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Original Article

ORIGINAL ARTICLE

Cyclic AMP response element-binding protein is required in excitatory neurons in the forebrain to sustain wakefulness

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Abstract

The molecular and intracellular signaling processes that control sleep and wake states remain largely unknown. A consistent observation is that the cyclic adenosine monophosphate (AMP) response element-binding protein (CREB), an activity-dependent transcription factor, is differentially activated during sleep and wakefulness. CREB is phosphorylated by the cyclic AMP/protein kinase A (cAMP/PKA) signaling pathway as well as other kinases, and phosphorylated CREB promotes the transcription of target genes. Genetic studies in flies and mice suggest that CREB signaling influences sleep/wake states by promoting and stabilizing wakefulness. However, it remains unclear where in the brain CREB is required to drive wakefulness. In rats, CREB phosphorylation increases in the cerebral cortex during wakefulness and decreases during sleep, but it is not known if this change is functionally relevant to the maintenance of wakefulness. Here, we used the Cre/lox system to conditionally delete CREB in the forebrain (FB) and in the locus coeruleus (LC), two regions known to be important for the production of arousal and wakefulness. We used polysomnography to measure sleep/wake levels and sleep architecture in conditional CREB mutant mice and control littermates. We found that FB-specific deletion of CREB decreased wakefulness and increased non-rapid eye movement sleep. Mice lacking CREB in the FB were unable to sustain normal periods of wakefulness. On the other hand, deletion of CREB from LC neurons did not change sleep/wake levels or sleep/wake architecture. Taken together, these results suggest that CREB is required in neurons within the FB but not in the LC to promote and stabilize wakefulness.

Statement of Significance

Using conditional cyclic AMP response element-binding protein (CREB) knockout mice, we demonstrate that CREB is required in forebrain neurons but not noradrenergic locus coeruleus cells to drive and sustain wakefulness.

Key words: sleep; locus coeruleus; sleep deprivation; slow wave activity; CREM

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Introduction

Mapping of the neural circuits that control wakefulness and sleep began over 60 years ago, with many studies delineating specific neural systems controlling sleep/wake cycles in the past two decades [1]. Electrophysiological and behavioral studies have led to an improved understanding of the neurotransmitter systems, pathways, and cell firing patterns that regulate these states. Wake-promoting regions such as the locus coeruleus (LC) [2] send projections to the cerebral cortex, and activity in these regions produces electroencephalogram (EEG) desynchronization consistent with arousal and wakefulness [3]. This arousal-producing system is balanced by sleep-promoting areas that induce sleep and suppress wakefulness [4]. These two mutually inhibiting systems act in concert with circadian and homeostatic processes to properly regulate sleep/wake states [5]. The combination of lesion studies and more recently optogenetic and chemogenetics approaches has allowed the functional delineation of key sleep and wake regulatory circuits in the brain. However, much less is known about the intracellular processes underlying sleep/wake regulation [6]. Genetic manipulations and immunohistochemical measurements have identified the cyclic AMP response element-binding protein (CREB) signaling pathway and CREB-regulated transcription as potential regulators of sleep/wake states. The CREB signaling pathway has been extensively characterized in the context of synaptic plasticity and memory formation [7–13]. Interestingly, some studies also suggest that CREB signaling is also essential for the proper regulation of sleep/wake states. In flies, CREB activity is inversely related to rest [14]. Mice lacking two isoforms of CREB show reduced wakefulness and increased sleep [15]. However, it is unclear where in the brain CREB plays a critical role to drive wakefulness. CREB activity and expression of CREB downstream target genes are increased in the cortex following periods of wakefulness [16]. These changes in the cortex require inputs from the LC [17], and mice lacking dopamine beta-hydroxylase (DBH), the enzyme required for norepinephrine synthesis, show reduced wakefulness and wake fragmentation [18]. Here, we examine the role of CREB in the cortex and in the LC in promoting and stabilizing wakefulness using conditional knockout (cKO) approaches and polysomnography.

Methods

Animals

Forebrain CREB cKO (FB CREB cKO) mice were produced by crossing animals expressing Cre recombinase driven by the calcium/calmodulin-dependent protein kinase II (CAMKII) promoter [19] to mice carrying a floxed allele of *Creb1* [20]. Males and females were used for both FB CREB cKO and control groups. Mice lacking CREB in noradrenergic neurons were produced by crossing mice expressing Cre recombinase driven by the DBH promoter [21] to mice carrying a floxed allele of Creb1AZ [20]. Experimental CREB cKO animals were male and female mice homozygous for the floxed *Creb1* allele and hemizygous for Cre recombinase. Control mice were either hemizygous for Cre recombinase or carried floxed alleles of *Creb1*. Both males and females were used for both conditional knockout and control groups. Animals were maintained on a 12 hour light/12 hour dark cycle with lights on (Zeitgeber time [ZT] 0) at 07:00 am. Food and water were available ad libitum. All animal care and experiments were approved by the Institutional Animal Care and Use Committee of the University of Pennsylvania and conducted in accordance with the National Institute of Health guidelines.

Surgery

Animals were implanted with EEG and electromyogram (EMG) electrodes under isoflurane anesthesia. Electrodes were held in place with dental cement (Ketac, 3M, St Paul, MN). Electrodes consisted of Teflon-coated wires (Cooner wires, Chatsworth, CA) soldered to gold socket contacts (Plastics One, Roanoke, VA) and pushed into a six-pin plastic plug (363 plug, Plastics One). The contacts were cemented to the plug using dental cement. Animals were connected to amplifiers using light-weight cables (363, Plastics One) attached to a rotating commutator (SLC6, Plastics One). All recordings were obtained using a parietal electrode (medial lateral ±1.5 mm, anterior/posterior –4 mm from bregma) referenced to an electrode over the cerebellum (1.5 mm posterior of lambda). Mice were allowed to recover from surgery for a minimum of 2 weeks. During the second week of recovery, mice were acclimated to the cables and to the recording chambers.

