Genetic Evidence for a Role of CREB in Sustained Cortical Arousal

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INTRODUCTION

The cyclic-AMP response element binding protein (CREB) is an activity-dependent transcription factor that is activated by phosphorylation at serine 133 by the cyclic AMP/protein kinase A (cAMP/PKA) signaling pathway, the nerve growth factor signaling pathway, a Ca2+/calmodulin-dependent pathway, and a mitogen-activated protein (MAP) kinase regulated pathway (reviewed in Lonze and Ginty 2002). When phosphorylated, CREB promotes the transcription of a variety of target genes including zif268 and BDNF (Lonze and Ginty 2002; Mayr and Montminy 2001). Much work has focused on a role for CREB in memory storage and synaptic plasticity (Silva et al. 1998), and evidence suggests that CREB may play a role in sleep/wake regulation in mammals. We therefore examined the effects of low levels of CREB on the regulation of sleep/wake states in mammals. We utilized genetically modified mice that lack the α and Δ isoforms of the CREB protein (CREB α/Δ mice; Hummler et al. 1994). These mice have deletion of two of the three major isoforms of CREB, resulting in the marked reduction of CREB protein levels to 15% of control levels in all brain regions examined (Walters and Blendy 2001). The remaining isoform, CREBβ, is incapable of binding to a perfect CRE consensus site (Walters and Blendy 2001), thus making these mice a useful model for determining if the CREB protein is involved in the behavioral regulation of sleep/wake states. We not only studied total amounts of sleep and wake to compare with results in Drosophila, but also took advantage of the more detailed phenotyping of sleep available in mice. We examined the effects of this mutation on sleep state architecture, spectral content of the electroencephalogram in wakefulness and different sleep states, and the response to sleep deprivation to assess sleep homeostasis. Our results show that, as in Drosophila, CREB acts in mammals to promote the duration of the active state, in this case wakefulness. We further demonstrate that CREB directly affects the electrophysiological properties of arousal because CREB-deficient mice have reduced θ-power in both wakefulness and REM sleep.

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METHODS

Mouse breeding/genotyping

To control for effects of genetic background, CREB αΔ homozygous and wild-type mice were obtained from an F1 cross of C57BL/6J and 129/SvEvTac CREB αΔ heterozygous mice (Graves et al. 2002). The CREB αΔ mutation (Hummler et al. 1994) was backcrossed in a heterozygous state for 4–9 generations to 129/SvEvTac mice and 8–13 generations to C57BL/6J mice. Genotyping was performed by PCR as described (Walters and Blendy 2001).

Mouse handling

Food and water were provided ad libitum. Mice were maintained on a 12:12 light/dark cycle with lights on at 7 A.M. (ZT 0) and lights off at 7 P.M. (ZT 12). Temperature in the mouse cage was maintained at 25 ± 2°C. All animal care and experiments were carried out in accordance with National Institutes of Health guidelines and were fully approved by the Institutional Animal Care and Use Committee of the University of Pennsylvania.

Surgery

Wild-type (n = 9 female and 8 male mice) and CREB αΔ (n = 8 female and 10 male mice) mice were surgically implanted with four electroencephalographic (EEG) and two electromyographic (EMG) electrodes. Surgery was performed as described previously (Veasey et al. 2000). EEG electrodes were implanted at 2 mm lateral to Bregma and 3 mm caudal to Bregma (for 2 caudal EEG electrodes; al. 2000). EEG electrodes were implanted at 2 mm lateral to Bregma and 1 mm rostral (for 2 rostral EEG electrodes) and 2 mm lateral to Bregma and 3 mm caudal to Bregma (for 2 caudal EEG electrodes; Veasey et al. 2000).

Sleep recording

Recordings were performed as described previously (Veasey et al. 2000), except EEG signals were filtered at 0.3–35 or at 0.3–300 Hz (1/2 amplitude, 6 dB/octave) and EMG signals were filtered at 1–100 Hz (12A5 amplifier, Astro-Med).

