Suppression of preoptic sleep-regulatory neuronal activity during corticotropin-releasing factor-induced sleep disturbance

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Gvilia I, Suntsova N, Kumar S, McGinty D, Szymusiak R. Suppression of preoptic sleep-regulatory neuronal activity during corticotropin-releasing factor-induced sleep disturbance. Am J Physiol Regul Integr Comp Physiol 309: R1092–R1100, 2015. First published September 2, 2015; doi:10.1152/ajpregu.00176.2015.—Corticotropin releasing factor (CRF) is implicated in sleep and arousal regulation. Exogenous CRF causes sleep suppression that is associated with activation of at least two important arousal systems: pontine noradrenergic and hypothalamic orexin/hypocretin neurons. It is not known whether CRF also impacts sleep-promoting neuronal systems. We hypothesized that CRF-mediated changes in wake and sleep involve decreased activity of hypothalamic sleep-regulatory neurons localized in the preoptic area. To test this hypothesis, we examined the effects of intracerebroventricular administration of CRF on sleep-wake measures and c-Fos expression in GABAergic neurons in the median preoptic nucleus (MnPN) and ventrolateral preoptic area (VLPO) in different experimental conditions. Administration of CRF (0.1 nmol) during baseline rest phase led to delayed sleep onset and decreases in total amount and mean duration of non-rapid eye movement (NREM) sleep. Administration of CRF during acute sleep deprivation (SD) resulted in suppression of recovery sleep and decreased c-Fos expression in MnPN/VLPO GABAergic neurons. Compared with vehicle controls, intracerebroventricular CRF potentiated disturbances of both NREM and REM sleep in rats exposed to a species-specific psychological stressor, the dirty cage of a male conspecific. The number of MnPN/VLPO GABAergic neurons expressing c-Fos was reduced in the CRF-treated group of dirty cage-exposed rats. These findings confirm the involvement of CRF in wake-sleep cycle regulation and suggest that increased CRF signaling in the brain (i) negatively affects homeostatic responses to sleep loss, (ii) exacerbates stress-induced disturbances of sleep, and (iii) suppresses the activity of sleep-regulatory neurons of the MnPN and VLPO.

CRF: sleep homeostasis; stress; preoptic hypothalamus

CORTICOTROPIN-RELEASING FACTOR (CRF), the primary central neuropeptide regulator of endocrine, autonomic, and behavioral responses to stress (reviewed in Refs. 2 and 12) has been implicated in wake-sleep cycle regulation in both normal (6, 7, 9, 25, 30) and stress conditions (34, 43). Exogenously administered CRF results in increased wakefulness and reduced non-rapid eye movement (NREM) sleep in humans (3), rats (14, 15), and mice (33). Administration of CRF receptor agonists also promotes wakefulness, suppresses NREM sleep, and can impact REM sleep as well (8). The wake-promoting effects of restraint stress in rats are attenuated by administration of CRF-receptor 1 antagonist (10). Contextual fear and inescapable shock cause REM sleep suppression that is potentiated by CRF and prevented by CRF antagonists (42).

Experimental evidence suggests that CRF-mediated alterations in the wake-sleep cycle involve excitatory effects on at least two important arousal systems, noradrenergic neurons (NA) in the locus coeruleus and hypothalamic orexin/hypocretin (HCT) neurons (26, 31, 32, 43, 44). But, it is not known whether CRF also acts on sleep-promoting neuronal systems.

The preoptic hypothalamus is considered an important sleep-regulatory region (35, 40) with the highest densities of sleep-active neurons in the median preoptic nucleus (MnPN) and ventrolateral preoptic area (VLPO). Both MnPN and VLPO exhibit sleep-associated c-Fos immunoreactivity (Fos-IR) (21, 37) and subsets of neurons in these nuclei have elevated discharge rates during spontaneous sleep compared with wake, as demonstrated by unit-recording studies (38, 39). Sleep-related c-Fos-IR is predominantly expressed in MnPN GABAergic cells and in GABAergic/galaninergic VLPO neurons (19, 20, 36). Our recent studies report that sleep-active neurons in the MnPN/VLPO exhibit increased activity in situations of high homeostatic sleep pressure, i.e., in response to sleep deprivation (SD), suggesting the role of the MnPN/VLPO in sleep homeostatic regulation (1, 23, 24).