EEG recordings and analysis

EEG/EMG signals were recorded over a 24 hour baseline period, during which animals were undisturbed. The next day, animals were sleep deprived (SD) for 6 hours, starting at lights on (ZT 0) using gentle handling [22] and allowed to recover for 18 hours. EEG/EMG signals were sampled at 256 Hertz (Hz) and filtered at 0.5–30 Hz and 1–100 Hz, respectively, with 12A5 amplifiers (Astro-Med, West Warwick, RI). Data acquisition and visual scoring were performed using SleepSign software (Kissei Comtec, INC, Japan). EEG/EMG recordings were scored in 4-second epochs as wake, non-rapid eye movement (NREM), or rapid eye movement (REM) by a trained experimenter blind to genotype. Epochs containing movement artifacts were included in the state totals and architecture analysis but excluded from subsequent spectral analysis. Spectral analysis was performed using a fast Fourier transform (FFT; 0.5–20 Hz, 0.125 Hz resolution). EEG spectra were computed over 24 hours. A few of the recordings had too many movement artifacts to process for FFT over 24 hours. These recordings were excluded from the 24-hour FFT analysis. NREM EEG spectra were computed in 2 hour windows for the baseline and 1 hour windows to the recovery period following SD. NREM slow-wave activity (SWA) was normalized to the last 4 hours of the light phase of the baseline day for each animal as previously described [23].

Immunohistochemistry

Mice were anesthetized with isoflurane and transcardially perfused with ice-cold phosphate buffered saline (PBS), followed by ice-cold 4.0% paraformaldehyde using a peristaltic perfusion pump (Rainin Instruments, Oakland, CA). Fixed brains were dissected, postfixed overnight, and cryoprotected in 30% sucrose. Brains were frozen and cryosectioned coronally at a thickness of 30 um into PBS. Floating sections were blocked for 1 hour at room temperature in 2% normal donkey serum (NDS, Vector

Laboratories, Burlingame, CA) in 0.3% Triton X-100 PBS. Sections were first processed for CREB immunostaining with nickel ammonium sulfate intensification of 3,3'-diaminobenzidine and then processed for tyrosine hydroxylase (TH). Sections were incubated overnight at 4°C in 2% NDS, 0.1% Triton X-100 in PBS with anti-CREB primary antibody (1:3,000; 9197 Cell Signaling, Danvers, MA), 2 hours at room temperature with biotinylated donkey anti-Rabbit secondary antibody in 0.1% Triton X-100 PBS (1: 500; Jackson ImmunoResearch, Newmarket Suffolk, England), and 1.5 hours at room temperature with ABC in 0.1% Triton X-100 (1:500; Vector Laboratories, Burlingame, CA). Sections were washed 3 × 10 minutes with PBS between each incubation. The same procedure was followed for TH immunostaining using anti-TH antibody (1:10,0000, Millipore, Billerica, MA). For fluorescent immunohistochemistry, fluorescein isothiocyanate-conjugated anti-rabbit IgG secondary antibodies (1:1,000, Chemicon, Temecula, CA) were used. Sections were mounted on slides and imaged using a microscope or confocal microscope. Cell counting and optical density measurements were conducted using ImageJ. Six sections (three from each side) from three to four animals were used for all quantifications of CREB levels.

Quantitative real-time PCR

Cortical samples were collected into 500 uL of RNAlater (Ambion, Austin, TX) at ZT 16 and flash frozen. All collections were done under red light to avoid any potential light pulse effects. The list of transcripts examined was based on previous reports showing that CREB is required for the expression of these genes [24]. Preparation of messenger ribonucleic acid and complementary deoxyribonucleic acid(cDNA) synthesis were conducted as previously described [25]. PCR reactions were prepared in 96-well optical reaction plates (Applied Biosystems, Foster City, CA). Each well contained 10 uL of cDNA and 1uL of Taqman probes and 9 uL of TaqMan Fast Universal PCR Master Mix (Applied Biosystems, Foster City, CA). Six biological and three technical replicates were used. Data were normalized to *Hprt1* (probe ID: Mm0145399-m1), *Tuba4a* (Mm00849767-S1), and *Gapdh* (Mm99999915_g1). Fold change was calculated from the delta cycle threshold (ΔCt) cycle threshold values with corrections for standard curve data from each gene and housekeeping gene expression levels for each sample. For each sample, ΔCt was calculated against the mean for the sample set of that gene. Next, each of these values was corrected with the slope of the standard curve for the relevant probe to account for efficiency. This corrected value was normalized to the ΔCt from housekeeping genes for each sample to account for input variability. Fold change is equal to two raised to the difference between experimental and control Ct values. Student's *t*-tests were used to compare groups.

Statistics

Student's *t*-tests were used to compare wake, NREM, and REM sleep levels averaged over 24 hours. Multivariate analysis of variance (MANOVA) was used on the proportion of time spent in each state during the light phase, the dark phase, and the 8 × 3-hour time points across 24 hours, followed by Tukey studentized range tests to compare genotype groups. The same procedure was applied to the number of bouts and the average bout duration to compare groups. MANOVA was also used to compare the difference in total sleep time, NREM, and REM sleep between the baseline and recovery periods following 6 hours of SD. Tukey studentized range tests were used to analyze raw EEG spectra for wake, NREM, and REM sleep. Student's *t*-tests were used to analyze theta peak frequency for EEG spectra. NREM SWA during the 4 × 2-hour bins was analyzed using repeated-measures ANOVA followed by Tukey studentized range tests.

Results

CREB is required in excitatory neurons in the FB to drive and sustain wakefulness

FB CREB cKO mice were generated by crossing a Cre-expressing line driven by the CaMKII promoter [19] to animals carrying a floxed allele of *Creb1* [20] (Figure 1, A). The expression of *Creb1* and CREB target genes *Nr4a1* and *Gadd45b* was markedly reduced in the cortex of FB CREB cKO animals (Table 1). In contrast, the expression of cAMP response element modulator (CREM), another member of the same family of transcription factors, was increased in conditional CREB mutants (Table 1), which is consistent with previous manipulations decreasing *Creb1* expression [26]. CREB protein expression was substantially decreased in the cortex of FB CREB cKO (Figure 1, B–F; $p = .005$). We used EEG/EMG recordings to measure wake, NREM, and REM sleep in conditional CREB mutant animals and control littermates. During the 24 hours baseline period, animals were undisturbed and recording began at lights on (ZT 0). FB CREB cKO animals showed decreased wakefulness over 24 hours (Figure 2, A; $t(26) = 4.15$, $p = .0003$) and during light and dark cycle (Figure 2, A; $F(2,25) = 13.98$, $p < .0001$).

These changes in wakefulness were most pronounced during the early period of the dark phase (Figure 2, B; $F(8,19) = 4.04$, $p = .0059$; Tukey post hoc tests). Decreased wakefulness was accompanied by increased NREM sleep in FB CREB cKO compared with control over 24 hours ($t(26) = 4.24$, $p = .0003$) and during the light/dark phase (Figure 2, C; $F(2,25) = 12.81$, $p = .0001$). Analysis over 3-hour bins across light/dark revealed a large increase during the early part of the dark phase in particular (Figure 2, D; $F(8,19) = 4.22$, $p = .0047$). REM sleep levels were unchanged over 24 hours ($t(26) = 0.24$, $p = .8116$) or during the light or dark phases (Figure 2, E; $F(2,25) = 1.39$, $p = .2701$). MANOVA across 3-hour bins revealed more subtle changes in REM sleep during the dark phase (Figure 2, F; $F(8,19) = 3.45$, $p = .0128$).