Recording procedures and sleep scoring

Mice were allowed ≥9 days of recovery following surgery prior to the beginning of baseline recordings. Data from 1 day of baseline recording are presented for each mouse. Mouse records were scored in 10-s epochs as wake, nonrapid eye movement (NREM), or rapid eye movement (REM) sleep by observation of the EEG signal and the EMG signal for one baseline day (24 h). After baseline recording, mice were totally sleep deprived for 6 h from ZT 0 to ZT 6 (Franken et al. 1999). Mice were sleep deprived by gentle stroking in an attempt to reduce the stress of the deprivation procedure (Leduox et al. 1996). Recording was then continued for another 18 h. Sleep-wake states were scored visually as described (Veasey et al. 2000) by a trained observer blind to the genotype of the animal. Waking was defined as a predominance (within a 10-s epoch) of fast, desynchronized waves and a high-EMG amplitude. NREM sleep was defined as a predominance of higher amplitude EEG waves, consistent with either α- or δ-waveforms, with a medium EMG signal, and REM sleep was defined as a high-frequency EEG with a significant predominance of θ-waves and a very low EMG amplitude (Veasey et al. 2000). All values are shown as mean ± SE. Because of loss of signal quality in some animals after sleep deprivation, the final group sizes for sleep deprivation analysis were n = 7 female and 7 male wild-type mice and n = 6 female and 10 male CREB αΔ mice. Because no differences were seen between data from male and female mice, data from these groups were pooled for all analyses.

Assessment of sleep/wake amounts and microstructure analysis

Percentages of wake, NREM, and REM sleep were calculated in eight 3-h blocks for the 24-h baseline period. Differences were analyzed with a mixed-model ANOVA to allow between- and within-group comparisons for main effects of genotype, time period, condition (baseline or post sleep deprivation), and their interaction (Hendricks et al. 2000). A Bonferroni correction was applied in the post-hoc comparisons.

Sleep microstructure from baseline recordings was characterized by determining the length of wake bouts, NREM and REM sleep bouts, and total sleep bouts, as well as the numbers of wake, NREM, REM, and total sleep bouts. Wake bouts were defined as ≥30 s of continuous wake; NREM sleep bouts were defined as ≥30 s of continuous NREM; REM sleep bouts were defined as ≥30 s of continuous REM sleep, and total sleep bouts were defined as ≥30 s of continuous NREM or REM sleep (Veasey et al. 2000; http://rhbase.med.upenn.edu/mouse/sleep/analysis.htm). All values are shown as mean ± SE. Differences between wild-type and mutant mice in sleep microstructure for the baseline period were analyzed with a Student’s t-test (Microsoft Excel 2000).

EEG spectral analysis

We performed a fast-Fourier transform (FFT) on the EEG signals over the 24-h baseline period for all subjects recorded with filter settings for EEG signals set between 0.3 and 35 Hz (n = 7 male and 4 female wild-type and 4 male and 6 female CREB αΔ mice). Overall EEG power spectra were analyzed by determining the average power in the δ (1–4 Hz), θ (4–8 Hz), and α (10–14 Hz) frequency bins (Franken et al. 1998). The results obtained from the FFT analysis for wake, NREM, and REM sleep were normalized as a weighted percentage of total power between 1 and 25 Hz (Franken et al. 1998). The mean and SE of the power spectra were determined in each frequency bin for relative values and differences were analyzed between CREB αΔ and wild-type mice using a Student’s t-test (Microsoft Excel 2000). Differences between CREB αΔ and wild-type mice in the time course of NREM δ-power rebound after sleep deprivation were analyzed by using a mixed model ANOVA (Hendricks et al. 2000). Because of loss of signal quality in some animals after sleep deprivation, the final group sizes for the power analysis after sleep deprivation were n = 6 male and 3 female wild-type mice and 4 male and 4 female CREB αΔ mice.