We hypothesized that increased CRF signaling in the brain negatively affects the functional activity of GABAergic neurons in the MnPN and VLPO. To test the hypothesis in this present study, we examined the effects of intracerebroventricular administration of CRF on the patterns of 1) spontaneous sleep, 2) recovery sleep following acute SD, 3) sleep following exposure to a psychological stressor, and 4) c-Fos-IR in MnPN/VLPO GABAergic neurons in the conditions of post-SD and post-stress sleep.

MATERIALS AND METHODS

Animals and Experimental Environment

Male Sprague-Dawley rats (n = 52) weighing 280–320 g at the beginning of the experiments, were acclimated to a 12:12-h light-dark cycle (lights on at 0800). The rats were housed individually in environmental chambers (Fisher Scientific, Pittsburgh, PA). Food and water were available ad libitum, and ambient temperature was maintained at 23 ± 0.5°C. All experiments were approved by the Animal Care and Use Committee at the Veterans Affairs Greater Los Angeles Health Care System and were conducted in accordance with the (U.S.) National Research Council’s Guide for the Care and Use of Laboratory Animals.

Substances

Stock solution of CRF (Peninsula Laboratories) was prepared in pyrogen-free saline. Aliquots of this stock solution were stored at

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Surgical and Postsurgical Procedures and Recordings

All surgical procedures were done under anesthesia (keta-
mine + xylazine: 80:10 mg/kg ip) and aseptic conditions. Details of the surgical procedures were described previously (22, 23, 24). In brief, rats were implanted with EEG and dorsal neck electromyogram (EMG) electrodes for recording sleep-wake behavior. In addition, rats were implanted with a microinjection guide cannula (22G stainless-steel tube, in the lateral ventricle at coordinates, AP = −0.92 mm; L = 1.4 mm, and H = −3 mm), which was sealed with a removable obdurator. The patency and free drainage of the intracerebroventric-
ular cannulas were assessed by administering ANG II (200 ng, human ANG II octapeptide; Peninsula Laboratories, San Carlos, CA) before and after the experiments; angiotensin elicits a drinking response mediated by structures in the preoptic area (18). Placement of the cannula in the right lateral ventricle was histologically confirmed at the end of the experiment.

Starting from the 5th day after surgery, animals were connected to a recording cable suspended above them by a counter-weighted beam and were adapted to the recording procedure within a week, for 5 or 6 h each day. Rats were habituated for intracerebroventricular injec-
tions by daily handling. Rats assigned to experiment 2.2 were also adapted to gentle handling-induced SD procedures, as described previously (23, 24). During the experiments, EEG and EMG signals were recorded continuously using an electrophysiological recording system (15A94 Quad Neuroamplifiers; Grass Technologies; Astro-
Med Industrial Park, West Warren, RI). EEG potential were band-pass filtered (0.3–30 Hz), and EMG data were high-pass filtered (10–100 Hz). The signals were digitized at 256-Hz sampling rate and stored on a computer’s hard drive. For manual scoring of sleep-wake, the EEG and EMG channels were displayed on the computer screen using Spike 2 software (CED System; Cambridge Electronic Design, Cambridge, UK).

Exposure to Acute Species-Specific Psychological Stressor: Cage Exchange Paradigm

Each rat assigned to experiment 2 was placed into a dirty cage, which was previously occupied by another male rat for 1 wk, and left undisturbed until the end of the experimental protocol. It has been reported that the exposure to a species-specific psychological stressor (being inescapably surrounded by the territory that has been marked by another male rat) results in difficulty initiating sleep post-stress exposure (4). After the initial period of insomnia, the animals fall asleep but exhibit difficulties with maintaining sufficient sleep (in-
creased wakefulness, increased sleep fragmentation, and decreased NREM sleep). This model of sleep disturbances was developed to reproduce sleep perturbations observed in human stress-induced insomnia, in which the typical stressor is a self-sustaining psychological state (4).