CREB is required in the FB to sustain wakefulness

FB cKO mice showed an increased number of wake bouts during the light and dark phase (Figure 3, A; $F(2,24) = 5.38$, $p = .0017$). In addition, the average duration of wake bouts was reduced in FB CREB cKO mice (Figure 3, B; $F(2,24 = 18.85$, $p < .0001$). The number of NREM bouts also increased in FB CREB cKO (Figure 3, C; $F(2,24) = 8.55$, $p = .0016$). However, the average duration of NREM bouts was unchanged by CREB deletion in the FB (Figure 3, D; $F(2,24) = 2.17$, $p = .1364$). The number of REM bouts was

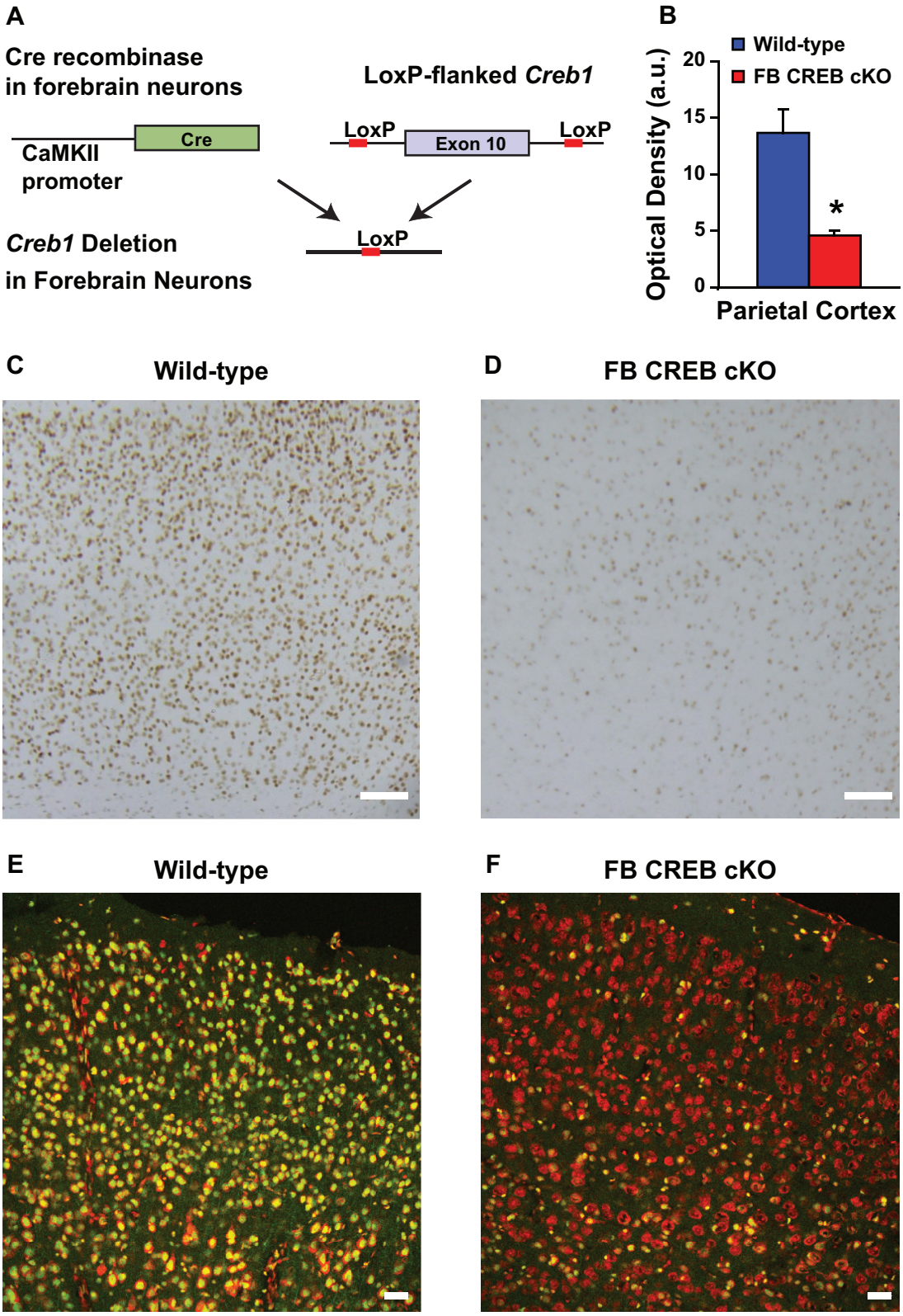

Figure 1. FB CREB cKO animals show reduced CREB expression in the cortex. (A) FB CREB cKO animals were generated using the Cre/lox system. (B) CREB protein levels were quantified using optical density in the parietal cortex of FB CREB cKO and wild-type littermates. (C and D) Representative immunohistochemical stains using an anti-CREB antibody (brown nuclei) of the cortex for wild-type (C) and FB CREB cKO (D) mice. (E and F) Sections from wild-type (E) and FB CREB cKO (F) cortex tissue were imaged using fluorescent microscopy for CREB (green) and propidium iodide (red). The coordinates for all images are as follows from the bregma: anterior/posterior (AP): −1.5; dorsal/ventral (DV): −0.5; medial/lateral (ML): 1. *$p < .05$.

Table 1. FB CREB cKO mice show reduced expression of CREB target genes in the cortex during the active phase

Gene	Fold change	P-value	Probe ID
Dec 1	1.07 ± 0.05	.283	Mm00478593-m1
Dec 2	1.07 ± 0.07	.387	Mm00470512-m1
Arc	0.84 ± 0.17	.579	Mm01204954_g1
BdnfIV	0.89 ± 0.08	.339	Mm00432069_m1
Creb1	**0.34 ± 0.01**	**<.0001**	**Mm01254160-m1**
Crem	**3.89 ± 0.27**	**<.0001**	**Mm01230944-g1**
Egr1	1.1 ± 0.20	.691	Mm00656724_m1
Fos	0.97 ± 0.13	.907	Mm00487425_m1
Gadd45b	**0.76 ± 0.04**	**.048**	**Mm00435123-m1**
Nr4a1	0.84 ± 0.16	.411	Mm00439358_ml
Nr4a2	**0.4 ± 0.09**	**.005**	**Mm00443056_ml**
Nr4a3	0.78 ± 0.10	.129	Mm00450074_ml

Tissue from the cortex was collected at ZT 16 from FB CREB cKO and control mice ($n = 6$). Gene expression was measured using quantitative polymerase chain reaction. Transcripts changed by deletion of CREB are shown in bold. Mean ± s.e.m.

unchanged by CREB deletion (**Figure 3, E**; $F(2,24) = 1.29$, $p = .2928$), and the average duration of REM bouts was decreased in CREB cKO mice only during the light phase (**Figure 3, F**; $F(2,24) = 5.41$, $p = .0115$, Tukey post hoc test). Taken together, these results suggest that CREB is required in the FB to sustain wakefulness.

