Current Biology 24, 693–698, March 17, 2014 ©2014 Elsevier Ltd All rights reserved http://dx.doi.org/10.1016/j.cub.2014.02.011

Report

Selective Activation of Cholinergic Basal Forebrain Neurons Induces Immediate Sleep-wake Transitions

Yong Han,[1] Yu-feng Shi,[1] Wang Xi,[1] Rui Zhou,[1] Zhi-bing Tan,[1] Hao Wang,[1] Xiao-ming Li,[1] Zhong Chen,[1] Guoping Feng,[2] Minmin Luo,[3] Zhi-li Huang,[4] Shumin Duan,[1,*] and Yan-qin Yu[1,*]

[1]Department of Neurobiology, Key Laboratory of Medical Neurobiology of Ministry of Health of China, Key Laboratory of Neurobiology, Zhejiang University School of Medicine, Hangzhou, Zhejiang 310058, China

[2]Department of Neurobiology, Duke University Medical Center, Durham, NC 27710, USA

[3]National Institute of Biological Sciences, Beijing 102206, China

[4]Department of Pharmacology, Shanghai Medical College, Fudan University, Shanghai 200032, China

Summary

The basal forebrain (BF) plays a crucial role in cortical activation [1, 2]. However, the exact role of cholinergic BF (ch-BF) neurons in the sleep-wake cycle remains unclear [3, 4]. We demonstrated that photostimulation of ch-BF neurons genetically targeted with channelrhodopsin 2 (ChR2) was sufficient to induce an immediate transition to waking or rapid eye movement (REM) sleep from slow-wave sleep (SWS). Light stimulation was most likely to induce behavioral arousal during SWS, but not during REM sleep, a result in contrast to the previously reported photostimulation of noradrenergic or hypocretin neurons that induces wake transitions from both SWS and REM sleep. Furthermore, the ratio of light-induced transitions from SWS to wakefulness or to REM sleep did not significantly differ from that of natural transitions, suggesting that activation of ch-BF neurons facilitates the transition from SWS but does not change the direction of the transition. Excitation of ch-BF neurons during wakefulness or REM sleep sustained the cortical activation. Stimulation of these neurons for 1 hr induced a delayed increase in the duration of wakefulness in the subsequent inactive period. Our results suggest that activation of ch-BF neurons alone is sufficient to suppress SWS and promote wakefulness and REM sleep.

Results

Photoactivation of ch-BF Neurons in ChR2-EYFP Transgenic Mice

To confirm that the expression of channelrhodopsin 2 (ChR2) was restricted to cholinergic neurons, we carried out immunohistochemical detection of choline acetyltransferase (ChAT) in the basal forebrain (BF) of ChAT-ChR2-EYFP transgenic mice (ChAT mice). We found that out of 532 EYFP neurons examined, 97.6% ± 0.9% were ChAT positive. Conversely, 94.9% ± 1.0% of ChAT-positive cells also expressed EYFP (Figures S1A–S1C available online).

*Correspondence: duanshumin@zju.edu.cn (S.D.), yanqinyu@zju.edu.cn (Y.-q.Y.)

To test the function of ChR2 expressed in cholinergic BF (ch-BF) neurons, we performed whole-cell recordings in brain slices (Figures S1D and S1E). Application of 30 ms photostimulation at 5–20 Hz reliably induced repetitive bursts of action potentials in ChR2-expressing ch-BF neurons (Figure S1E). Furthermore, most ch-BF neurons were Fos activated after in vivo photostimulation (Figure S1F). Together, these results suggested that ChR2 was selectively expressed in ch-BF neurons of the ChAT mice and was driven by blue light with high temporal precision.

Stimulation of ch-BF Neurons during SWS Causes Immediate Transitions to Wakefulness or REM Sleep

To establish a correlation between stimulation strength and the sleep-wake behavioral responses, we applied 30 ms photostimulation at 10 or 20 Hz for 1–15 s in the inactive period (13:00 to 17:00) and calculated the probability of slow-wave sleep (SWS)-to-waking transitions within 15 s after the onset of light stimulation. We found that light stimulation at 20 Hz was more effective in promoting waking events than 10 Hz (Figure S2A), consistent with the previous report that ch-BF neurons fire bursts at ~20 Hz during the waking state and rapid eye movement (REM) sleep [5]. As the duration of stimulation increased, the probability of SWS-to-waking transitions also increased. The cumulative probability contribution of SWS-to-waking transitions induced by 20 Hz light stimulation is shown in Figure S2B. We thus used 30 ms photostimulation at 20 Hz for 15 s in the subsequent experiments.