Experimental Paradigm

In experiment 1.1, rats received intracerebroventricular injection of either saline (n = 6,6) or two different doses of CRF (0.05 nmol and 0.1 nmol; n = 6/dose) at zeitgeber time (ZT) 6 (i.e., at 1400) followed by the recording of undisturbed wake-sleep for 8 h. This experiment identified an intracerebroventricular dose of CRF (0.1 nmol) that, in our hands, evoked moderate effects on spontaneous NREM sleep of relatively short duration and that was subsequently studied under conditions of acute SD (experiment 1.2) and acute exposure to psychological stress (experiments 2.1/2.2).

Experiment 1.2 was designed to determine whether increased CRF signaling in the brain can negatively impact compensatory sleep rebound following acute SD and sleep-related c-Fos expression in preoptic GABAergic neurons. To produce a condition of elevated homeostatic pressure for sleep at the time of CRF administration, we applied previously used protocol of acute SD, at ZT1-3 (i.e., from 0900 until 1100), which led to the elevation of sleep homeostatic pressure during SD and compensatory sleep rebound following the SD protocol. At ZT2.5, 90 min after the SD protocol started, one group of rats (n = 6) was intracerebroventricularly injected with CRF (0.1 nmol), while the other group (n = 6) was administered saline. Following the injections of CRF, all rats were maintained on the SD protocol for an additional 30 min. After the cessation of SD at ZT3, the rats were recorded for a 90-min recovery sleep followed by cardiac perfusion at ZT4.5 (i.e., at 1230). Brain sections cut through the MnPN and VLPO were processed for double immunostaining for c-Fos and glutamic acid decarboxylase (GAD).

Experiment 2.1 was designed to determine whether exogenous CRF can exacerbate behavioral responses to a psychological stressor: exposure to the cage that was previously occupied by another male rat. At ZT1 (0900), 10 rats received intracerebroventricular injections of either CRF (0.1 nmol, n = 5) or saline (n = 5) followed by immediate placement into dirty cages and EEG/EMG recordings for 8 h following the cage transfer.

In experiment 2.2, rats were subjected to intracerebroventricular injections of either CRF (0.1 nmol; n = 6) or saline (n = 6) at ZT1 followed by placement into dirty cages and EEG/EMG recordings. In 5.5 h following the transfer to a dirty cage (at ZT6.5, i.e., 1430), the rats were given a lethal dose of pentobarbital sodium followed by thoracotomy and transectional perfusion. Brain sections cut through the MnPN and VLPO were processed for c-Fos/GAD immunostaining.

Immunohistochemistry

Under deep pentobarbital sodium anesthesia (100 mg/kg), animals were transcardially perfused, and brain tissue was processed for double immunostaining for c-Fos, the protein product of the immediate-early gene c-fos (13, 28), and for glutamic acid decarboxylase (GAD), a marker of GABAergic cells using the protocol described in our previous studies (23, 24). In brief, the sections were processed for c-Fos protein staining first. Sections were incubated overnight in a rabbit anti-c-Fos primary antiserum (ABC; Vector Laboratories, Burlingame, CA) for 1.5 h at room temperature, followed by reaction with avidin-biotin complex (ABC; Vector Elite kit; 1:200; Vector Laboratories, Burlingame, CA) and developed with nickel-diaminobenzidine tetrahydrochloride (NiDAB). To stain for GAD, we used mouse anti-GAD67 monoclonal antibody (MAB5406, lot no. 25030191; Chemicon, Temecula, CA). Sections were incubated into the primary antibody (1:300) at 4°C, over 48 h, and then processed with biotinylated anti-mouse IgG (BA-2001; 1:500; Vector Laboratories) followed by a reaction with avidin-biotin complex (avidin/biotin blocking kit, 1:100; Vector Laboratories) and developed with DAB.

Data Analysis

Sleep-wake cycle analysis. Sleep-wakefulness states of the rats were determined in 10-s epochs by an experienced scorer blind to the experimental condition and group identity of the animal. Wake-sleep stages were defined according to the criteria described previously (22, 23, 24).