Deletion of CREB in the FB reduces sleep homeostasis

Following the baseline day, animals were sleep deprived for 6 hours using gentle handling [15, 22, 27] starting at lights on, and mice were allowed to recover for the subsequent 18 hours. FB CREB cKO animals showed lower NREM sleep rebound when released from 6 hours of sleep deprivation compared with wild-type littermates (Figure 4, A; genotype: $F(1,13) = 6.02$, $p = .0290$; time: $F(7,13) = 16.94$, $p < .0001$; time by genotype: $F(7,13) = 2.81$, $p = .0907$). In contrast, REM sleep rebound was not altered by deletion of CREB from the FB neurons (Figure 4, B; genotype: $F(1,10) = 0.05$, $p = .8296$; time: $F(7,13) = 9.14$, $p < .0001$; genotype by time interaction: $F(7,13) = 0.02$, $p = .9743$). SWA, the power in the delta (0.5–4 Hz) frequency range during NREM sleep, is a marker of sleep pressure and sleep intensity [23, 28, 29]. SWA was normalized to the last 4 hours of the light phase during the baseline period for each animal [23, 30, 31]. During the baseline period, SWA was highest at lights on and decreased over the course of the light phase (Figure 4, C; time: $F(3,14) = 31.04$, $p < .0001$). FB CREB cKO showed reduced SWA during the baseline day (genotype: $F(1,14) = 16.33$, $p = .0012$; time by genotype interaction: $F(3,14) = 2.62$, $p = .0635$). Following sleep deprivation, SWA was highest when the animals were allowed to sleep (ZT 6) and decreased during recovery sleep (Figure 4, D; time: $F(3,14) = 101.76$, $p < .0001$). SWA was lower for FB CREB cKO (genotype: $F(1,14) = 22.97$, $p = .0003$; time by genotype interaction: $F(3,14) = 8.82$, $p = .0001$). These results indicate that CREB deletion reduces sleep rebound following sleep deprivation and prevents the accumulation of SWA, a marker of sleep pressure.

Deletion of CREB from the FB neurons does not change EEG spectral content

We examined EEG spectral content using FFT of wake, NREM, and REM sleep (Figure 5). In particular, we quantified power in the theta (6–10 Hz) range during wakefulness, a marker of arousal, as well as power in the delta range during NREM sleep, an indicator of sleep pressure. We found no difference in absolute theta power during wake or REM sleep (Table 2). Delta power during NREM was also similar comparing conditional CREB KO animals to controls.

A separate cohort of animals was used to examine the circadian activity patterns in FB CREB cKO ($n = 6$) and control ($n = 8$) animals. Mice showed rhythmic activity patterns under 12 hour light/12 hour dark conditions and under constant darkness. Deletion of CREB did not affect the period (control Tau = 23.54 ± 0.04; cKO Tau = 23.64 ± 0.08; $p = .202$).

Expression of Cre recombinase in the FB neurons produces subtle changes in sleep/wake

The control animals in the aforementioned studies did not express Cre recombinase. To address this potential caveat, we directly examined the potential impact of Cre recombinase expression alone on sleep/wake patterns. A separate cohort of animals was run through polysomnography to examine the potential impact of Cre recombinase expression on sleep/wake. Cre recombinase expression produced a subtle decrease in wakefulness and an increase in NREM sleep that was only evident when the data are analyzed over 24 hours (Supplemental Figure 1). The expression of Cre recombinase did not impact sleep/wake architecture (Supplemental Figure 2) or EEG spectral content (Supplemental Figure 3; Table 3).

CREB is not required in the LC to sustain wakefulness

We used the DBH promoter [21] to confer the deletion of CREB to noradrenergic neurons (LC CREB cKO, Figure 6, A). LC CREB cKO mice showed reduced expression of CREB in neurons that were positive for TH in the LC (Figure 6, B–D; 86% reduction, $p = 1.19 \times 10^{-5}$). The amount of wakefulness, NREM sleep, and REM sleep was not affected by deletion of CREB in noradrenergic neurons (Supplemental Figure 4). Sleep architecture (Supplemental Figure 5) or EEG spectral power (Supplemental Figure 6; Table 4) was not altered by CREB deletion in LC neurons. Lastly, sleep homeostasis was intact in LC conditional CREB KO animals (Supplemental Figure 7). Taken together, these results demonstrate that deletion of CREB from noradrenergic cells of the LC did not alter sleep/wake states.

Discussion

The goal of this study was to begin to functionally identify brain regions, where CREB is important for the induction and maintenance of wakefulness. Activity in noradrenergic neurons of the LC can desynchronize cortical EEG and produce arousal [2, 3, 32, 33]. Wake-promoting regions share the cerebral cortex as a common output, and cortical activity is ultimately predictive of behavioral state [34, 35]. We used cKO approaches to separately test the role of CREB in these two key components of the wake-promoting neural circuitry. Our results demonstrate that CREB is required in excitatory neurons in the FB but not in the LC to sustain wakefulness. The lack of phenotype in LC conditional CREB knockouts is consistent