We found that photostimulation delivered 12 s after the onset of SWS during the inactive period frequently caused an immediate SWS-to-waking transition within 15 s light stimulation in the ChAT mice (Figures 1A, 1B, and 2A and Movie S1). Along with the immediate SWS-to-waking behavioral transition and electromyographic (EMG) activity after photostimulation, the cortical electroencephalogram (EEG) power also changed, characterized by decreased slow-wave (delta, 0.5–4 Hz) and increased theta-wave (4–10 Hz) activity (Figure 1B and Movie S1). In some cases, an SWS-to-REM transition occurred during the 15 s photostimulation, characterized by a frequency shift to faster components of the EEG power without apparent behavioral arousal and EMG activity (Figures 1C and 2A and Movie S2). The photostimulation of ch-BF neurons during SWS also reduced the duration of the SWS episode in the ChAT mice (Figure 2B). Light stimulation during the active period (22:00 to 01:00) similarly induced an increased transition of SWS-to-waking and SWS-to-REM, as well as reduced SWS duration in ChAT mice (Figures 2C and 2D).

Since light stimulation during SWS promoted transitions to both wakefulness and REM sleep, we examined whether the light-induced transitions have a preference for either wakefulness or REM sleep, as compared with natural transitions from SWS (transitions after the end of SWS episodes without light stimulation in ChAT mice or control light stimulation in wild-type [WT] mice). We found that the ratios of SWS transitions to wakefulness and REM sleep were around 80% and 20%, respectively, either when these transitions were induced by light or occurred naturally (Figure 2E). These results suggest

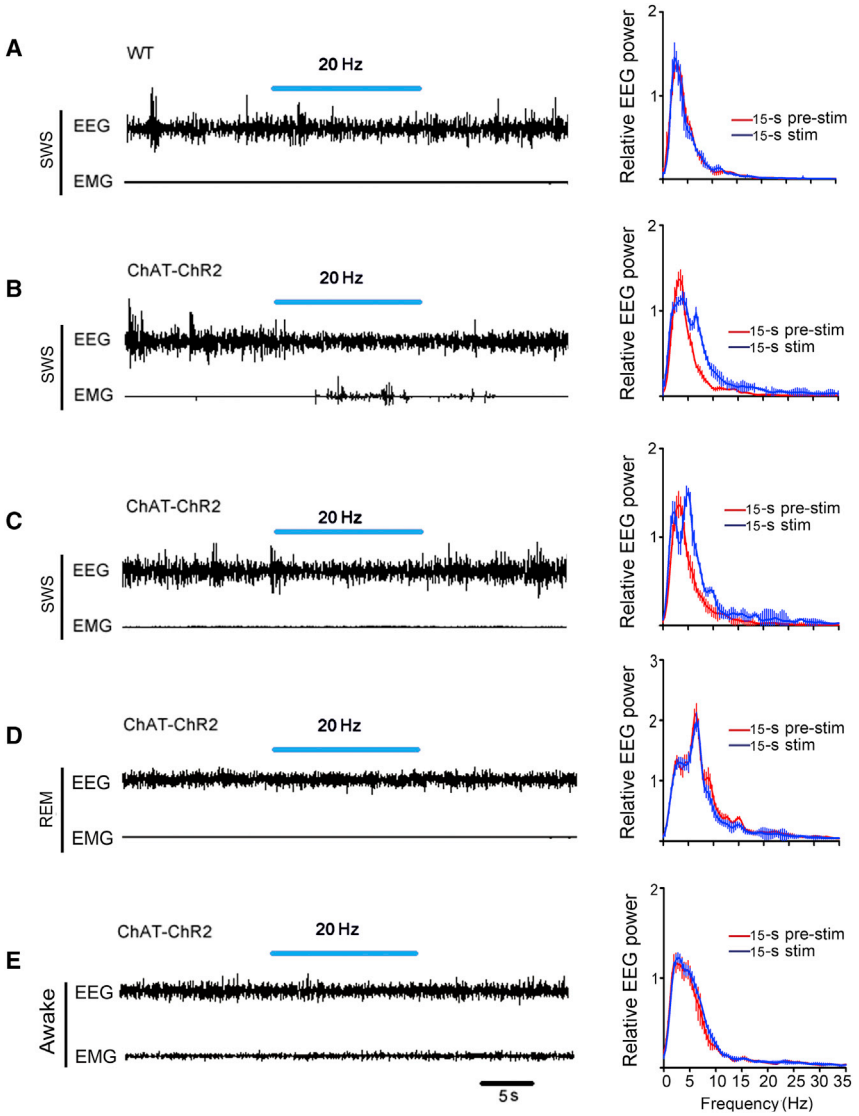

Figure 1. Photostimulation of ch-BF Neurons Causes Immediate SWS-to-Waking or SWS-to-REM Transitions in ChAT Mice during the Inactive Period

(A–C) Left: representative EEG and EMG recordings showing that photostimulation (blue bars, 30 ms pulses at 20 Hz for 15 s) applied during SWS induced a transition to waking (B) or REM sleep (C) in a ChAT mouse, but not in a WT mouse (A). Right: relative cortical EEG power (0.38 Hz binned frequencies, 15 s duration) before (red trace) and after (blue trace) the onset of photostimulation in ChAT mice (n = 7 mice). Ten (A), seven (B), and eight (C) stimulations per mouse were performed.

