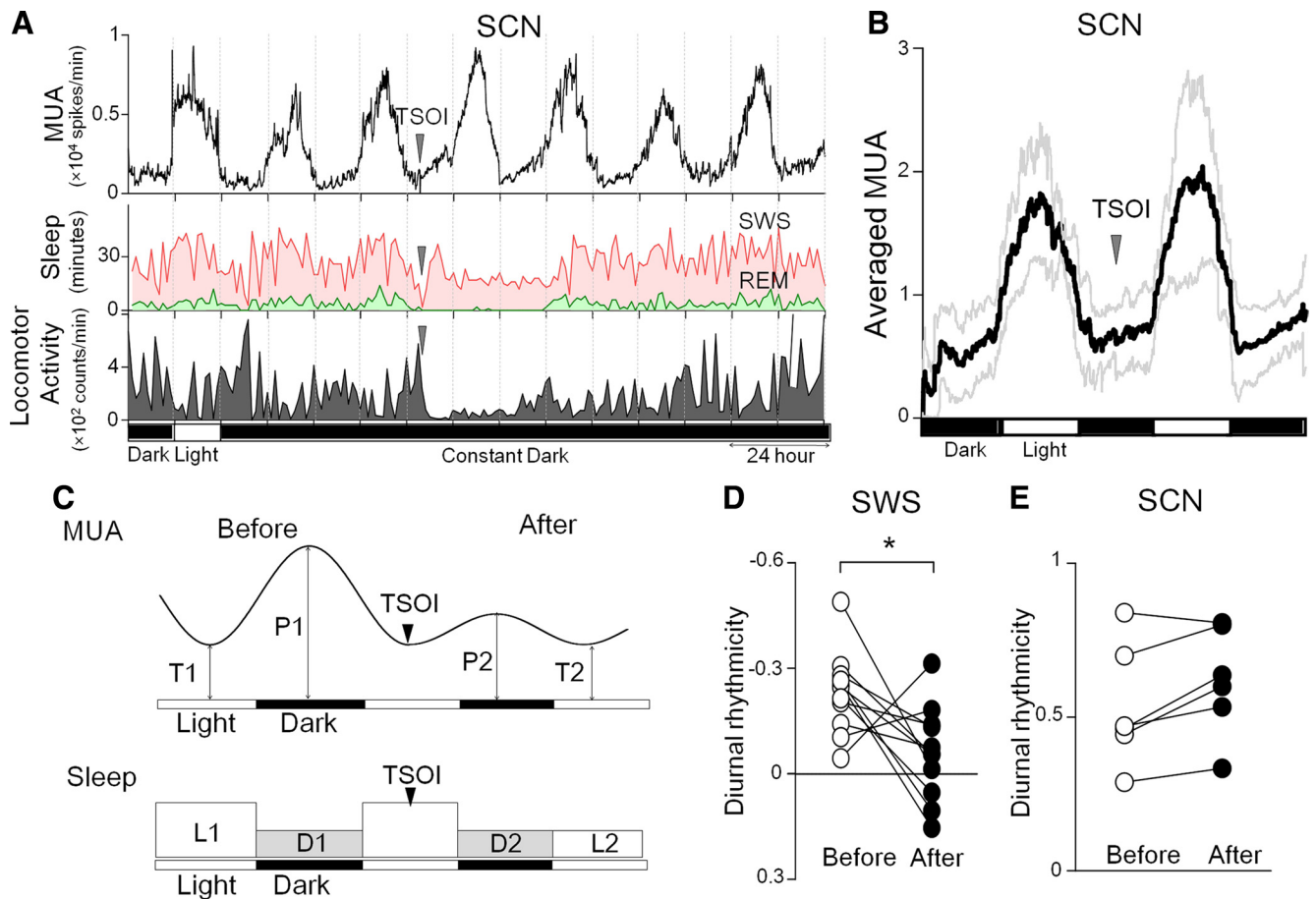
## Materials and Methods

**Animals (rat/mouse).** All experiments were performed under the approval of the local animal care/review committees of the University of Tokyo and RIKEN Brain Science Institute. Full-grown male Sprague Dawley rats (at least 12 weeks of age, 350 g at the time of surgery; Japan SLC) and C57 BL/6 mice (> 8 weeks of age; 30 g at time of surgery; Japan SLC) were used in this study. They were housed (25°C, 55% humidity) under 12 h light (8 A.M.–8 P.M.)/12 h dark (8 P.M.–8 A.M.) lighting conditions (200 lux at cage level).

**Sleep–wake state (rat).** Behavioral states of rats were assessed using electroencephalogram (EEG) and electromyogram (EMG) recordings. Bipolar EEG electrodes consisting of two stainless steel wires were placed on the dura over the frontal cortex (3.0 mm anterior to the bregma, 1.5 mm lateral to the midline). For EMG recordings, two stainless steel wires were inserted into the neck muscle. EEG and EMG signals, which were continuously recorded throughout the experiments, were amplified, bandpass filtered (0.5–30 Hz) and sampled at 100 Hz. Using EEG power spectra analyses (BIMUTAS, Kissei Comtec), three vigilance states—waking (W), slow-wave sleep (SWS), and rapid eye movement sleep (REM)—were determined visually based on standardized EEG/EMG criteria for rodents (Miyamoto et al., 2003).

**Neuronal activity recording (rat).** Compared with mice, rats are more suitable for implantation of multiple MUA electrodes because of their larger body size. Local *in vivo* neuronal activity (MUA) was obtained by bipolar recordings with contiguously aligned Teflon-coated platinum-iridium wires (bare diameter, 125  $\mu$ m; AM Systems) (Nishihara et al., 1994). The positions were determined using the following parameters: anteroposterior (AP), anterior to bregma; mediolateral (ML), lateral to the midline; dorsoventral (DV), depth from the dura; and the angle in the coronal plane. The electrodes were inserted into the brain and aimed at the suprachiasmatic nucleus (AP,  $-1.1$  mm; ML, 1.1 mm; DV, 8.8 mm depth; angle, 6°), subparaventricular zone (AP,  $-1.3$  mm; ML, 1.2 mm; DV, 8.5 mm; angle, 6°), frontal cortex (AP, 3.0; ML, 1.5; DV, 1.1 mm), centrolateral thalamic nucleus (AP,  $-3.14$ ; ML, 2.1; DV, 5.26 mm; angle, 8°), medial septum/diagonal band (AP, 0.48; ML, 1.10; DV, 7.1 mm; angle, 8°), substantia innominata (AP,  $-0.92$ ; ML, 2.5; DV, 8.4 mm), preoptic area (AP,  $-0.6$ ; ML, 1.2; DV, 8.5 mm; angle, 2°), pontine reticular nucleus (oral part) (AP,  $-8.0$ ; ML, 2.2; DV, 7.2 mm; angle, 6°), caudate putamen (AP, 0.70; ML, 2.8; DV, 5.2 mm), and hippocampal CA1 region (AP,  $-3.6$ ; ML, 1.4; DV, 2.61 mm).