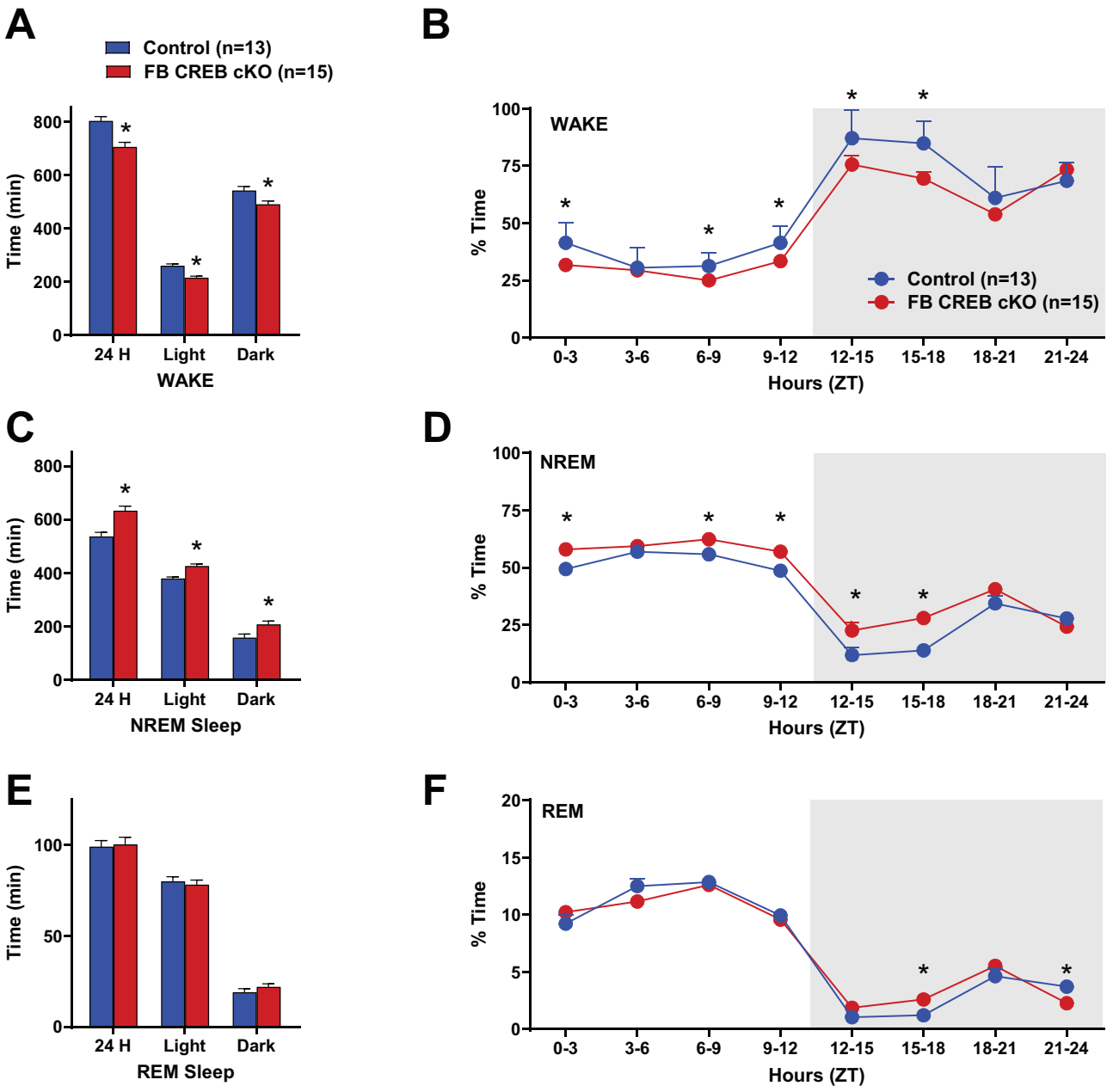

Figure 2. Deletion of CREB in the FB neurons reduces wakefulness. (A) Total time spent (minutes) in wakefulness over 24 hours and during the light and dark phases separately. (B) The proportion of time in wake in 3-hour bins over a single 24 hour light/dark period. (C) Total time in NREM sleep. (D) Percentage of time in NREM sleep in FB CREB cKO and control mice. (E) Total time spent in REM sleep over 24 hours and during each circadian period. (F) The proportion of time in REM sleep displayed in 3-hour epochs across the light/dark cycle. ZT is represented on the *x*-axis. Lights come on at ZT 0. Mean +/− s.e.m. *compares FB CREB cKO to controls ($p < .05$).

with previous research demonstrating that manipulations of the LC often lead to no changes in baseline sleep/wake states [36, 37]. On the other hand, the architecture of wakefulness was uniquely disrupted and fragmented by deletion of CREB in FB neurons. Interestingly, although the number of NREM bouts increased in FB CREB cKO animals, NREM sleep architecture was largely unaffected suggesting that reductions in wakefulness were replaced by bouts of NREM sleep in these animals. These experiments point to a role for CREB in sustaining wakefulness in the FB excitatory neurons. Several regions were impacted by the cKO in these studies, including the cortex and hippocampus. Further studies would be needed to further delineate which region(s) within the FB contribute the most to sustaining wake via CREB signaling.

We included a control experiment where Cre recombinase only was expressed in the FB neurons. Cre-expressing animals showed a slight decrease in wakefulness and a concomitant increase in NREM sleep. These findings indicate that part of the phenotype associated with the deletion of CREB in the FB neurons could be due to Cre recombinase expression alone. However, it is noteworthy that animals expressing Cre recombinase in FB neurons had normal sleep and wake architecture, suggesting that the inability to sustain wakefulness in FB CREB cKO animals can be solely attributed to CREB deletion in these