For experiment 1.1, mean total amounts of wakefulness, NREM sleep, and REM sleep (as percentage of total recording time) were calculated for consecutive 1-h intervals across the 8-h EEG/EMG recordings. In addition, we calculated the latencies of the first onset and mean durations of NREM sleep and REM sleep episodes. EEG records of consecutive 10-s epochs of NREM sleep were subjected to a fast-Fourier transform routine to obtain EEG power spectra in the delta frequency range [0.3–4.0 Hz, representing EEG slow-wave...
activity (SWA)]. Epochs containing EEG artifacts, which were recognized during visual scoring, were excluded from spectral analysis. The average SWA value for all NREM sleep epochs subjected to spectral analysis was determined for each animal, and individual group mean SWA values were calculated. The same sleep-wake parameters were measured for assessing the impact of intracerebroventricular CRF on 1) post-SD recovery sleep in experiment 1.2 and 2) stress-induced alterations in sleep-wake cycle in experiments 2.1 and 2.2.

**Cell counts.** Neurons that were single labeled for c-Fos-IR, single labeled for GAD-IR, and double labeled for c-Fos/GAD-IR (see Fig. 1, A and B) were counted by an individual who was blind to the experimental condition of the animals, using the Neurolucida computer-aided plotting system (MicroBrightField, Williston, VT). MnPN and VLPO section outlines were drawn under ×20 magnification. c-Fos- and GAD-immunoreactive neurons (IRNs) were mapped in the section outlines under ×400 magnification. Cell counts were calculated for rostral and caudal parts of the MnPN, VLPO core, and extended VLPO, as described in our previous studies (23, 24). The location and size of different counting boxes are shown in Fig. 1, C–E.

**Statistical Analysis**

All results are reported as means ± SE. Sleep-wake data were averaged through consecutive 1-h intervals of the entire recording (for experiments 1.1 and 2.1) or for the 90-min recordings preceding the animals’ perfusion (for experiments 1.2 and 2.2). One-way non-repeated-measures ANOVA was calculated for behavioral stage percentages across animals that were injected with either different doses of CRF or saline and allowed undisturbed sleep-wake time postinjection in experiment 1.1. Significance of the differences between individual group means was assessed by Newman-Keuls post hoc tests. Student’s unpaired t-test was used to assess differences between the treatment and control groups in the measured sleep parameters and in the numbers of single c-Fos-IR, single GAD-IR, or double c-Fos/GAD-IR cells in experiments 1.2, 2.1, and 2.2. P < 0.05 was considered to be significant for all tests.

**RESULTS**

**CRF-Induced Alterations in Spontaneous Wake-Sleep Cycle, Experiment 1.1**

Hour-by-hour changes in the percentages of time spent in wakefulness, NREM sleep, and REM sleep following intracerebroventricular administration of CRF (0.05 nmol and 0.1 nmol) are shown in Fig. 2. One-way ANOVA across 1-h time blocks revealed that the higher dose of CRF (0.1 nmol) resulted in significant changes in total times spent in wakefulness and NREM sleep. Specifically, during the first 2 h postinjection, CRF-injected rats vs. the controls exhibited significant increases in the percentage of time spent in wakefulness (see Fig. 2A) and decreases in the percentage of time spent in NREM sleep.

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**Fig. 1.** Representative examples of double immunostaining for c-Fos protein and glutamic acid decarboxylase (GAD) (A and B) and positions of counting boxes within the median preoptic nucleus (MnPN) and ventrolateral preoptic area (VLPO) (C–E). A and B: photomicrographs show c-Fos single-labeled (▲) and GAD double-labeled (arrows) neurons within rostral MnPN from a saline-treated (▲) and a CRF-treated (▲) rat that were permitted undisturbed sleep following acute SD in experiment 1.2. c-Fos protein is stained black and confined to the nucleus. GAD-immunoreactive neurons are stained brown, and the staining is evident throughout the soma and the proximal dendrites. Scale bar: 50 μm. C–E: section outlines through rostral MnPN (C), caudal MnPN (D), and VLPO (E). The rostral MnPN grid (1) was a 600 × 600 μm square centered on the apex of the third ventricle rostral to the decussation of the anterior commissure (AP; −0.26 mm). D, the caudal MnPN grid (2) was a 300 × 600 μm rectangle placed dorsal to the third ventricle at the level of the decussation of the anterior commissure (AP; −0.1 mm). E, the VLPO counting grids were placed bilaterally (AP; −0.3 to −0.7 mm) and included core and extended VLPO boxes (23, 24). The VLPO core box (3) was a 300 × 300 μm square placed along the base of the brain, with its far border 400 μm lateral to the lateral edge of the optic chiasm. The medial extended VLPO box (4), 400 × 300 μm, was placed medial to the VLPO core. The dorsal extended VLPO box (5), 200 × 300 μm, was positioned above the VLPO core and medial extended VLPO boxes and centered over their border. Och, optic chiasm; CA, anterior commissure.
sleep (see Fig. 2B). During this time period, CRF caused no significant changes in the percentage of time spent in REM sleep (see Fig. 2C). As shown in Fig. 2, CRF-treated rats exhibited a tendency of increased sleep after ZT12, the dark/active phase. The comparison (unpaired t-test) of all CRF-treated rats (n = 12) with the controls (n = 6) revealed significant differences in the percentages of time spent in NREM sleep [t(16) = 3.8, P < 0.01] and REM sleep [t(16) = 2.5, P < 0.05] for ZT13-14, suggesting a delayed sleep rebound.