Rats were allowed at least 1 week to recover from the surgery, after which their electrodes were connected to head-stage buffer amplifiers



**Figure 2.** Persistent SCN oscillations in the absence of a diurnal sleep–wake rhythm upon acute depletion of brain 5-HT. **A**, The SCN circadian MUA is preserved under constant darkness, whereas the circadian sleep rhythm and locomotor activity are severely disrupted following 5-HT depletion (arrowheads, TSOI, 20 units i.p.). MUA, hourly sleep time (minutes per hour), and locomotor activity were simultaneously recorded from the same rat. Note that the light:dark cycle was switched to constant darkness before TSOI injection. Magenta trace, SWS; green trace, REM (middle panel). **B**, Average of normalized MUA rhythms in the SCN (mean  $\pm$  SD,  $n = 6$ ). The MUA at each time point was normalized by the total number of spikes before or after TSOI injection (2.5 days), and the MUAs were then averaged. Black/white bars, 12:12 h dark/light phase. **B** includes data from **A** (under light: dark cycle,  $n = 5$ ; under constant darkness,  $n = 1$ ). All data are aligned to clock time and averaged. **C**, MUA rhythmicity indices (top panel) were calculated using the following equations: before TSOI =  $(\text{MUA peak1} - \text{MUA trough1}) / (\text{MUA peak1} + \text{MUA trough1})$ ; after TSOI =  $(\text{MUA peak2} - \text{MUA trough2}) / (\text{MUA peak2} + \text{MUA trough2})$ ; where the SCN and frontal cortex MUA peaks were measured during the light and dark phases, respectively, and SCN MUA valleys were obtained from the dark phase (see Materials and Methods). P1, MUA peak1; T1, MUA trough1. The maximal fluctuation was scored as 1, and a lack of difference between the peak and trough was scored as 0. Similarly, the diurnal rhythmicity of sleep is expressed as  $(\text{SWS}_D - \text{SWS}_L) / (\text{SWS}_L + \text{SWS}_D)$ , where  $\text{SWS}_D$  is the amount of SWS during the dark phase (D1), and  $\text{SWS}_L$  is the amount of SWS during the light phase (L1). **D**, Population analysis of diurnal rhythmicity for SWS time.  $\text{SWS} = -0.23 \pm 0.04$  (before TSOI) versus  $-0.06 \pm 0.04$  (after TSOI), 11 rats;  $t = 2.60$ ,  $df = 10$ , paired  $t$  test,  $*p = 0.027$ . **E**, Population analysis of diurnal rhythmicity for MUA in the SCN:  $0.53 \pm 0.08$  (before TSOI) versus  $0.62 \pm 0.07$  (after TSOI), 6 rats;  $t = 0.77$ ,  $df = 10$ ,  $t$  test,  $p = 0.46$ .

(TL084, Texas Instruments) on the skull. Buffer amplifiers were connected to a counterbalanced commutator (MT GIKEN) via a flexible cable, and the rats were allowed to move freely in the recording chamber.

The neuronal activity of the SCN and other brain regions was amplified using a differential amplifier filtered (bandpass, 300–3000 Hz) and fed through a window discriminator (Nihon Kohden) that detected action potentials crossing a preset threshold. To detect the MUA diurnal amplitude and state dependence, we set the threshold of the window discriminator over a range of 20–50 spikes/s for practical purposes to keep the spike counts within the measurable range of the pulse counter. This threshold corresponded to 4 or 5 SDs of the MUA signals (ranging from 30 to 100  $\mu\text{V}$ ). Amplified signals and detected spikes were displayed on an oscilloscope, and the numbers of spikes per 50 s were counted by a pulse counter (MET1100; Nihon Kohden) and stored for further processing. This duration of sampling time was sufficient to evaluate the diurnal rhythmicity and sleep–wake state dependence. Occasionally, we used 1 s sampling to more closely examine the relationship between sleep/wake states and MUA. Usually three or four bipolar electrodes were implanted simultaneously, although the number of active electrodes that stably detected MUA rhythms over multiple days varied. Only recordings that were stable and showed minimal baseline drift over at least 1 week

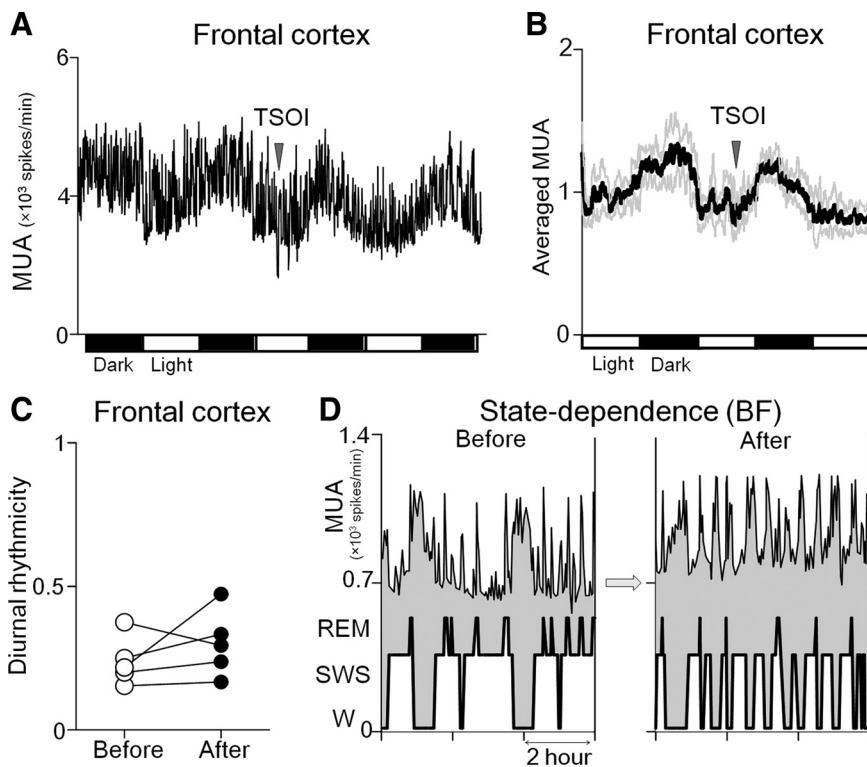
were included in the analysis. In total, MUA recordings from each rat experiment were obtained for 3 weeks or more.

**Enzyme and drug administration (rat).** Tryptophan side chain oxidase type I (TSOI; 20 units) was diluted in 3 ml of saline and injected intraperitoneally under light diethyl ether anesthesia. Control subjects received saline alone. Para-chlorophenylalanine methyl ester hydrochloride (Wako) with Tween 20 in saline (300 mg/kg) or  $\alpha$ -methyl-tyrosine (150 or 250 mg/kg; Wako) were also given intraperitoneally. For the TSOI experiment, the rats were deprived of food, but not water, to minimize tryptophan intake the night before and during the experiments. The total duration of food deprivation was restricted to within 3 days in the experiments. Although food deprivation ( $< 3$  days) per se did not affect diurnal rhythmicity (Nakamaru-Ogiso et al., 2012), we continuously monitored each rat's condition and behavior using EEGs and an infrared camera and evaluated the body weight daily.