(D and E) Left: representative traces of EEG and EMG recordings with a single bout of photostimulation (30 ms pulses at 20 Hz for 15 s) during REM sleep (D) and wakefulness (E) in ChAT mice. Right: relative EEG power (15 s) before (red) and after (blue) the onset of photostimulation in REM sleep (D) or awake (E) mice.

See also Figures S1 and S2.

that light stimulation may only facilitate the SWS transition to the next episode of wakefulness or REM sleep, but does not change the fate of the transition. Interestingly, the duration of the light-induced wakefulness or REM sleep was significantly shorter than natural wakefulness or REM sleep (Figure 2F).

The EEG power of the induced wakefulness and REM sleep during 15 s photostimulation was compared with 15 s natural wakefulness or REM sleep sampled around the same time (inactive period) on the previous day. We found that the total theta power (4–10 Hz) of the light-induced waking or REM sleep was not significantly different from that of natural waking or REM sleep. However, when the theta component was divided into two bands, we found that the low theta power (4–7 Hz) of the light-induced REM sleep was slightly increased, whereas the high theta power (7–10 Hz) of the light-induced waking or REM sleep was significantly decreased as compared with natural wakefulness or REM sleep (Figures S3A and S3B). The high theta range has been associated with active exploratory behavior and attentive wakefulness, while the delta and low theta ranges have been associated with increased sleep pressure and drowsiness during wakefulness [6].

Photostimulation of Ch-BF Neurons during the Waking State and REM Sleep Prolonged Their Durations

To further explore the roles of ch-BF neurons in controlling sleep and wakefulness, we applied photostimulation 12 s after the onset of the awake or REM state. Interestingly, stimulation during REM sleep failed to induce an immediate transition to wakefulness in the ChAT mice (Figures 1D, 2G, and 2H and Movie S3). On the contrary, the probability of a REM-to-waking transition during the 15 s stimulation period was evidently lower in the ChAT mice than in the WT mice (Figure 2G), possibly due to a light-induced increase in the duration of the REM sleep episode (Figure 2H). Similarly, stimulation applied during wakefulness extended the duration of wakefulness (Figures 1E and 2H). There was no detectable frequency shift in the theta waves of REM sleep or wakefulness during the 15 s stimulation (Figures 1D and 1E).

Long-Term Effects of Photostimulation of Ch-BF Neurons

To investigate whether continuous photostimulation of ch-BF neurons has long-term effects on sleep architecture, we analyzed the amount of wakefulness, SWS, and REM sleep per hour during the 24 hr after 1 hr of photostimulation (30 ms pulses at 20 Hz for 15 s, once per minute). We found that stimulation during the inactive period (07:00 to 08:00) in the ChAT mice increased the amount of wakefulness and decreased the amount of SWS at a latency of several hours (Figures 3A and 3B). The stimulation increased the total duration of wakefulness and decreased the total duration of SWS during the subsequent 48 hr. There was no significant difference in the total duration of REM sleep between ChAT and WT mice (Figures 3C and 3D).

Interestingly, we found that photostimulation of ch-BF neurons during the active period (21:00 to 22:00) also increased wakefulness and decreased SWS in the subsequent inactive period (Figures 4A and 4B). The amount of