Administration of the 0.1-nmol dose of CRF also resulted in significant delay in the onset of sleep states and fragmentation of NREM sleep. The latencies of the first NREM sleep and REM sleep episodes in CRF-injected vs. saline-treated rats were 37 ± 3.1 vs. 21.7 ± 2.6 min [t(10)-3.8, P < 0.01] and 58.3 ± 2.2 vs. 41.1 ± 2.5 min [t(10)-5.1, P < 0.001], respectively. Intracerebroventricular administration of CRF significantly reduced the duration of NREM sleep episodes compared with saline and had no significant effects on REM sleep bout duration or EEG SWA during NREM sleep (Table 1).

Table 1. The effect of ICV CRF (0.1 nmol) on sleep consolidation, Experiment 1.1

<table>
<thead>
<tr>
<th>Postinjection Time Periods</th>
<th>Mean Duration of NREM Sleep Episodes, min</th>
<th>Mean Duration of REM Sleep Episodes, min</th>
<th>EEG SWA, μV²</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Saline-Injected Rats</td>
<td>CRF-Injected Rats</td>
<td>Saline-Injected Rats</td>
</tr>
<tr>
<td>ZT6–7</td>
<td>5.8 ± 1.4</td>
<td>1.8 ± 0.2**</td>
<td>1.4 ± 0.3</td>
</tr>
<tr>
<td>ZT7–8</td>
<td>3 ± 0.1</td>
<td>2.1 ± 0.2*</td>
<td>1.9 ± 0.1</td>
</tr>
<tr>
<td>ZT8–9</td>
<td>2.7 ± 0.2</td>
<td>1.8 ± 0.2*</td>
<td>1.8 ± 0.3</td>
</tr>
<tr>
<td>ZT9–10</td>
<td>3.1 ± 0.4</td>
<td>1.3 ± 0.1**</td>
<td>1.5 ± 0.2</td>
</tr>
<tr>
<td>ZT10–11</td>
<td>2.6 ± 0.3</td>
<td>1.4 ± 0.3**</td>
<td>1.4 ± 0.2</td>
</tr>
<tr>
<td>ZT11–12</td>
<td>1.7 ± 0.2</td>
<td>1.5 ± 0.2</td>
<td>0.8 ± 0.3</td>
</tr>
<tr>
<td>ZT12–13</td>
<td>2.5 ± 0.5</td>
<td>1.5 ± 0.2</td>
<td>1.3 ± 0.5</td>
</tr>
<tr>
<td>ZT13–14</td>
<td>1.9 ± 0.3</td>
<td>1.6 ± 0.2</td>
<td>1.761.4 ± 52.1</td>
</tr>
</tbody>
</table>