**Locomotor activity (rat).** The signal output of each rat movement sensor (Actomonitor, Medical Agent) or EMG was amplified, and the locomotor activity was quantified by counting the number of signal peaks crossing a preset threshold.

**Data analysis (diurnal rhythmicity index) (rat/mouse).** The diurnal rhythmicity index based on Michelson's contrast is versatile, able to





**Figure 3.** Night-dominant MUA rhythm in the cortex and state-dependence after 5-HT depletion. **A**, Frontal cortex MUAs also retain a strong diurnal rhythm after TSOI injection (arrowhead, left panel). Black/white bars, 12:12 h dark/light phase. **B**, Average of normalized MUA rhythms in the frontal cortex (mean  $\pm$  SD,  $n = 5$ ). **C**, Population analyses of diurnal MUA rhythmicity in the frontal cortex:  $0.24 \pm 0.04$  (before TSOI) versus  $0.30 \pm 0.05$  (after TSOI), 5 rats;  $t = 1.12$ ,  $df = 4$ , paired  $t$  test,  $p = 0.33$ . **D**, The ultradian, state-dependent neuronal activity also remains unaffected by TSOI injection (6 h recording in BF).

quantify rhythmicity during transient processes and indifferent to MUA absolute values, and it produces limited values ranging from  $-1$  to  $1$  (Silver and Stryker, 1999). One represents the highest contrast, and 0 represents no contrast. The indices were calculated using MUA values from the day before and the day after TSOI injection (see Fig. 2C). First, we smoothed the MUAs using a linear moving average (for 30 min, 5 min steps). We then picked the highest MUA peak (P1) at midnight (6 h after lights off, between 1:00 A.M. and 3:00 A.M.) and the lowest at midday (6 h after lights on, between 1:00 P.M. and 3:00 P.M.). The diurnal index before TSOI was taken as  $(P1 - T1)/(P1 + T1)$ . Similarly, the MUA peaks (P2) at midnight and the MUA troughs (T2) after TSOI administration were used to create diurnal indices after TSOI  $[(P2 - T2)/(P2 + T2)]$ . In the case of the SCN MUA, the peaks were sampled at midday.

The amount of SWS or REM sleep during the dark phase (i.e., D1) and the light phase (L1) (12 h each) was similarly used to calculate rhythmicity indices  $[(D1 - L1)/(D1 + L1)]$  before and after TSOI (or drug) application. We analyzed single days before and after the TSOI injection. We also calculated the average MUAs of the SCN, cortex, and BF/POA. Because the absolute values of the MUAs varied substantially, even within the same brain region, the individual MUAs at each time point were normalized by the total number of neuronal spikes before or after the TSOI injection (2.5 days), and the MUAs were then averaged. These averaged MUAs were amenable to the statistics of the rhythmicity index.

**Drug microinjection into the basal forebrain (mouse).** To test whether the observed effects of 5-HT depletion induced by TSOI or *p*-chlorophenylalanine (PCPA) were due to secondary melatonin depletion, we also used C57BL/6 mice, which lack the key enzyme for melatonin synthesis from 5-HT. Like rats, mice show clear diurnal sleep wake patterns. Male mice (8 weeks, 30 g at time of surgery; Japan SLC) were microinjected with ritanserin. Ritanserin (12.5  $\mu$ g; Wako) was dissolved in 0.5  $\mu$ l of vehicle solution (10% DMSO in saline) by sonication before use and slowly injected with a micro-syringe via a polyethylene tube connected to an injection cannula (guide cannula 6 or 7 mm in length,

internal cannula tip located 1 mm below the guide tube; *Plastics One*). During the injection, each mouse was lightly anesthetized with halothane. The tip of the cannula was positioned just above the basal forebrain region (AP, 0.8 mm; LM, 0 mm; DV, 3.0 mm). Screw EEG electrodes were implanted into the parietal cortex (AP,  $-2$ ; LM, 1.5), and reference electrodes were placed in the cerebellum.

After at least 1 week of recovery from the implant surgery, continuous EEG and EMG recordings were initiated (telemetry system, Data Sciences International) and stored (Vital Recorder, Kissei Comtec). Sleep–wake states were scored automatically using computer software (SleepSign, Kissei Comtec). The criteria for sleep scoring (4-s epoch, 256-Hz sampling) were based on the amplitude and frequency of the EEG/EMG recordings. The sleep criteria were set using baseline data (before drug application) from individual mice and applied to the experimental data (after drug treatment). After confirming the sleep–wake diurnal rhythm, we tested the effect of the drug on sleep–wake cycles.

**Anatomical localization of the recording electrodes.** At the end of the experiment, each animal (rat/mouse) was anesthetized with an overdose of sodium pentobarbiturate, and its brain was removed. Frozen or fixed (4% paraformaldehyde) sections (40  $\mu$ m thick) were subjected to Nissl staining to identify the recording site.

## Results

### Diurnal rhythmicity and state dependence of neuronal activity across brain regions

In the BF/POA, the MUA increased during waking states (W) or rapid eye movement (REM) sleep and decreased during slow-wave sleep (SWS) (Fig. 1A). Comparable to these findings in the BF/POA, prominent MUA changes were associated with sleep–wake states in most rat brain regions observed, including thalamus and brainstem (Fig. 1A,B), which participate directly in sleep–wake control as part of the reticular activating system. State-dependence of MUA varied from region to region. But the MUA associated with W or REM was usually more prominent in thalamus and much less significant in the SCN (Deboer et al., 2003) (Fig. 1B). In addition, the MUA in various other brain regions (basal ganglia, limbic system) also exhibited diurnal rhythms (Fig. 1B). Daytime-dominant circadian neuronal activity is a hallmark of the SCN in nocturnal and diurnal rodents (Inouye and Kawamura, 1979; Deboer et al., 2003). In contrast, night-dominant MUA rhythms are prevalent in brain regions outside the SCN (Inouye and Kawamura, 1979; Nakamura et al., 2008) (Fig. 1B). We observed variability of state dependence in degree or directionality (i.e., W active, SWS active; Fig. 4A) and diurnal activity in amplitude/waveform, despite a clear diurnal MUA in each region (e.g., SCN, cortex, BF/POA) (see Figs. 2B, 3B, 5B).