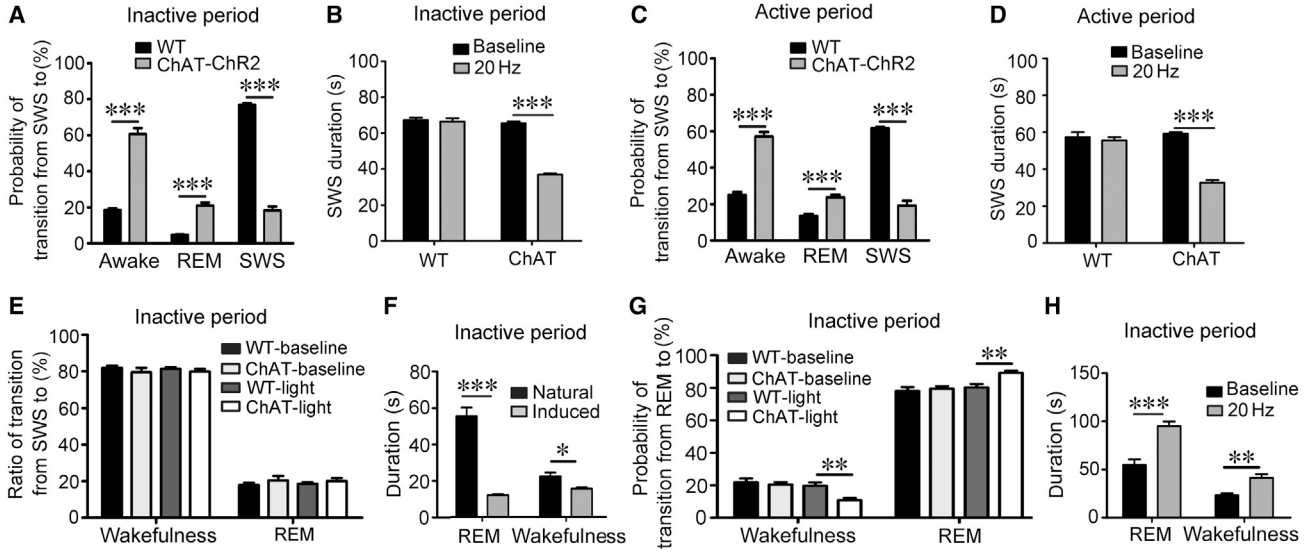

Figure 2. Statistical Analyses of SWS-to-Waking and SWS-to-REM Transitions Induced by Photostimulation of ch-BF Neurons

(A and C) Probabilities of transitions from SWS to awake, REM sleep, or maintained SWS during a 15 s stimulation (30 ms pulses at 20 Hz) period in the inactive period (A) and active period (C). Twenty stimulations per mouse were performed. n = 7 mice in each group.

(B and D) Averaged SWS durations in WT and ChAT mice with (gray columns) or without (black columns, baseline) 20 Hz stimulation (30 ms pulses for 15 s) delivered 12 s after the onset of SWS during the inactive period (B; 13:00 to 17:00) and the active period (D; 22:00 to 01:00). Twenty-five stimulations per mouse were performed. n = 7 mice in each group.

(E) Transition ratio to wakefulness or REM sleep after the end of SWS in a single intact sleep-wake cycle in ChAT or WT mice with or without 15 s photostimulation during the inactive period (13:00 to 17:00). The baseline (natural transition) was calculated by analysis of the probability of a transition to wakefulness or REM sleep after the end of SWS in ChAT and WT mice about 24 hr before stimulation. Twenty stimulations per mouse were performed. n = 6 mice in each group.

(F) Episode duration of natural REM sleep and wakefulness or those induced by photostimulation from SWS. Twenty-five stimulations per mouse were performed. n = 6 mice.

(G) The probability of a transition from REM sleep to wakefulness or maintaining REM sleep during 15 s photostimulation in ChAT and WT mice during the inactive period (13:00 to 17:00). The baseline was calculated by analysis of the probability of a transition from REM sleep to wakefulness or maintaining REM sleep about 24 hr before stimulation. Twenty stimulations per mouse were performed. n = 6 mice.

(H) Episode duration of REM sleep and wakefulness before and during photostimulation in WT and ChAT mice with (gray columns) or without (black columns, baseline) 20 Hz stimulation. Twenty-five stimulations per mouse were performed. n = 7 mice.

Data are represented as mean ± SEM. *p < 0.05, **p < 0.01, ***p < 0.001, two-tailed Student's t test between each group. See also Figure S3.

REM sleep was only slightly decreased at 09:00 (Figure 4A). After 1 hr of stimulation during the active period, the total duration of wakefulness increased and that of SWS decreased in the subsequent 24 hr in the ChAT mice. There was no significant difference between ChAT and WT mice in the total duration of REM sleep after stimulation in the active period (Figures 4C and 4D).

Discussion

Although BF cholinergic neurons have long been thought to play important roles in cortical activation and sleep-wake behavior [2, 7], inconsistent results have been reported. Microinjection of neurotensin into the BF, a procedure proposed to relatively selectively stimulate cholinergic neurons, induces cortical activation characterized by decreased SWS, enhanced REM sleep, and increased time in a quiet waking state with low EMG activity [3]. On the other hand, microinjection of the cholinergic agonist carbachol into the BF, which may affect both cholinergic and noncholinergic neurons, has been reported either to suppress the REM sleep induced by the pontine application of carbachol [8] or to evoke muscle atonia [4]. Lesions of cholinergic BF neurons in rats by immunotoxin 192 immunoglobulin G-saporin has been shown to significantly suppress cortical activation as defined by reduced high-frequency EEG activity, but does not affect the overall sleeping and waking behavior [1]. However, more-recent studies have shown only minor EEG changes after extensive BF cholinergic-specific lesions [9, 10].

In the BF, the cholinergic neurons comprise only ~5% of the total cell population, while GABAergic neurons account for ~35%, and glutamatergic neurons ~55% [11]. Therefore, stimulation of the BF may have different effects on sleep-wake behavior because of the activation of heterogeneous neurons. We found that the selective activation of ch-BF neurons was sufficient to evoke immediate SWS-to-waking transitions (Figures 1B, 2A, and 2C and Movie S1) and prolong wakefulness (Figures 2H, 3 and 4), providing a direct causal link between ch-BF neurons and both cortical activation and waking behavior, as judged by the increased EMG activity and behavioral arousal.