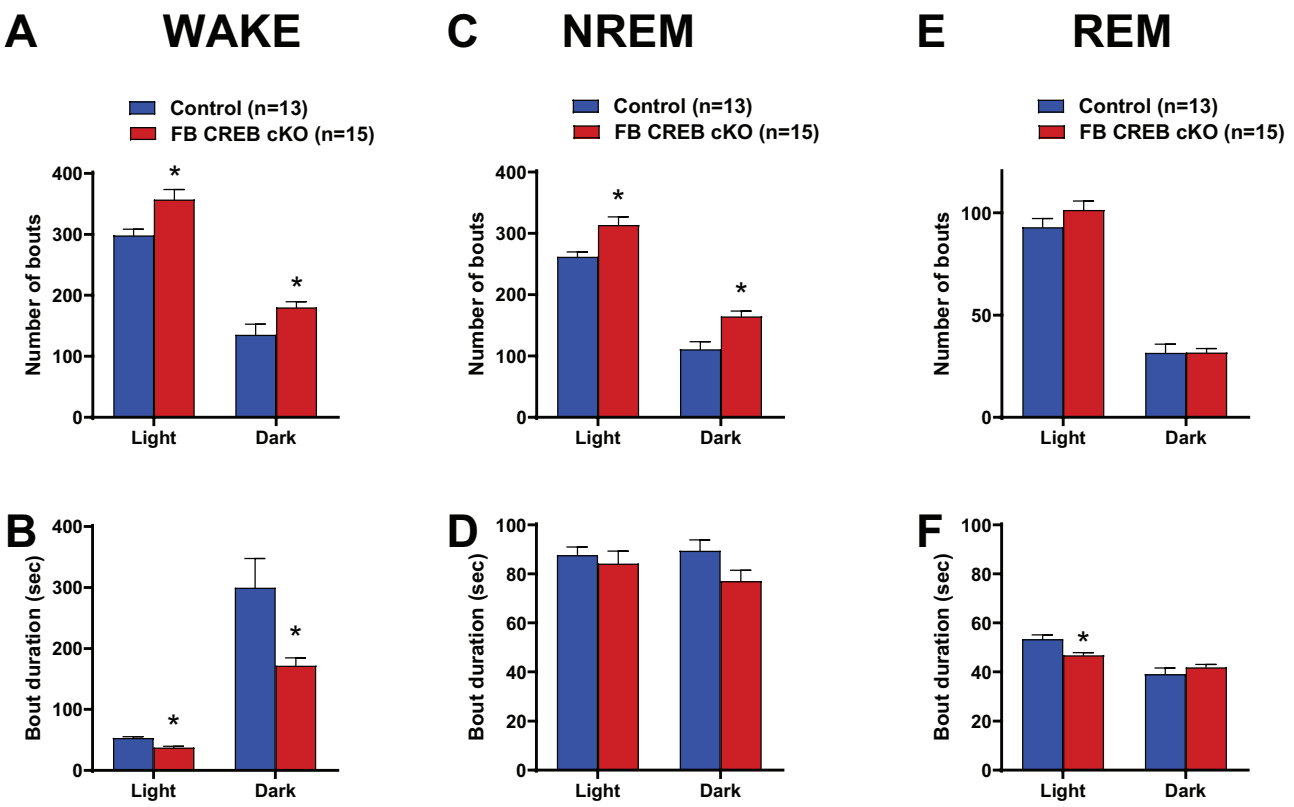

Figure 3. FB CREB cKO mice are unable to sustain wakefulness. The number of bouts and average bout duration for wake (A and B, respectively), NREM (C and D), and REM sleep (E and F) during the light and dark phase. Mean ± s.e.m.; *$p < .05$.

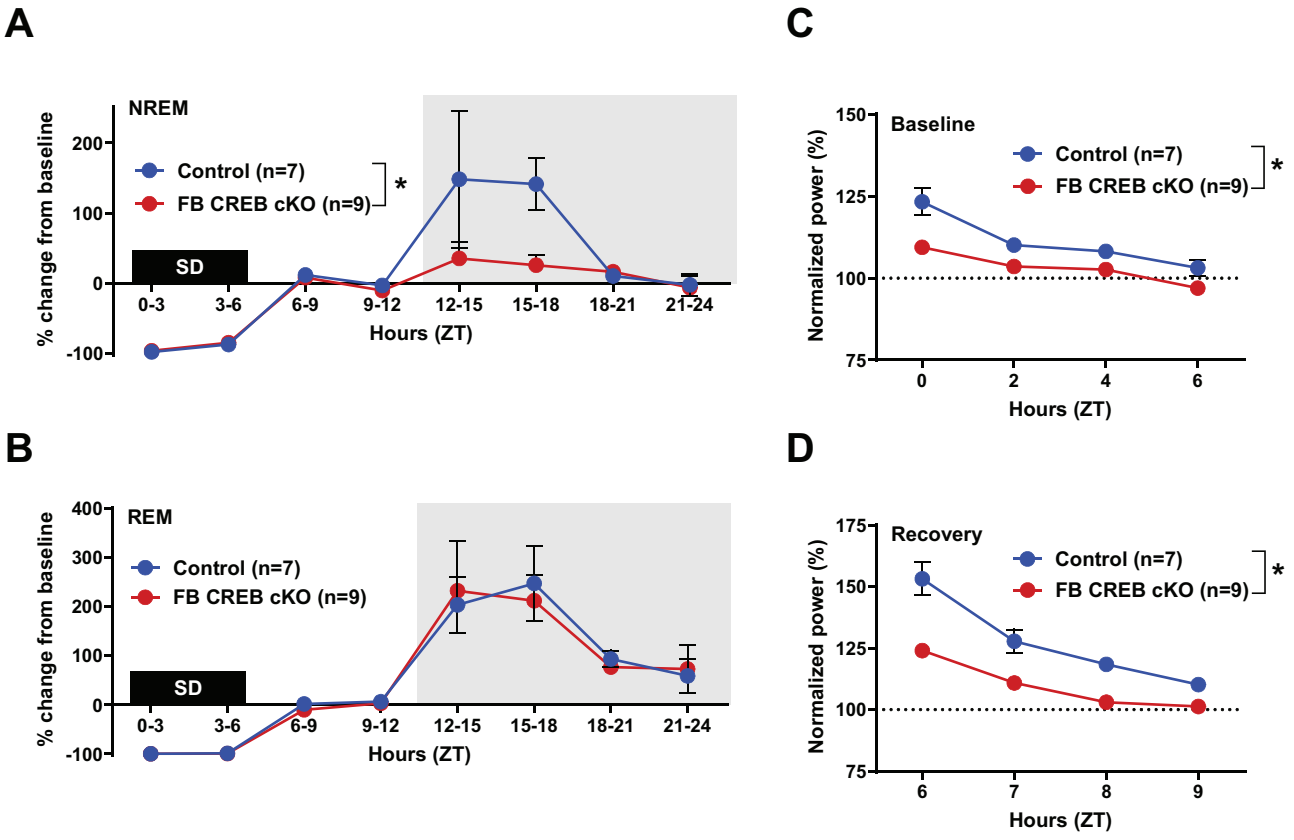

Figure 4. The FB deletion of CREB decreases sleep homeostasis. Animals were sleep deprived using gentle handling for 6 hours (ZT 0–6, labeled SD). Shown is the percent change in NREM (A) and REM sleep time (B) between the recovery and baseline periods. (C) SWA expressed as percent baseline during the baseline light period. (D) SWA during recovery following 6 hours SD. Mean ± s.e.m., *$p < .05$.