### Decoupling of sleep–wake cycles from SCN circadian activity during 5-HT depletion

To further explore the integration of circadian and ultradian MUA rhythms, we acutely perturbed the diurnal rhythm of rat sleep–wake cycles via systemic 5-HT depletion. Brain 5-HT was transiently reduced by depleting tryptophan in the blood (Nakamaru-Ogiso et al., 2012). Since tryptophan is a precursor of

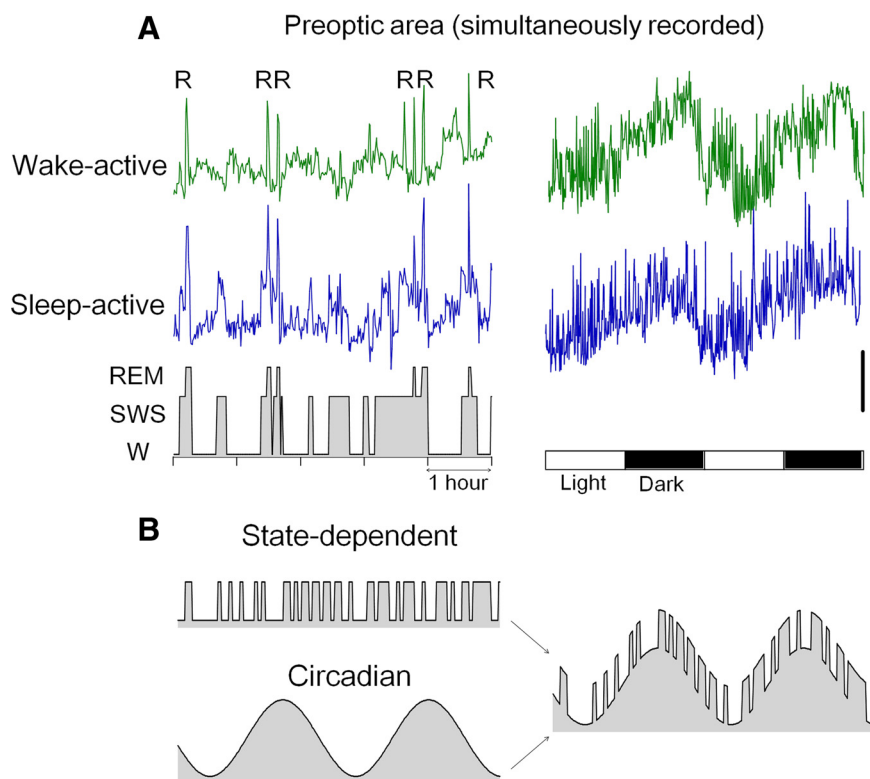
brain 5-HT, to minimize the uncontrolled intake of tryptophan from the diet, rats were deprived of food during these experiments. Briefly (<3 days), food-deprived control rats show a robust sleep–wake diurnal rhythm (Nakamaru-Ogiso et al., 2012). Intraperitoneal injection of the tryptophan-degrading enzyme TSOI then resulted in a 70–90% depletion of brain 5-HT (Nakamaru-Ogiso et al., 2012). Subsequently, locomotor activity was diminished, sleep patterns were fragmented, and the daily rhythm of sleep–wake cycles was lost for ~36 h in TSOI-treated rats under normal light–dark cycles (Fig. 2A). These results are consistent with a previous report showing that chronic tryptophan depletion lowered the levels and circadian amplitudes of wheel-running activity (Kawai et al., 1994).

Because SCN neurons express several 5-HT receptor subtypes and receive dense 5-HT innervation, we determined whether 5-HT depletion altered SCN oscillatory function (Herzog et al., 1998; de la Iglesia et al., 2000; Nakamura et al., 2002; Ohta et al., 2005). As expected (reviewed by Morin and Allen, 2006), the circadian MUA rhythm of the SCN remained intact, even when the rat was maintained in constant darkness (Fig. 2A). The normalized SCN MUAs from all rats ( $n = 6$ ) were averaged, revealing robust oscillations in the SCN (Fig. 2B) after 5-HT depletion. All SCN experiments, except for Figure 2A, were performed under light:dark condition throughout the experiments. To quantify the degree of diurnal MUA rhythmicity and sleep, we calculated a diurnal rhythmicity index (Fig. 2C) in which a value of 1 represents the highest contrast between MUA peak and trough activity, and a value of 0 represents no difference. Statistical analyses confirmed that the diurnal SWS rhythm in particular was lost following 5-HT depletion (Fig. 2D), whereas the diurnal MUA rhythm in the SCN was unchanged (Fig. 2E). These findings demonstrated that sleep–wake regulation is uncoupled from SCN rhythmicity after 5-HT depletion.

MUA in the frontal cortex maintained night-dominant rhythms after 5-HT depletion (Fig. 3A–C), while the diurnal rhythm of sleep based on EEGs from frontal cortex was lost after 5-HT depletion (Fig. 2D) (Nakamaru-Ogiso et al., 2012). The state-dependent neuronal activity and the magnitude of that activity remained unchanged in the BF (Fig. 3D). In addition, the diurnal MUA in other brain regions, such as the striatum, was unaffected by TSOI treatment. We observed wake–active patterns in most MUA recordings. However, a few sleep–active recordings were also obtained. Both wake–active and sleep–active MUAs showed dark-dominant diurnal activities (Fig. 4A). MUA patterns thus appear to consist of separable circadian and state-dependent components (Fig. 4B), in line with previous findings in the SCN (Deboer et al., 2003) (Fig. 4B).