Reported are the mean durations of non-rapid eye movement (NREM) and rapid eye movement (REM) sleep episodes, and the values of EEG slow-wave activity (SWA, represented by power density in the 0.3- to 4.0-Hz band) in ICV saline- and CRF-injected rats during consecutive 1-h intervals postinjection. Values are expressed as means ± SE; n = 6 in each group. ZT, zeitgeber time. Microinjection of CRF resulted in significant shortening of NREM sleep episodes during the first [t(10)-2.8, P < 0.05], second [t(10)-4.1, P < 0.01], third [t(10)-3.5, P < 0.01], fourth [t(10)-4.0, P < 0.01], and fifth [t(10)-3.2, P < 0.05] h postinjection. *P < 0.01, **P < 0.05.
Table 2. Sleep-wake characteristics of saline- and CRF-injected rats during post-SD recovery period, Experiment 1.2

<table>
<thead>
<tr>
<th>ZT3-4.5</th>
<th>Saline-Injected SD Rats</th>
<th>CRF-Injected SD Rats</th>
</tr>
</thead>
<tbody>
<tr>
<td>% Wakefulness</td>
<td>43.1 ± 5.5</td>
<td>76.2 ± 5.8**</td>
</tr>
<tr>
<td>% NREM sleep</td>
<td>44.4 ± 5.1</td>
<td>22 ± 4.9*</td>
</tr>
<tr>
<td>% REM sleep</td>
<td>12.5 ± 1.9</td>
<td>1.8 ± 0.9*</td>
</tr>
<tr>
<td>First NREM sleep onset latency, min</td>
<td>18.5 ± 3.9</td>
<td>41.8 ± 6.3**</td>
</tr>
<tr>
<td>Mean duration of NREM sleep episodes, min</td>
<td>4.9 ± 0.6</td>
<td>1.9 ± 0.2**</td>
</tr>
<tr>
<td>First REM sleep onset latency, min</td>
<td>33 ± 3.4</td>
<td>60.2 ± 4.8*</td>
</tr>
<tr>
<td>Mean duration of REM sleep episodes, min</td>
<td>2.5 ± 0.3</td>
<td>0.8 ± 0.2*</td>
</tr>
<tr>
<td>EEG SWA, µV²</td>
<td>6,834.5 ± 68.2</td>
<td>1,795.8 ± 76.9**</td>
</tr>
</tbody>
</table>

Values are expressed as means ± SE; n = 6 in each group. SD, sleep deprivation; EEG SWA, slow-wave activity represented by power density in the 0.3- to 4.0-Hz band. CRF-treated vs. saline-injected rats exhibited significantly different % of time spent in wakefulness [t(10) = 2.7; P < 0.01], NREM sleep [t(10) = 3.3; P < 0.001] and REM sleep [t(10) = 3.2; P < 0.001]. Also, intracerebroventricular CRF resulted in delayed onset of both NREM sleep [t(10) = 3.1, P < 0.01] and REM sleep [t(10) = 4.6, P < 0.001] first episodes. decreases in the mean duration of NREM sleep [t(10) = 4.5, P < 0.001] and REM sleep [t(10) = 5.1, P < 0.001] episodes, and suppression of EEG SWA. All the calculations were done for a 90-min recording period that followed the termination of ± SD. *P < 0.001, **P < 0.01.

CRF-Mediated Changes in Post-SD Recovery Sleep and the Expression of c-Fos in MnPN and VLPO GABAergic Neurons, Experiment 1.2

During the SD period, both saline- and CRF-injected rats exhibited less than 4% of time spent in NREM sleep (3.6 ± 0.4% and 2.8 ± 0.2%, respectively), and no REM sleep was achieved. After the cessation of SD protocol, the CRF-treated rats exhibited a significantly higher percentage of wakefulness time, a lower percentage of both NREM sleep and REM sleep times, delayed onset of sleep states, fragmented NREM sleep, and REM sleep and decreased EEG SWA compared with saline-injected rats (Table 2). These two groups of rats also differed in the numbers of c-Fos-IRNs determined in preoptic sleep-regulatory sites (see Fig. 3). The numbers of single c-Fos+ cells in the MnPN and VLPO of CRF-treated SD rats were significantly higher compared with saline-injected rats (Fig. 3A). Single GAD+ cell counts did not differ between the groups (Fig. 3B), but the numbers of dual Fos+GAD+ neurons in the MnPN and VLPO were significantly lower in CRF-treated rats compared with the controls (Fig. 3C).