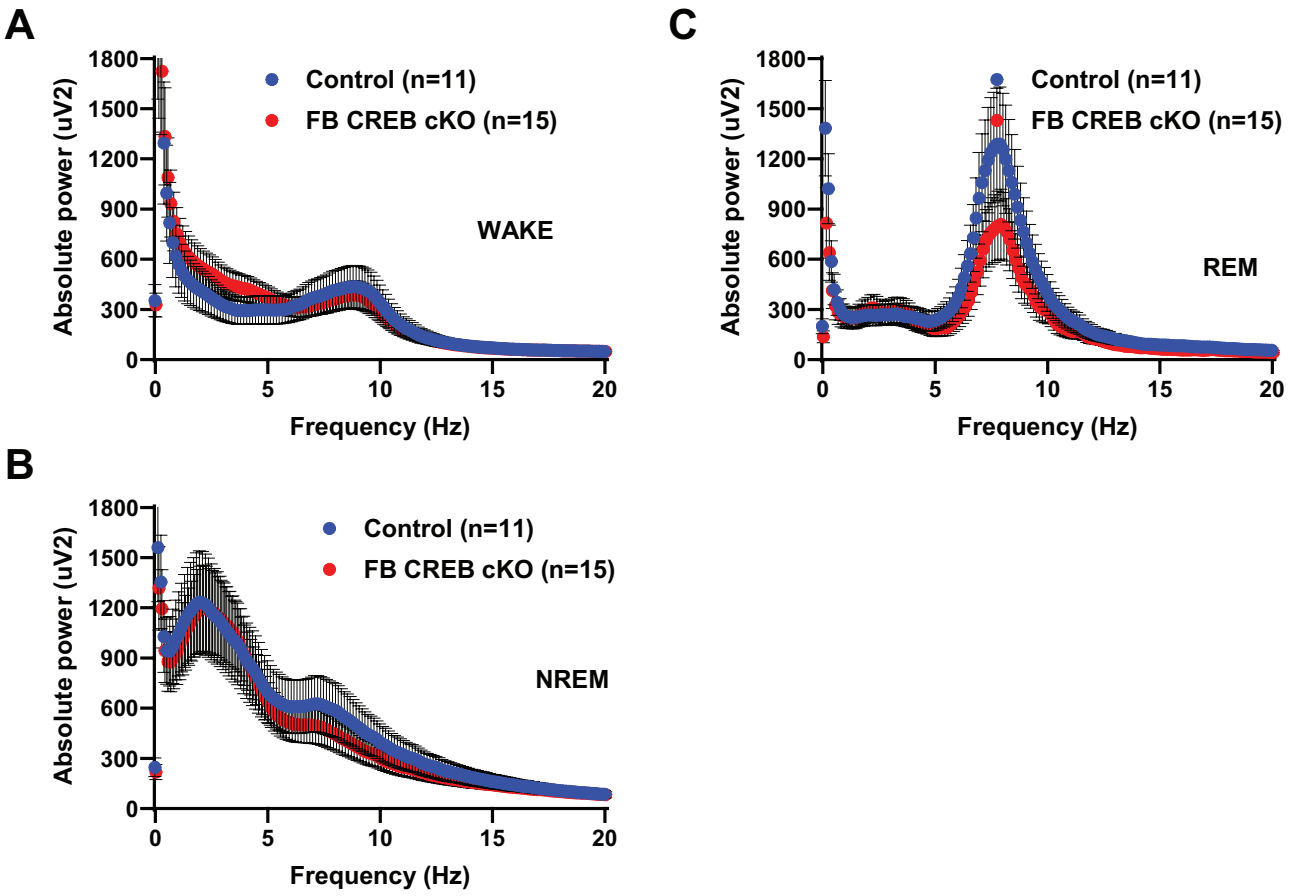

Figure 5. Deletion of CREB in the FB does not alter EEG spectral content. EEG spectra were computed using FFT over 24 hours. Absolute power is shown for wake (A), NREM (B), and REM (C) sleep. Mean ± s.e.m.

Table 2. CREB deletion in the FB does not alter EEG spectral power

	Control (n = 11)	FB CREB cKO (n = 15)	P-value
Theta (Wake)	384.47 ± 111.61	339.05 ± 68.61	.7183
Delta (NREM)	1083.25 ± 260.15	1059.57 ± 220.02	.9451
Theta (REM)	816.09 ± 219.28	613.30 ± 132.42	.4116

Table 3. EEG spectral power is not affected by the expression of Cre recombinase in the FB

	Control (n = 7)	FB Cre (n = 9)	P-value
Theta (Wake)	125.05 ± 21.79	120.02 ± 8.56	.8174
Delta (NREM)	398.7 ± 78.16	428.65 ± 41.95	.7249
Theta (REM)	215.59 ± 46.07	179.03 ± 13.98	.4123

neurons. The subtle but statistically significant change in sleep/wake levels in Cre recombinase-expressing animals underscores the need to thoroughly examine potential behavioral alterations elicited by the expression of the Cre recombinase enzyme in the brain for each Cre-expressing mouse line. We decided not to undertake the same experiments for the LC cKO animals because these mice showed no change in sleep/wake levels or architecture.

We used acute sleep deprivation to test the possibility that reduced and fragmented wakefulness resulted from increased sleep pressure in conditional CREB KO animals. Chronic heightened sleep pressure is characterized by enhanced sleep homeostatic responses as shown in chronic sleep restriction experiments [38] or in animal models with genetic manipulations resulting in increased sleep homeostasis [14, 39, 40]. FB CREB cKO mice had blunted NREM sleep rebound following 6 hours of sleep deprivation. SWA was reduced both during the baseline period and during recovery from acute sleep deprivation. It is possible that conditional CREB mutant mice did not reach the same levels of sleep pressure as control animals due to the increased opportunity to discharge SWA during the active period. Interestingly, the SWA levels reached by FB CREB cKO animals following 6 hours of enforced wakefulness are comparable to baseline SWA in control animals at lights on during the baseline day. These findings are consistent with the hypothesis that reduced SWA in CREB mutant mice stems from the wake fragmentation during the active period, which prevents the accumulation of SWA. Hence, our findings indicate that CREB is not directly involved in sleep homeostasis and that CREB does not serve a restorative function during sleep.