Interestingly, we found that light stimulation of ch-BF neurons evoked wakefulness only from SWS but not from REM sleep (Figures 1D, 2G, and 2H), in contrast to the results obtained from photostimulation of noradrenergic neurons in the locus coeruleus or hypocretin neurons in the lateral hypothalamus, which induce wake transitions from both SWS and REM sleep [12–14]. Furthermore, activation of ch-BF neurons promoted SWS-to-REM transitions (Figures 2A and 2C) and prolonged the REM sleep episodes (Figure 2H).

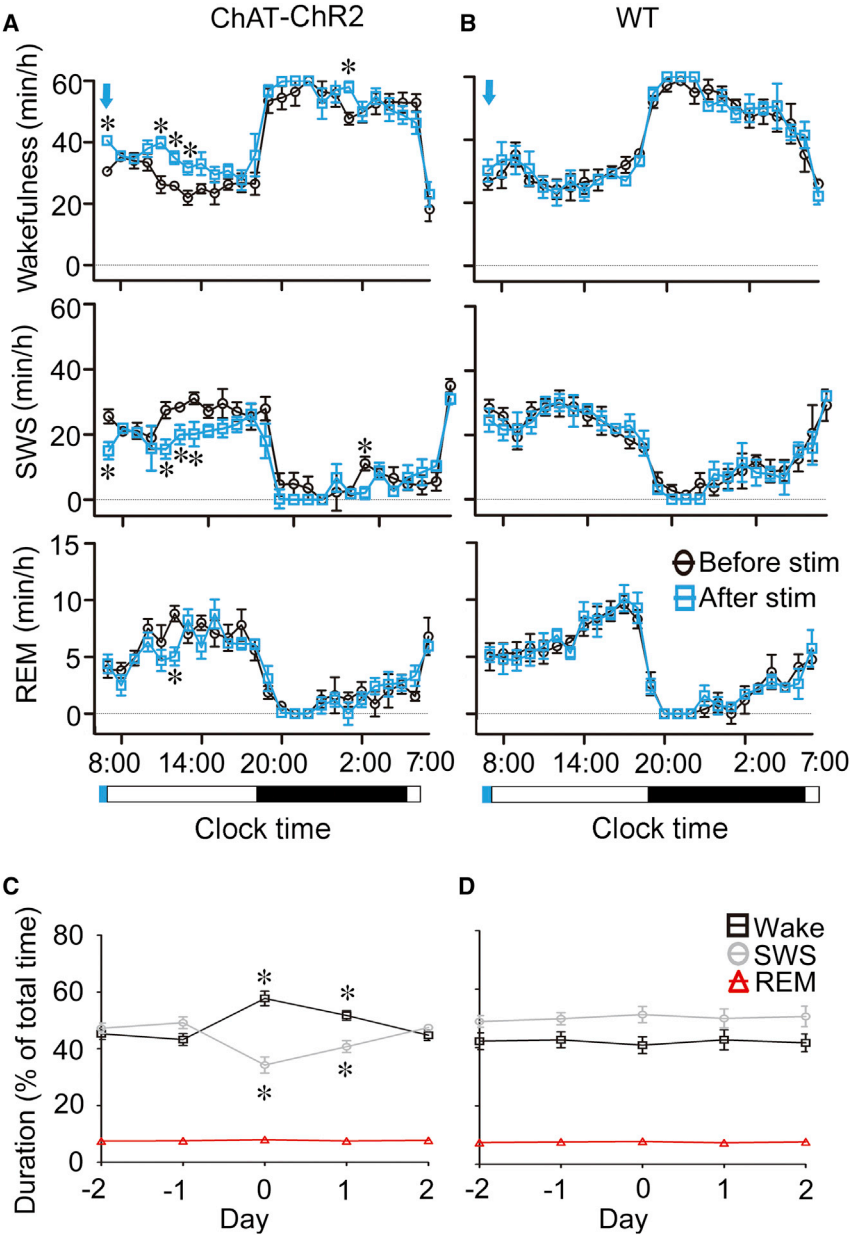

Figure 3. Long-Term Effects of 1 hr Photostimulation of ch-BF Neurons during the Inactive Period

(A and B) Time spent in wakefulness (top), SWS (middle), and REM sleep (bottom) during the 24 hr period after 1 hr of photostimulation (30 ms pulses at 20 Hz for 15 s, once per min) in the inactive period (07:00 to 08:00) in ChAT (A) and WT (B) mice. Arrows indicate delivery of stimulation. Each symbol represents the mean time per hour in each stage. Black and white bars at the bottom of panels depict the active and inactive periods, respectively. n = 5 mice. *p < 0.05, two-tailed Student's t test between groups (before and after stimulation).