RESULTS

CREB-deficient mice have decreased wakefulness and increased NREM sleep

We measured levels of wake, NREM, and REM sleep in CREB-deficient and wild-type mice using EEG and EMG recordings. Over 24 h, CREB αΔ mice had decreased wakefulness compared with their wild-type littermates (Fig. 1A; 707.9 ± 28.5 vs. 594.6 ± 19.3 min for wild-type and CREB αΔ mice, respectively; F[1, 33] = 11.48; P < 0.01). Decreases in wakefulness were accompanied by increases in NREM sleep in CREB αΔ mice (Fig. 1B; 650.0 ± 22.1 vs. 754.1 ± 21.3 min for wild-type and CREB αΔ mice, respectively; F[1, 33] = 10.52; P < 0.01). There was no significant difference in the amount of REM sleep. Thus the overall change in wakefulness was on the order of 2 h per day or 16%.

Both CREB αΔ and wild-type mice showed significant changes in wakefulness and NREM sleep across time (wake: F[7,231] = 32.14; P < 0.0001; NREM sleep: F[7,231] = 28.12; P < 0.0001), indicating that there were diurnal varia-
tions in wake and NREM sleep levels. The differences between CREB αΔ and wild-type mice in wakefulness and NREM sleep depended on time of day (genotype × time interaction: wake: \( F[7,231] = 2.26; P < 0.05 \); NREM sleep: \( F[7,231] = 2.35; P < 0.05 \)). Wild-type and CREB αΔ mice were most different in levels of wake and NREM sleep during the dark period (Fig. 1). In particular, CREB αΔ mice had significantly increased NREM sleep and decreased wakefulness during the first half of the dark period, ZT 12–18 (\( P < 0.01 \)). During this 6-h period, the reduction in wakefulness was 62 min from a baseline in wild-type mice of 248.1 ± 10.1 min, a difference of 25%. The difference in wakefulness was most remarkable during the 3-h ZT 15–18 time point, where the reduction in wakefulness was 39 min, a difference of 32%. REM sleep was not statistically different between CREB αΔ and wild-type mice over 24 h, although there was a trend toward increased REM sleep in the CREB αΔ mice (81.4 ± 5.8 vs. 91.2 ± 6.2 min for wild-type and CREB αΔ mice, respectively; \( F[1,33] = 1.58; P > 0.05 \)). A diurnal variation in REM sleep across the day and night was evident in both mutant and wild-type mice (\( F[7,213] = 22.53; P < 0.0001 \)) and CREB αΔ had significantly more REM sleep during the ZT 15–18 time point (\( P < 0.01 \)).

Thus over 24 h CREB αΔ mice have almost 2 h of increased NREM sleep at the expense of wakefulness, mainly during the first half of the dark period when wild-type mice exhibit maximal levels of wakefulness. These results suggest that CREB αΔ mice exhibit deficits in their ability to maintain these maximal levels of wakefulness. Generalized motor impairments could contribute to the reduction in wakefulness. However, open field activity, home cage activity monitored over 24 h, as well as swim speed, of CREB αΔ mice have increased NREM sleep across 24 h compared with wild-type mice (\( P < 0.01 \)). C: CREB αΔ mice do not have statistically reliable alterations in REM sleep across 24 h (\( P > 0.05 \)), although there is a trend to increased REM sleep in the CREB αΔ mice.