#### Selective reduction of diurnal MUA in the BF/POA region during 5-HT depletion

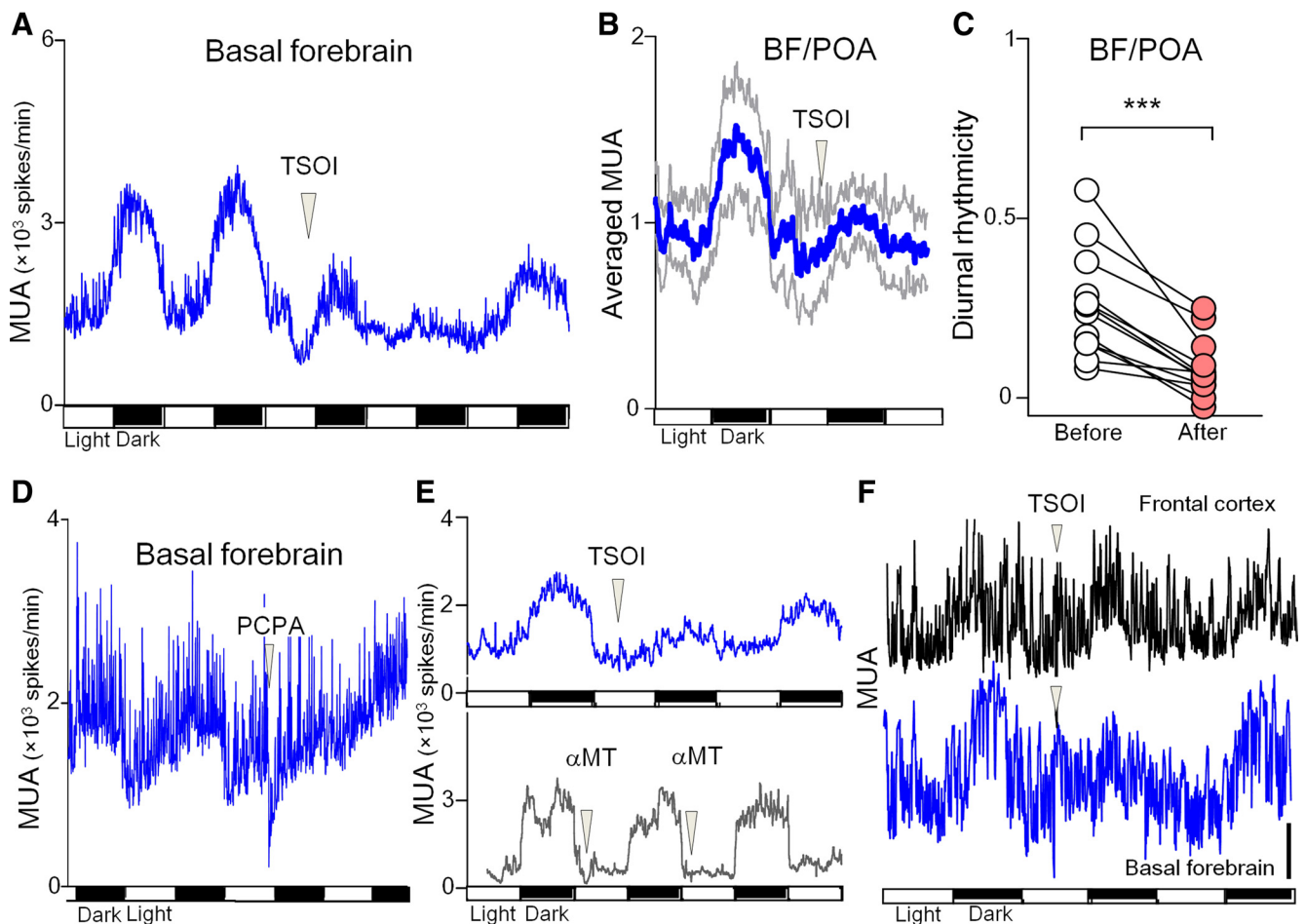
To determine the specific brain regions responsible for the loss of diurnal sleep rhythm, we focused on MUA patterns in the BF/



**Figure 4.** Diurnal and state-dependent components of MUA. **A**, Diurnal rhythms of MUA in the preoptic area with opposite state dependence. Representative MUAs were simultaneously recorded from the preoptic area of the same rat. One MUA showed the W/REM active pattern (green line, left panel), another MUA displayed the SWS/REM active pattern (blue line, left panel), and both MUAs showed diurnal rhythmicity (right panel). The green curve was arbitrarily shifted upward for visual comparison (scale bar,  $10^3$  spikes/min). **B**, Schematic representation of neuronal activity comprised of both circadian and state-dependent components.

POA. We found that the MUA rhythms in the BF/POA were severely disrupted following TSOI injection (Fig. 5A,B). The response to 5-HT depletion was complex: the BF/POA MUA failed to increase during the dark phase in many cases (e.g., Fig. 5A,E), whereas other MUAs showed unusual increases during the light phase and complementary decreases during the dark phase. The reduction of diurnal rhythmicity in the BF/POA was significant (Fig. 5C), and the reduction of MUA rhythmicity coincided with sleep fragmentation and decreased locomotor activity. Both wake–active and sleep–active MUAs reduced dark-dominant diurnal rhythmicity after TSOI. Therefore, we treated them together for the calculation of the diurnal rhythmicity index. The MUA rhythmicity gradually recovered after 48 h as brain 5-HT levels returned to normal (Nakamaru-Ogiso et al., 2012).

We further tested the effects of a 5-HT synthesis inhibitor PCPA (300 mg/kg i.p.), which also causes extensive 5-HT depletion in the brain. Consistent with observations following 5-HT depletion with TSOI, both the diurnal MUA rhythmicity in the BF/POA and diurnal sleep–wake cycles were disrupted following PCPA administration [ $0.217 \pm 0.028$  (rhythm index before PCPA) vs  $0.063 \pm 0.055$  (rhythm index after PCPA), mean  $\pm$  SEM,  $t$  value = 3.46,  $df = 5$ , paired  $t$  test,  $p = 0.0181$ , 6 recording sites, 3 rats] (Fig. 5D). These data underscore the importance of the 5-HT system for diurnal MUA rhythmicity in the BF/POA and in global diurnal sleep–wake cycles. As a control for other monoamine neurotransmitters, we depleted catecholamines using a supramaximal dose of  $\alpha$ -methyl- $p$ -tyrosine, which inhibits tyrosine hydroxylase, and we observed no subsequent change in the MUA patterns of the BF/POA (Fig. 5E).



**Figure 5.** Collapsed MUA rhythms in the BF/POA upon 5-HT depletion. **A**, Typical loss of diurnal MUA rhythm in the BF region (medial septum/diagonal band) upon 5-HT depletion (arrowhead, TSOI, 20 unit i.p.). Black/white bars, 12:12 h dark/light phase. **B**, Average MUA rhythms in the BF/POA (mean  $\pm$  SD, 12 recording sites, 8 rats). **C**, Population diurnal rhythmicity index from the BF/POA:  $0.26 \pm 0.04$  (before TSOI) versus  $0.08 \pm 0.02$  (after TSOI); 12 recording sites, 8 rats,  $t = 6.03$ ,  $df = 11$ , paired  $t$  test,  $***p < 0.001$ . **D**, Diurnal MUA rhythmicity in the BF/POA is reduced following systemic administration of the serotonin synthesis inhibitor PCPA (300 mg/kg i.p.). **E**, Diurnal MUA in the BF region (substantia innominata) is reduced upon 5-HT depletion (top, TSOI), but not after catecholamine depletion [bottom,  $\alpha$ -methyl-tyrosine ( $\alpha$ -MT); first injection, 150 mg/kg; second injection, 250 mg/kg]. Data were taken from the same recording site of the same rat. **F**, Simultaneous recordings of MUA in frontal cortex and medial septum/diagonal band (MSDB) from the same rat (scale bar, 500 spikes/min). The MSDB reduced its MUA rhythmicity while it was preserved in the cortex, suggesting regional specificity of 5-HT depletion.

The changes in MUA rhythms in the BF/POA were striking and reproducible. Simultaneous recordings from the frontal cortex and basal forebrain (medial septum/diagonal band) (Fig. 5F) confirmed the regional selectivity of changes in the MUA following brain 5-HT depletion. In contrast, diurnal brain temperature rhythm did not change in the BF/POA following TSOI injection (Nakamaru-Ogiso et al., 2012), which suggests that thermoregulation and metabolic activity are preserved in the brain.