(C and D) Total time spent per day in wakefulness, SWS, and REM sleep in ChAT (C) and WT (D) mice. Photostimulation was delivered on day 0 for 1 hr. n = 5 mice. *p < 0.05, two-tailed Student's t test between baseline and stimulation.

Data are shown as mean ± SEM.

These results indicate that ch-BF neurons work in a way different from other arousal-promoting systems.

Cholinergic neurons in the laterodorsal tegmental nucleus (LDT) and pedunculopontine tegmental nucleus (PPT) of the brainstem, which mainly project to the thalamus and to a lesser extent to the BF [15], have been suggested to play a critical role in REM sleep and are regarded as "REM-on" neurons [16, 17]. Notably, although EYFP expression was strong and stable in the BF, very weak or no expression of EYFP was found in the LDT/PPT in the ChAT mice we used (Figure S4). The result that most of the cholinergic BF neurons were Fos positive after BF photostimulation (Figure S1F) was also consistent with the light-induced direct activation of cholinergic neurons in the BF, rather than the secondary effects of activation of cholinergic terminals from LDT/PPT neurons projecting to the BF.

The results that the ratio of light-induced transitions from SWS to wakefulness or to REM sleep did not significantly differ from that of natural transitions (Figure 2E) indicate that the main effect of cholinergic BF neuron activation is to terminate SWS so that the transition to the next episode is permitted, whereas the type of transition (to wakefulness or to REM sleep) may be determined by cooperation of the ch-BF neurons with other arousal-sleep control systems [7, 13, 14, 18–23]. It has been suggested that cholinergic neurons stimulate cortical activation in association with muscle tone when orexinergic neurons are also active, but stimulate cortical activation with muscle atonia when orexinergic neurons are silent [7]. Further studies are required to address this issue.

Sustained stimulation (1 hr) of ch-BF neurons had a delayed long-term effect on sleep and wakefulness, which occurred mainly during the inactive period, whether the stimulation was applied during the active or inactive period. Sleep is known to be regulated by two basic physiological processes: sleep homeostatic need and the circadian rhythm [24]. The circadian rhythm did not change in the sustained light-stimulated mice (Figures 3 and 4). Furthermore, sleep homeostasis regulation cannot explain this delayed change, because sleep deprivation during 1 hr of light stimulation should increase rather than decrease the SWS during the poststimulation period. A waking drive or the arousal system has recently been suggested as the third component regulating sleepiness, and insomnia is considered to be a disorder of hyperarousal [25–28]. Thus, the elevated arousal level induced by the sustained light stimulation of ch-BF neurons may contribute to this delayed prolongation of wakefulness and decrease of SWS during the poststimulation period.

Supplemental Information

Supplemental Information includes Supplemental Experimental Procedures, four figures, and three movies and can be found with this article online at http://dx.doi.org/10.1016/j.cub.2014.02.011.