CREB αΔ mice have alterations in sleep microstructure

In addition to altering the overall amount of sleep, a CREB deficiency may also alter sleep architecture by altering the duration or frequency of sleep bouts. To determine the nature of the increase in NREM sleep in CREB αΔ mice, we mea-
sured the length of each wake, NREM, REM, and total sleep bout (defined as 30 s or more of wake, NREM, REM, and NREM plus REM sleep, respectively) as well as the number of wake, NREM, REM, and total sleep bouts in wild-type and CREB αΔ mice. Over the 24-h baseline period, the average sleep bout length was not altered by the CREB mutation (data not shown). Because baseline sleep differences were seen mainly during the dark period, we analyzed sleep microstructure during the light and dark periods separately. For the light period, when there were no major differences in the total amounts of wakefulness and sleep, CREB αΔ mutants showed an increase in the number of REM sleep bouts (34.9 ± 2.4 vs. 42.2 ± 0.9 REM bouts for wild-type and CREB αΔ mice, respectively; P < 0.05) but no other significant changes in bout numbers or length of bouts. During the dark period, when differences in the total amount of NREM sleep and wakefulness are observed, the number of NREM sleep bouts was increased in CREB αΔ mice (Fig. 2A; P < 0.05), as was the number of wake bouts (although this did not reach statistical significance; Fig. 2A; P = 0.07) and the number of REM bouts (although this did not quite reach statistical significance; Fig. 2A; P = 0.052). The length of the average NREM sleep bout did not differ between CREB αΔ and wild-type mice (Fig. 2B), but the length of the average wake bout was significantly decreased in CREB αΔ mice (Fig. 2B; P < 0.05), as was the length of the average REM bout (Fig. 2B; P < 0.05). These results indicate that increased NREM sleep during the dark period in CREB αΔ mice is accounted for by an increase in the number of NREM sleep bouts, whereas decreased wakefulness is accounted for by shorter wake bouts. These results also indicate that CREB αΔ mice are not able to maintain a cortical activated state (i.e., wakefulness and REM sleep) for the same amount of time as can wild-type mice.

**CREB-deficient mice exhibit decreased θ-power**

Different sleep/wake states are characterized by prominent frequencies in the EEG spectrum. To examine differences in the EEG power spectrum between CREB αΔ and wild-type mice, we performed a FFT analysis on the baseline 24 h to calculate the power (μV²; 1 to 25 Hz) of the EEG signal for each subject. There was no detectable difference in the total (1 to 25 Hz) EEG power between CREB αΔ and wild-type mice (3241 ± 824 and 2390 ± 1017 μV² for wild-type and CREB αΔ mice, respectively; P > 0.05), although this may be due to variability in these measures of absolute spectral power. Because of this, we determined relative power by expressing the spectral power in wake, NREM, and REM sleep as a weighted percentage of total power between 1 and 25 Hz (Franken et al. 1998). This analysis indicated that CREB αΔ mice had a decrease in the level of θ-power during wake (Fig. 3A) and REM sleep (Fig. 3C) and a decrease in the level of α power during REM sleep (Fig. 3C) compared with wild-type mice.

**Response to sleep deprivation in CREB-deficient mice**

To examine the homeostatic response to sleep deprivation (Borbely 1982), CREB αΔ and wild-type mice were totally sleep deprived for 6 h, starting at ZT 0, using the technique of gentle handling (Franken et al. 1999; Ledoux et al. 1996). Over the 18 h after the end of sleep deprivation (ZT 6–24) and during the light and dark periods examined separately, the mixed model ANOVA showed that there were no overall differences in the number of NREM sleep and wakefulness in wake, NREM, or REM sleep as a result of sleep deprivation (wake: F[1,28] = 2.41; NREM: F[1,28] = 1.39; REM: F[1,28] = 2.04; P’s > 0.05; Fig. 4). The effectiveness of the sleep deprivation period was evaluated and found to be similar in CREB αΔ and wild-type mice in that both had increases in NREM sleep δ-power that declined over the 18-h post sleep-deprivation period (F[5,15] = 3.02; P < 0.05; data not shown). Despite these overall similarities in the responses of CREB αΔ and wild-type mice to 6 h of sleep deprivation, CREB αΔ mice had significantly more NREM and REM sleep rebound and a corresponding decrease in wakefulness during the first 3 h of the dark period after sleep deprivation (ZT 12–15; wake rebound: −9.0 ± 6.9 vs. −18.5 ± 5.76 min for wild-type and CREB αΔ mice; NREM sleep rebound: 6.1 ± 6.1 vs. 12.1 ± 5.35 min for wild-type and CREB αΔ mice, respectively; REM sleep rebound: 3.0 ± 1.2 vs. 6.5 ± 1.0 min for wild-type and CREB αΔ mice, respectively; Fig. 4; P’s < 0.01). These results show that CREB αΔ and wild-type mice both possess a sleep homeostatic response. However, the larger differences we found in the baseline amounts of NREM sleep and wakefulness are not reflected in similar differences in the homeostatic response to sleep deprivation. The differences after sleep deprivation are temporally limited and are largest at the start of the dark period when baseline differences were greatest.
DISCUSSION