This phenotype of FB CREB cKO animals is similar to that of animals constitutively lacking two isoforms of CREB [15] and of animals lacking norepinephrine [18]. In the cortex, the increase in phosphorylated CREB (pCREB) that occurs during wakefulness requires LC inputs [17]. Increased cortical norepinephrine during wakefulness [33] could lead to increase in pCREB via beta-adrenergic Gs and cAMP/protein kinase A (PKA) activation

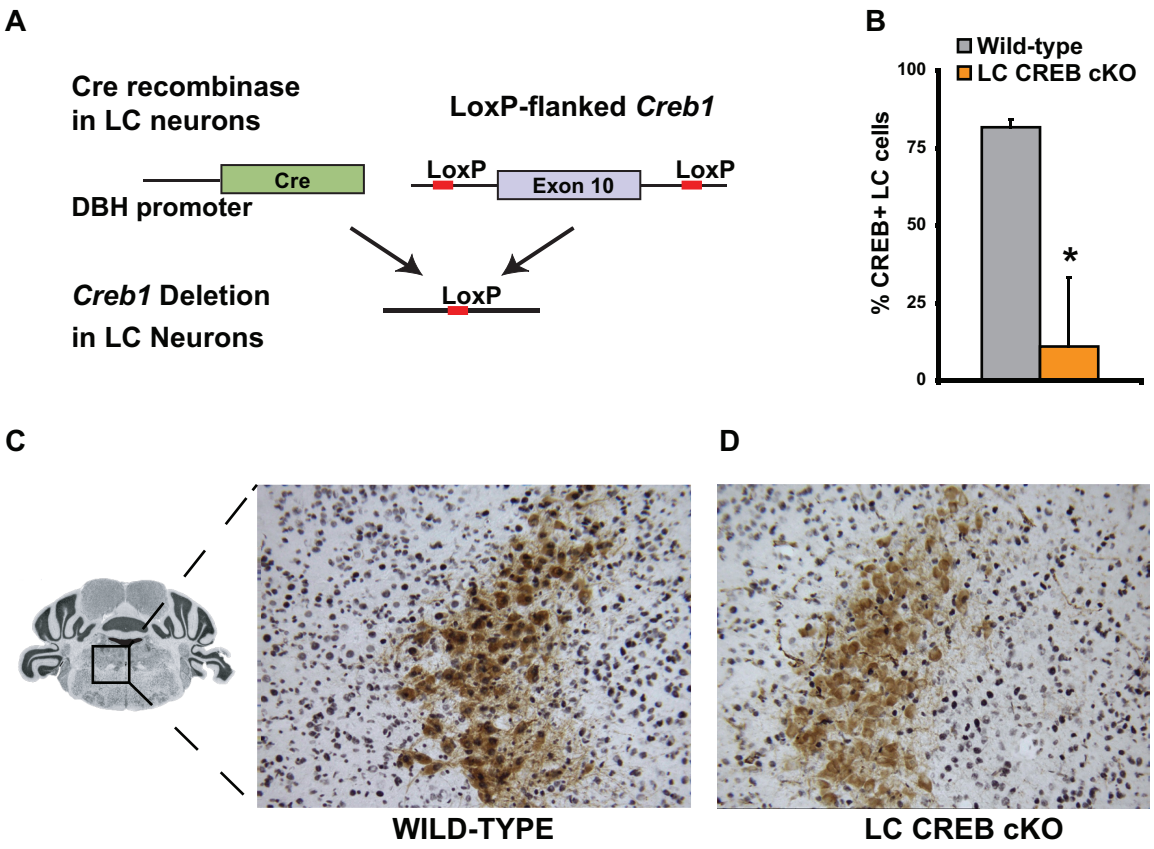

Figure 6. LC CREB cKO show reduced CREB expression in LC neurons. (A) The Cre/lox system was used to restrict the deletion of CREB to noradrenergic neurons. (B) The percentage of noradrenergic (TH positive) neurons also labeled for CREB is reduced in DBH CREB cKO animals. (C) and (D) Section representing where the images were taken (bregma –5.5 anterior/posterior [AP]). Representative photomicrograph of LC sections stained using an anti-TH antibody (brown cells) and anti-CREB antibody (blue nuclei) for wild-type (C) and mutant (D) mice. Mean ± s.e.m. $^{*}p < .05$.

Table 4. EEG spectral power is not affected by deletion of CREB from noradrenergic neurons

	Control (n = 6)	LC CREB cKO (n = 7)	P-value
Theta (Wake)	64.78 ± 5.43	53.72 ± 3.23	.4863
Delta (NREM)	216.30 ± 20.03	196.1 ± 19.25	.8147
Theta (REM)	148.00 ± 13.96	127.10 ± 8.23	.6052

and signaling. Another wake-promoting mechanism that converges onto the cortex is peptidergic hypocretin/orexin inputs from the hypothalamus [35, 41–44]. Activation of Gq-coupled orexin-2 receptors could also lead to increased pCREB via intracellular calcium release. These neurons also send broad projections to other cortical layers, and their activation, in turn, could result in broad cortical activity. Deletion of CREB in the cortex causes wake fragmentation, suggesting that the converging neural wake-promoting systems require CREB downstream signaling to produce wakefulness.

Activity-dependent CREB transcription is regulated by a large set of signaling pathways. One example of this is PKA, which has long been known to be a key part of CREB-mediated transcription involved in synaptic plasticity and memory formation [45, 46]. In a *Drosophila* model of Huntington's disease, animals showed increased wake and decreased sleep. This phenotype was at least partly dependent on increased PKA activity [47]. These findings are consistent with our current report and previous work in flies and mice supporting the idea that CREB is wake promoting. Another important regulator of CREB-mediated transcription is the protein kinase ERK (extracellular signal-regulated kinase). Deletion of *Erk1* and *Erk2* genes that encode ERK protein leads to increased wake and decreased sleep. ERK phosphorylation is thought to be critical for transducing waking experience and associated plasticity into sleep. Thus, the disruptions in sleep and wake caused by ERK manipulations have been interpreted as deficits in wake-induced plasticity, which ultimately impacts the quantity and quality of sleep [48, 49]. Taken together, these studies demonstrate that CREB signaling as well as several upstream regulators of CREB activity are involved in the regulation of sleep and wakefulness. Further studies are needed to establish the region specificity of these observations and the complex interactions of these molecules in the context of sleep/wake and related neural plasticity.

Consistent with previous reports, we found that the deletion of CREB in the FB was accompanied by compensatory increased expression of CREM [20, 21, 26, 50–52]. CREM is nearly undetectable in the brain under normal conditions, and this marked compensatory increase has been shown to partly restore CREB function [20]. We also found reduced expression of CREB target genes *Nr4a2* and *Gadd45b* in CREB cKO mice. Interestingly, the activity-dependent expression of these transcripts requires CREB activity in the hippocampus and striatum [24]. Further studies are needed to determine whether these changes in

gene expression are functionally relevant to the maintenance of wakefulness. This work suggests that the arousal system efferents to the FB promote wakefulness via the activation of CREB signaling. Overall, our combined results have emphasized the importance of CREB signaling within the FB neurons in promoting and sustaining wakefulness. Further studies are needed to refine where in the forebrain CREB is required for the maintenance of wakefulness and which effector genes mediate arousal in these cells.

Supplementary Material

Supplementary material is available at *SLEEP* online.

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