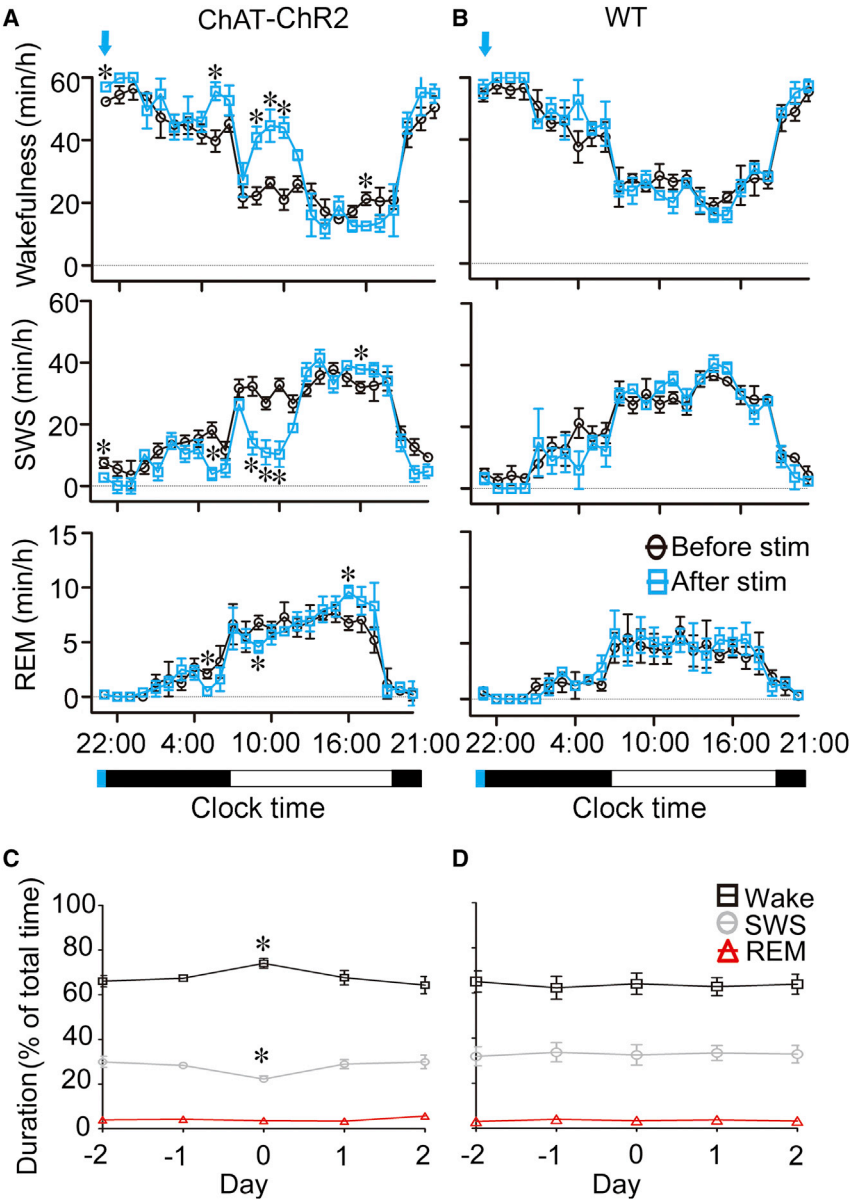

Figure 4. Long-Term Effects of 1 hr Photostimulation of ch-BF Neurons during the Active Period

(A and B) Time spent in wakefulness (top), SWS (middle), and REM sleep (bottom) during the 24 hr period after stimulation (30 ms pulses at 20 Hz for 15 s, once per min) in the active period (21:00 to 22:00) in ChAT (A) and WT (B) mice. Arrows indicate delivery of stimulation. Each symbol represents the mean time per hour in each stage. n = 5 mice. *p < 0.05, two-tailed Student's t test between groups (before and after stimulation).

(C and D) Total time spent in wakefulness, SWS, and REM sleep in each day in ChAT (C) and WT (D) mice. Photostimulation was delivered on day 0 for 1 hr. n = 5 mice. *p < 0.05, two-tailed Student's t test between baseline and stimulation.

Data are shown as mean ± SEM.

Acknowledgments

We thank I.C. Bruce for discussions and reading the manuscript. This work was supported by grants from the Major State Basic Research Program of China (2011CB504400), the National Key Technology R&D Program of the Ministry of Science and Technology of China (2012BAI01B08), the National Natural Science Foundation of China (91232000, 81371458, 30870834, 31190060, 81221003, and 30730037), the Program of Introducing Talents of Discipline to Universities, and the Zhejiang Provincial Natural Science Foundation of China (Y2110057).

Received: November 4, 2013
Revised: January 11, 2014
Accepted: February 4, 2014
Published: March 6, 2014

References

1. Berntson, G.G., Shafi, R., and Sarter, M. (2002). Specific contributions of the basal forebrain corticopetal cholinergic system to electroencephalographic activity and sleep/waking behaviour. Eur. J. Neurosci. *16*, 2453–2461.
2. Brown, R.E., Basheer, R., McKenna, J.T., Strecker, R.E., and McCarley, R.W. (2012). Control of sleep and wakefulness. Physiol. Rev. *92*, 1087–1187.
3. Cape, E.G., Manns, I.D., Alonso, A., Beaudet, A., and Jones, B.E. (2000). Neurotensin-induced bursting of cholinergic basal forebrain neurons promotes gamma and theta cortical activity together with waking and paradoxical sleep. J. Neurosci. *20*, 8452–8461.
4. Nishino, S., Tafti, M., Reid, M.S., Shelton, J., Siegel, J.M., Dement, W.C., and Mignot, E. (1995). Muscle atonia is triggered by cholinergic stimulation of the basal forebrain: implication for the pathophysiology of canine narcolepsy. J. Neurosci. *15*, 4806–4814.
5. Lee, M.G., Hassani, O.K., Alonso, A., and Jones, B.E. (2005). Cholinergic basal forebrain neurons burst with theta during waking and paradoxical sleep. J. Neurosci. *25*, 4365–4369.
6. Zant, J.C., Rozov, S., Wigren, H.K., Panula, P., and Porkka-Heiskanen, T. (2012). Histamine release in the basal forebrain mediates cortical activation through cholinergic neurons. J. Neurosci. *32*, 13244–13254.
7. Jones, B.E. (2008). Modulation of cortical activation and behavioral arousal by cholinergic and orexinergic systems. Ann. N Y Acad. Sci. *1129*, 26–34.