The subparaventricular zone (SPZ) is a major output from the SCN (Deurveilher and Semba, 2005; Saper et al., 2005). We also observed night-dominant, baseline MUA rhythms in the SPZ (Fig. 6) (Nakamura et al., 2008). Similar to the BF/POA, these SPZ MUA rhythms were significantly abolished upon acute 5-HT depletion [ $0.364 \pm 0.040$  (rhythm index before TSOI) vs  $0.253 \pm 0.057$  (rhythm index after TSOI),  $t = 2.81$ ,  $df = 13$ , paired  $t$  test,  $p = 0.0146$ , 14 recording sites, 6 rats]. However, a substantial number of SPZ MUs also persisted after 5-HT depletion (Fig. 6A, B; MUs with a rhythmicity index ratio after TSOI vs before TSOI,  $>0.6$ , 6 recording sites,  $t = 0.33$ ,  $df = 6$ , paired  $t$  test,  $p = 0.756$ ). Simultaneously recorded MUs from the same rat revealed a differential SPZ response to 5-HT depletion (Fig. 6A, C), suggesting a coexistence of these two response types in the SPZ.

#### Local blockade of 5-HT signaling in the BF/POA disrupts diurnal SWS rhythm

Because the diurnal MUA rhythm in the BF/POA was specifically disrupted after 5-HT depletion in the brain, we hypothesized that the 5-HT signals in this region play a critical role in the regulation of diurnal sleep–wake rhythms. In addition, since melatonin is synthesized from 5-HT, we wondered whether the effects on sleep/wake cycles attributed to 5-HT depletion (by TSOI or PCPA) were actually secondary to “melatonin depletion.” To preclude this possibility, here we used the C57BL/6 mouse strain, in which a critical enzyme for melatonin precursor synthesis (arylalkylamine *N*-acetyltransferase) is missing, thus eliminating any melatonin effects (Kasahara et al., 2010). We investigated the effects of the local blockade of 5-HT neurotransmission by microinjection of the 5-HT<sub>2</sub> receptor antagonist ritanserin (Kantor et al., 2002) into the BF/POA of mice (Fig. 7).

Before the onset of the dark phase, when mice become more active and 5-HT release is higher, a small amount of ritanserin (12.5  $\mu$ g/10% DMSO in saline,  $<0.5 \mu$ l) was delivered into the medial septum/diagonal band of Broca (a basal forebrain region) via chronically implanted injection cannulae. On the first night after ritanserin administration, locomotor activity was decreased and the diurnal SWS rhythmicity was significantly reduced,

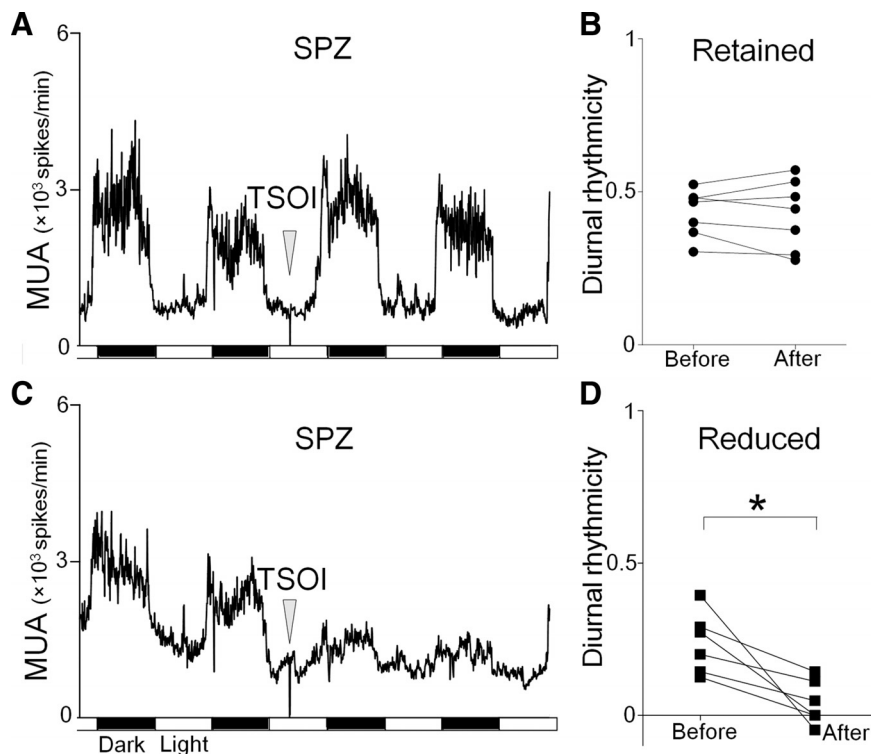


whereas REM sleep rhythmicity remained unaltered (Fig. 7*A,B*). The SWS diurnal rhythmicity of rats tended to be higher than that of mice, but this difference did not reach statistical significance ( $t$  test,  $t = 1.49$ ,  $df = 14$ ,  $p = 0.159$ ). Similarly, the magnitude of change following ritanserin in mice was not significantly different from that following TSOI in rats (before – after TSOI,  $0.172 \pm 0.066$ ; vehicle – ritanserin,  $0.071 \pm 0.016$ ;  $t$  test,  $t = 1.00$ ,  $df = 14$ ,  $p = 0.334$ ). Taken together, these results confirm in mice, as in rats, a crucial role for 5-HT signaling in the BF for diurnal rhythmicity.

## Discussion

Our data show that acute 5-HT depletion leads to a loss of diurnal sleep–wake cycles and a decrease of diurnal MUA rhythms in the BF/POA, whereas the SCN MUA activity is preserved. Local blockade of 5-HT neurotransmission in the BF/POA is sufficient to disturb diurnal sleep–wake cycles. Therefore, the diurnal MUA rhythm in the BF/POA is integral to coupling SCN circadian signals to ultradian sleep–wake cycles under serotonergic control (Fig. 7*C*).