The goal of these studies was to evaluate the role of CREB in determining the relative amounts of wakefulness and sleep in mammals. Because there are no good pharmacological tools to manipulate CREB directly, genetic studies are required to understand the function of this activity-dependent transcription factor in mammalian behavior. Our studies show that genetic alterations of CREB, in mice, change basic properties of sleep and wakefulness, supporting a conserved role for this protein in behavioral regulation. Our studies reveal that CREB mutant mice exhibit a sleep/wake phenotype similar to the rest/activity phenotype of CREB mutant Drosophila (Hendricks et al. 2001), supporting the idea that conserved molecular mechanisms underlie these phenotypes. Moreover, because more detailed phenotyping of state with electroencephalographic recording is possible in mice, we were able to extend these observations. Specifically, we found that the decrease in wakefulness was largely due to an inability of CREB mutant mice to maintain longer periods of wakefulness during the nocturnal active period. We also found that the electrophysiological properties of wakefulness, as well as REM sleep, were different in the CREB mutant mice as compared with wild-type mice, with the mutants having less \( \theta \)-power in these states. These observations support the hypothesis that CREB regulates the expression of genes that encode proteins that act to maintain cortical arousal.

To address our hypothesis, we used mutant mice that lack the \( \alpha \) and \( \Delta \) isoforms of CREB (Hummler et al. 1994). Although in rats it is known that CREB is activated by phosphorylation in cortical brain regions during wakefulness (Cirelli et al. 1996; Cirelli and Tononi 2000a), studies in other brain regions, particularly those more directly involved in sleep/wake regulation, have not yet been performed. To this end, we used mutant mice with globally decreased levels of CREB to create a study that was sensitive to changes in brain nuclei that are otherwise not experimentally accessible.

The most likely interpretation of our data, along with those in Drosophila, is that the activation of CREB, through phosphorylation via the cAMP/PKA/CREB signaling pathway (Abel et al. 1997; Hendricks et al. 2001; Ogasahara et al. 1981), is important for the maintenance of wakefulness. This activation of CREB is perhaps in response to one of the wake-active neurotransmitters (noradrenaline, serotonin, orexin, histamine, and acetylcholine; McGinty and Szymusiak...
There is specific evidence to support a role for noradrenaline. Unilateral neurotoxic lesions of the locus coeruleus in rats markedly reduces the levels of phosphorylated CREB in ipsilateral cortical neurons during wakefulness as compared with the contralateral side (Cirelli et al. 1996). Because the recently discovered orexin/hypocretin system has been shown to have extensive neural connections with many arousal-promoting areas, including the locus coeruleus (Aston-Jones et al. 2001; Sutcliffe and De Lecea 2002), one possibility is that the locus coeruleus, after input from the orexin/hypocretin system, influences cortical or wake-promoting neuronal groups through activation of the cAMP/PKA/CREB signaling pathway (Fig. 5). In turn, after phosphorylation, CREB may induce the expression of genes that help sustain wakefulness (Cirelli and Tononi 2000a). Studies showing that levels of phosphorylated CREB in wild-type mice (wild-type BL, filled squares) from ZT 6–24 and over the same 18 h in wild-type mice after sleep deprivation (Wild-type SD, open squares), Wake (A2), NREM (B2), and REM sleep (C2) are plotted over the baseline 18 h in CREB αΔ mice (CREB BL, filled circles) from ZT 6–24 and over the same 18 h in CREB αΔ mice after sleep deprivation (CREB SD, open circles). Wake, NREM, and REM sleep are plotted as percentage of total recording time ± SE in 3-h increments. CREB αΔ mice have larger changes from baseline in levels of wake and NREM and REM sleep during the time period ZT 12–15 compared with wild-type mice. *P < 0.01.