8. Baghdoyan, H.A., Spotts, J.L., and Snyder, S.G. (1993). Simultaneous pontine and basal forebrain microinjections of carbachol suppress REM sleep. J. Neurosci. *13*, 229–242.
9. Kaur, S., Junek, A., Black, M.A., and Semba, K. (2008). Effects of ibotenate and 192IgG-saporin lesions of the nucleus basalis magnocellularis/substantia innominata on spontaneous sleep and wake states and on recovery sleep after sleep deprivation in rats. J. Neurosci. *28*, 491–504.
10. Fuller, P.M., Sherman, D., Pedersen, N.P., Saper, C.B., and Lu, J. (2011). Reassessment of the structural basis of the ascending arousal system. J. Comp. Neurol. *519*, 933–956.
11. Gritti, I., Henny, P., Galloni, F., Mainville, L., Mariotti, M., and Jones, B.E. (2006). Stereological estimates of the basal forebrain cell population in the rat, including neurons containing choline acetyltransferase, glutamic acid decarboxylase or phosphate-activated glutaminase and colocalizing vesicular glutamate transporters. Neuroscience *143*, 1051–1064.
12. Adamantidis, A.R., Zhang, F., Aravanis, A.M., Deisseroth, K., and de Lecea, L. (2007). Neural substrates of awakening probed with optogenetic control of hypocretin neurons. Nature *450*, 420–424.
13. Carter, M.E., Yizhar, O., Chikahisa, S., Nguyen, H., Adamantidis, A., Nishino, S., Deisseroth, K., and de Lecea, L. (2010). Tuning arousal with optogenetic modulation of locus coeruleus neurons. Nat. Neurosci. *13*, 1526–1533.
14. Carter, M.E., Adamantidis, A., Ohtsu, H., Deisseroth, K., and de Lecea, L. (2009). Sleep homeostasis modulates hypocretin-mediated sleep-to-wake transitions. J. Neurosci. *29*, 10939–10949.
15. Losier, B.J., and Semba, K. (1993). Dual projections of single cholinergic and aminergic brainstem neurons to the thalamus and basal forebrain in the rat. Brain Res. *604*, 41–52.
16. Vetrivelan, R., Chang, C., and Lu, J. (2011). Muscle tone regulation during REM sleep: neural circuitry and clinical significance. Arch. Ital. Biol. *149*, 348–366.
17. Lu, J., Sherman, D., Devor, M., and Saper, C.B. (2006). A putative flip-flop switch for control of REM sleep. Nature *441*, 589–594.
18. Lazarus, M., Chen, J.F., Urade, Y., and Huang, Z.L. (2013). Role of the basal ganglia in the control of sleep and wakefulness. Curr. Opin. Neurobiol. *23*, 780–785.
19. McGinty, D.J., and Harper, R.M. (1976). Dorsal raphe neurons: depression of firing during sleep in cats. Brain Res. *101*, 569–575.
20. Parmentier, R., Ohtsu, H., Djebbara-Hannas, Z., Valatx, J.L., Watanabe, T., and Lin, J.S. (2002). Anatomical, physiological, and pharmacological characteristics of histidine decarboxylase knock-out mice: evidence for the role of brain histamine in behavioral and sleep-wake control. J. Neurosci. *22*, 7695–7711.
21. Steriade, M. (2004). Acetylcholine systems and rhythmic activities during the waking—sleep cycle. Prog. Brain Res. *145*, 179–196.
22. Boucetta, S., and Jones, B.E. (2009). Activity profiles of cholinergic and intermingled GABAergic and putative glutamatergic neurons in the pontomesencephalic tegmentum of urethane-anesthetized rats. J. Neurosci. *29*, 4664–4674.
23. Hassani, O.K., Lee, M.G., Henny, P., and Jones, B.E. (2009). Discharge profiles of identified GABAergic in comparison to cholinergic and putative glutamatergic basal forebrain neurons across the sleep-wake cycle. J. Neurosci. *29*, 11828–11840.
24. Borbély, A.A. (1982). A two process model of sleep regulation. Hum. Neurobiol. *1*, 195–204.
25. Riemann, D., Spiegelhalder, K., Feige, B., Voderholzer, U., Berger, M., Perlis, M., and Nissen, C. (2010). The hyperarousal model of insomnia: a review of the concept and its evidence. Sleep Med. Rev. *14*, 19–31.
26. Bonnet, M.H., and Arand, D.L. (2010). Hyperarousal and insomnia: state of the science. Sleep Med. Rev. *14*, 9–15.
27. Bakotić, M., and Radošević-Vidaček, B. (2012). Regulation of sleepiness: the role of the arousal system. Arh. Hig. Rada Toksikol. *63* (*Suppl 1*), 23–34.
28. Suzuki, A., Sinton, C.M., Greene, R.W., and Yanagisawa, M. (2013). Behavioral and biochemical dissociation of arousal and homeostatic sleep need influenced by prior wakeful experience in mice. Proc. Natl. Acad. Sci. USA *110*, 10288–10293.