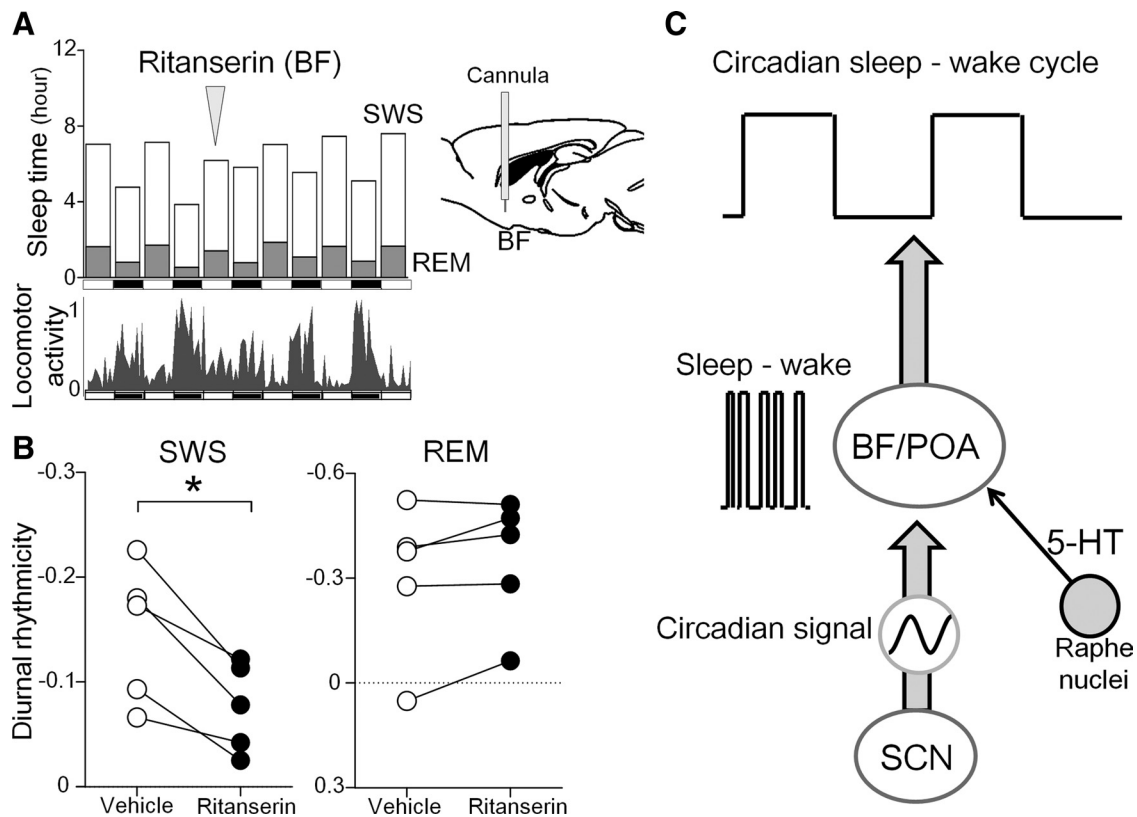
Sleep-active and wake-active neurons are reported to coexist in the BF/POA (Takahashi et al., 2009). We also observed primarily wake-active patterns and some sleep-active MUA recordings, consistent with a bias toward wake-active units in the basal forebrain (Hassani et al., 2009). This state dependence was not affected by 5-HT depletion (Fig. 3*D*), much like an earlier report demonstrating robust state-related neuronal activity in the thalamus (lateral geniculate nucleus) following local 5-HT depletion (Marks et al., 1989). It has been repeatedly demonstrated that circadian behavior is not dependent on 5-HT input by destruction of 5-HT neurons using electrical lesion (Block and Zucker, 1976) or neurotoxin (5,7-dihydroxytryptamine) (Smale et al., 1990; Morin and Blanchard, 1991). Furthermore, continuous SCN MUA rhythm after 5-HT reduction is expected, given the fact that SCN brain slices devoid of 5-HT innervation maintain circadian neuronal firing (Barnes et al., 2003; Ikeda et al., 2003; Yamaguchi et al., 2003; Tischkau et al., 2004; Hamada and Shibata, 2010). However, it was largely unknown whether SCN activity *in vivo* is maintained or disrupted in rats that have lost diurnal sleep/wake rhythm after 5-HT depletion, because behavioral arrhythmicity can result from desynchronization of individual SCN clock neurons (Ohta et al., 2005). In this respect, our finding that SCN activity was maintained after 5-HT depletion by TSOI in rats (Fig. 2) is significant and consistent with previous reports (Block and Zucker, 1976; Smale et al., 1990; Morin and Blanchard, 1991). Our data also indicate that other brain regions (i.e., BF/POA) outside the SCN mediate diurnal rhythms of sleep–wake and locomotor activity. However, our findings do not rule out the possibility that the function of specific subregions in the SCN (LeSauter and Silver, 1999) or diffusible factors mediating circadian signals could be impaired by 5-HT depletion (Silver et al., 1996; Kramer et al., 2001; Cheng et al., 2002).



**Figure 6.** MUAs of the subparaventricular zone, SPZ, are differentially affected by 5-HT depletion. **A**, Retention of MUA rhythm in the SPZ after TSOI injection. **B**, Population analyses of diurnal MUA rhythmicity in the SPZ. The ratio of rhythmicity index after TSOI to that before TSOI is  $>0.6$ ;  $0.43 \pm 0.03$  (before TSOI) versus  $0.43 \pm 0.04$  (after TSOI), 7 recording sites,  $t = 0.33$ ,  $df = 6$ , paired  $t$  test,  $p = 0.756$ . **C**, Reduction of MUA rhythm in the SPZ after TSOI injection (simultaneously recorded from the same rat as the one in **A**,  $>0.1$  mm apart). **D**, Population analyses of diurnal MUA rhythmicity in the SPZ. The ratio of rhythmicity index after TSOI to that before TSOI is  $<0.6$ .  $0.24 \pm 0.04$  (before TSOI) versus  $0.04 \pm 0.03$  (after TSOI), 6 recording sites,  $t = 3.46$ ,  $df = 5$ , paired  $t$  test,  $p = 0.0181$ .

In contrast to the SCN, night-dominant MUAs in the BF/POA reduced their rhythmicity (Fig. 5). This type of inverted diurnal pattern may potentially arise from excessive waking (enhanced MUA) during the night in rats, but this explanation seems unlikely. If the MUA consisted only of a state-dependent component, the diurnal patterns would be rectangular, similar to those found in the thalamus (Fig. 1*B*) where the diurnal MUA amplitudes are not correlated with the degree of state dependence. Moreover, night-dominant MUA rhythms were occasionally found in some brain regions, demonstrating the opposite state dependence (enhanced activity during sleep) (Fig. 4*A*). Thus, we hypothesize that the MUA patterns consist of separable circadian and state-dependent components (Fig. 4*B*), although it is unknown whether single neurons simultaneously encode both the circadian rhythm and state dependence or whether these two phenomena exist independently (Ono et al., 1987; Welsh et al., 2010). Since entrainment by a light:dark cycle may resist change of circadian sleep/wake rhythmicity after 5-HT depletion, a more stringent test under constant dark condition is required in the future to reveal roles of the 5-HT system in circadian regulation of MUA, sleep/wake cycles, locomotor activity, and photic influence (Morin and Allen, 2006).