Our model (Fig. 5) suggests that CREB acts downstream from norepinephrine to regulate wakefulness. It is difficult, however, to attribute these actions directly to CREB because the genetic alteration of CREB may act developmentally to alter the expression of other molecules involved in regulating wakefulness. To address this issue, we have begun molecular and neurochemical studies in the CREB αΔ mice. In our preliminary microarray studies examining change in gene expression in the cerebral cortex of CREB αΔ mice, we did not observe changes in the expression levels of genes encoding enzymes involved in the synthesis of wake-active neurotransmitters or their receptors (M. Mackiewicz, K. Hellman, J. A. Blendy, T. Abel, and A. Pack, unpublished data). Neurochemical studies have revealed that levels of norepinephrine measured using HPLC are not altered in the cerebral cortex of CREB αΔ mice (wild-type mice: 1060 ± 79 ng norepinephrine/g tissue, n = 3; CREB αΔ mice: 1275 ± 203 ng norepinephrine/g tissue, n = 4). Further, anatomical alterations and alterations in c-fos staining have not been observed in the CREB αΔ mutant mice on a B6/129 F1 hybrid background (J. A. Blendy, unpublished observations). Thus our data are consistent with the idea that CREB acts downstream of nor-
epinephrine to regulate wakefulness, and these observations may have applicability to human sleep disorders in which CREB function may be dysregulated. Future studies with mice that lack norepinephrine as well as mice in which CREB activity is conditionally regulated will be needed.

CREB may mediate the maintenance of cortical arousal by acting in the cortex as well as in one of the wake-active nuclei, including the brain stem serotonergic and noradrenergic nuclei, histaminergic nuclei within the hypothalamus, and potentially the amygdala (Charifi et al. 2000; McGinty and Szumusia 2000). Behavioral studies of CREB knockout mice suggest that CREB may be particularly important for amygdaloid function, because CREB knockout mice are impaired in contextual and cued fear conditioning (Bourtchouladze et al. 1994; Gass et al. 1998; Graves et al. 2002; Kogan et al. 1997), which are both amygdala-dependent tasks (Phillips and LeDoux 1992). In terms of sleep/wake regulation, alterations in amygdala function result in increased NREM sleep (Charifi et al. 2000), and shorter REM sleep episodes (Cheng et al. 1998; Morrison et al. 2000; Sanford et al. 1995), as observed in the CREB knockout mice. It seems unlikely, however, that this is the only area where CREB is playing a role. CREB-deficient mice have, as described above, reduced sleep homeostatic response to sleep deprivation between wild-type and mutant mice. One possibility is that longer periods of sleep deprivation will be needed to observe large differences. Alternatively, the recovery response to sleep deprivation might involve different molecular mechanisms than those regulating baseline levels of sleep and wakefulness. We found that the altered response to sleep deprivation in CREB knockout mice is specific to normally occurring peak levels of arousal during the active period, further supporting the idea that CREB is needed to maintain peak levels of arousal.

Circadian processes have also been proposed to play a role in regulating the sleep/wake cycle (Borbely 1982; Edgar et al. 1993). We found that the diurnal variation in sleep/wake cycles was not altered in CREB knockout mutants. Also, CREB knockout and wild-type mice do not differ in their circadian periods measured with wheel-running activity in constant darkness or in their ability to entrain to a light:dark cycle (data not shown). Although CREB has also been implicated in the response to phase-shifting light pulses (Ding et al. 1997; Gau et al. 2002), such alterations are unlikely to explain our findings of altered sleep/wake states during the dark period in the absence of external light pulses. Thus the alterations in wakefulness in CREB knockout mice that we report seem unlikely to arise from altered circadian processes.

Current models of sleep/wake regulation are generally based on the concept that there are sleep-promoting mechanisms that increase in intensity with increased duration of prior wakefulness (Borbely 1982). Our data, together with that from Drosophila (Hendricks et al. 2001), indicate that there are, in addition, molecular mechanisms that promote wakefulness that are activated by neurotransmitters such as noradrenaline. Compatible with this new concept is the observation that there are many genes upregulated during wakefulness (Cirelli and Tononi 2000a,b). Such wake-promoting mechanisms will need, in time, to be incorporated into new models of sleep/wake regulation.
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