Local ritanserin controls for the possible side effects of systemic TSOI-induced 5-HT depletion, as tryptophan depletion may also inhibit general protein synthesis and/or melatonin synthesis (Nakamaru-Ogiso et al., 2012). Notably, in the pineal body serotonin is transformed into melatonin, which also influences sleep and circadian rhythms. The C57BL/6 mice used in this



**Figure 7.** Focal 5-HT<sub>2</sub> receptor blockade in the BF disrupts diurnal SWS rhythm. **A**, Reduction of diurnal SWS rhythm and decreased locomotor activity upon microinjection of the serotonin antagonist ritanserin (arrowhead) into the BF region (inset). Note that REM sleep rhythmicity remains intact. **B**, Population analyses of diurnal rhythmicity for SWS and REM. SWS:  $-0.15 \pm 0.03$  (vehicle) versus  $-0.08 \pm 0.02$  (ritanserin); paired *t* test,  $t = 4.40$ ,  $df = 4$ ,  $p = 0.0117$ ; REM:  $-0.30 \pm 0.10$  (vehicle) versus  $-0.35 \pm 0.08$  (ritanserin);  $t = 1.94$ ,  $df = 4$ , paired *t* test,  $p = 0.1250$ . Data were obtained from the same mice ( $n = 5$ ). **C**, Schematic model. The SCN sends circadian clock signals to various brain regions including the BF/POA (and the raphe nucleus), which is a center of sleep–wake regulation. Under 5-HT control, the BF/POA integrates the circadian signal from the SCN with ultradian sleep–wake cycles to express the diurnal sleep–wake rhythm.

study lack a critical enzyme for melatonin synthesis (Kasahara et al., 2010). Therefore, the involvement of the melatonin system is precluded in this study. However, a comparison of C57BL/6 with C3H mice, which carry the intact enzymatic pathway, would be interesting to further determine a potential interaction between serotonin and melatonin systems in the circadian regulation of sleep–wake cycles.

Our data further indicate that the 5-HT input to the BF/POA via the excitatory action of 5-HT<sub>2</sub> receptors (Landolt and Wehrle, 2009) plays a critical role in the diurnal regulation of SWS and night-dominant locomotor activity. We cannot preclude the possibility that the effect of 5-HT blockade is mediated by regions outside the BF/POA, because we used a relatively large volume for microinjection experiments (Fig. 7A, B), and the drug could have spread to other hot spots beyond the BF/POA. However, we think that the effect of ritanserin can still be attributed to the BF/POA for several reasons. First, 5-HT<sub>2A/2C</sub> receptors are abundant in this region (Cornea-Hébert et al., 1999; Clemett et al., 2000). Second, our findings are consistent with previous reports that sleep–wake diurnal oscillations are reduced after selective cholinergic basal forebrain lesion (Kapás et al., 1996). Third, while SWS diurnal rhythmicity was reduced by the ritanserin injection, REM sleep diurnal rhythmicity, which is regulated by adjacent brain regions (Lu et al., 2002), was well preserved in the same mice. Our ritanserin experiment also suggests that the diurnal rhythmicity of REM sleep may be regulated by a different 5-HT receptor subtype or other brain regions. Collectively, our findings directly establish a connection between MUA rhythm in the BF/POA and

diurnal sleep–wake rhythm. Future studies involving conditional gene deletions (Mieda and Sakurai, 2011) in the mouse BF will be of great interest in this regard.

Most SCN neurons densely innervate the SPZ and travel further into the dorsomedial hypothalamus (DMH) (Deurveilher and Semba, 2005). Lesions of the ventral SPZ or DMH cause a disruption of the circadian sleep–wake rhythm and reduction of locomotor activity in rats, whereas brain temperature rhythm is not affected (Aston-Jones et al., 2001; Lu et al., 2001; Chou et al., 2003). Interestingly, the 5-HT-depleted rats in this study also maintained their diurnal brain–temperature rhythm (Nakamaru-Ogiso et al., 2012) while exhibiting a loss of diurnal sleep–wake rhythms and reduced locomotor activity. We thus hypothesize that the circadian signal from the SCN reaches the BF/POA via the ventral SPZ and DMH to regulate circadian sleep–wake cycles.

Indeed, we observed that MUA rhythms in the SPZ collapsed following 5-HT depletion, which suggests that the BF/POA may function in concert with the SPZ and DMH. As we found that MUAs in the SPZ differentially respond to 5-HT depletion (Fig. 6), individual/subregional SPZ cells may mediate SCN circadian signals separately (Lu et al., 2001). This may explain the persistence of the MUA (e.g., in the cortex) and brain temperature rhythms after 5-HT depletion despite a reduction of MUA rhythms in the BF/POA. It is also possible that selected synaptic connections in the SCN/SPZ or BF/POA are compromised by the acute drop in 5-HT. To confirm this, detailed mapping studies are needed to elucidate the temporal relationship between MUAs



of the SPZ, sleep/wake cycles, locomotor activity, and the response to 5-HT manipulation.

Based on our current results, we propose a straightforward model: circadian signals from the SCN are integrated with ultradian sleep–wake cycles generated in the BF/POA, and the 5-HT system may control the excitability of BF/POA neurons in a state-dependent manner (Fig. 7C). Alternatively, because SCN output to the raphe nuclei is relayed via the DMH (Deurveilher and Semba, 2008), and circadian rhythms of 5-HT release in the brain have been reported (Portas and McCarley, 1994; Dudley et al., 1998; Grossman et al., 2000), the SCN may also function through the raphe to regulate rhythms in other brain regions, including the BF/POA (Zant et al., 2011). Furthermore, 5-HT release is enhanced by motor activity (Gerin et al., 2008), and 5-HT has been suggested to mediate nonphotic phase shifting (Mistlberger et al., 1998; Glass et al., 2003). Thus, it is reasonable to suppose that feedback from rhythmic locomotor activity may also affect BF/POA and SPZ MUA.

Because 5-HT receptor subtypes exert excitatory (5-HT<sub>2</sub>) or inhibitory (5-HT<sub>1</sub>) action on postsynaptic neurons, 5-HT may directly regulate cell excitability in the BF/POA and SPZ. These regions, in turn, may affect circadian sleep–wake patterns. However, it is unclear whether 5-HT is only “permissive” for the expression of rhythmicity driven by the SCN or if the 5-HT system plays a more active “instructive” role. Future studies should therefore explore the relationships among individual cellular responses in the BF/POA, sleep–wake behavior and manipulation of 5-HT neurotransmission.

Basal forebrain regions have been implicated in sleep fragmentation and the homeostatic regulation of sleep (Kalinchuk et al., 2008; Matsuki et al., 2009). Therefore, a reduction in MUA may be linked to a failure to consolidate the sleep pattern. We speculate that the circadian oscillation of neuronal excitability in the BF/POA, which defines the sleep or wake state, is controlled by input signals from the SCN (Fig. 7C). Because 5-HT neurons are most active during the wake state (Jacobs et al., 2002), the excitatory influence of 5-HT<sub>2</sub> receptors should enhance the MUA in a positive feedback manner to consolidate the sleep–wake rhythm. Circadian neuronal activity across various brain regions may underlie other brain functions (Ruby et al., 2008; Phan et al., 2011) and disorders. This influence may be mediated through interactions with the brain 5-HT system, which is necessary for brain plasticity (Jitsuki et al., 2011) and has been implicated in psychiatric disorders (Takahashi et al., 2008; Wulff et al., 2010) such as depression and insomnia.

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