**Effect of Exogenous CRF on Acute Stress-Induced Alterations in Wake and Sleep, Experiment 2.1**

After being transferred to dirty cages, CRF-treated rats and control animals were awake for most of the next 1-h period (97.4 ± 1.9% and 91.8 ± 1.9%, respectively). The following few hours were characterized by significant differences in the total amounts and/or mean durations of wake-sleep states between the two groups of rats (see Figs. 4 and 5). As shown in Fig. 4, during the second, third, and fourth hour post-cage exchange, CRF-treated rats exhibited significantly higher percentage of time spent in wakefulness (see Fig. 4A) and lower percentage of time spent in NREM sleep (see Fig. 4B) compared with the control animals. The percentage of REM sleep total time in CRF-treated vs. saline-injected rats was significantly lower during the 3rd through 5th h following the placement into dirty cages (see Fig. 4C). The first 3 h after cage exchange, CRF-treated exhibited significantly longer episodes of wakefulness compared with control rats (Fig. 5A). Episodes of NREM sleep and REM sleep in CRF-treated rats were significantly shorter (compared with control rats) during the 3rd through 6th h post-cage exchange (see Fig. 5, B and C, respectively).

**Sleep-Related Expression of c-Fos in MnPN and VLPO GABAergic Neurons Following ICV CRF Combined with the Exposure of Rats to the Dirty Cage Paradigm, Experiment 2.2**

Expression of c-Fos in preoptic sleep-regulatory neurons was studied for the late stage of stress-induced wake-sleep...
Fig. 4. Mean percentages of time spent in wakefulness (A), NREM sleep (B), and REM sleep (C) during consecutive 1-h intervals following the exposure of intracerebroventricularly saline-injected (n = 5) and intracerebroventricularly CRF-treated (n = 5) rats to cage exchange at ZT1, experiment 2.1. CRF-treated vs. saline-injected rats exhibited a significantly higher percentage of time spent in wakefulness during the 2nd [t(8)–2.5, P < 0.05], 3rd [t(8)–12.3; P < 0.001], and 4th [t(8)–5.7, P < 0.001] h postexposure to the dirty cage paradigm. During the same time period, CRF-treated rats exhibited significantly lower % of time spent in NREM sleep, compared with the controls [during 2nd h postcage exchange: t(8)2.7, P < 0.05; 3rd h: t(8)10.1, P < 0.001; 4th h: t(8)3.9, P < 0.01], % of REM sleep time of CRF-treated vs. saline-injected rats was significantly increased during the 3rd [t(8)2.4, P < 0.05]; 4th [t(8)5.9, P < 0.001], and 5th [t(8)5.1, P < 0.001] h after cage exchange. Error bars represent means ± SEs. *P < 0.001, **P < 0.001, ***P < 0.05.

We found that intracerebroventricular administration of CRF in rats disrupted spontaneous sleep in the home cage, disrupted recovery sleep occurring after short-term sleep deprivation, and exacerbated sleep disturbances evoked by exposing male rats to a species-specific psychological stressor. Intracerebroventricular CRF also altered patterns of c-Fos expression in key preoptic hypothalamic sleep-regulatory nuclei during post-SD recovery sleep and following the exposure to a psychological stressor. While single Fos+ cell counts in the MnPN and VLPO were increased in CRF-treated vs. vehicle-treated rats in both conditions, numbers of GABAergic neurons immunopositive for c-Fos were reduced in these nuclei. Single GAD+ cell counts did not differ between the groups. These findings suggest that in addition to activation of arousal systems, CRF-induced sleep disturbance is associated with sup-

Fig. 5. Mean durations (in min) of wakefulness (A), NREM sleep (B), and REM sleep (C) episodes during consecutive 1-h intervals following the exposure of intracerebroventricularly saline-injected (n = 5) and intracerebroventricularly CRF-treated (n = 5) rats to cage exchange at ZT1, experiment 2.1. Mean duration of wake episodes in CRF-treated vs. saline-injected rats was significantly higher during the 1st [t(8)–4.1, P < 0.01], 2nd [t(8)–2.4, P < 0.05], and 3rd [t(8)–4.2, P < 0.01] h after cage exchange. NREM sleep episodes of CRF-treated rats were significantly shorter, compared with the controls, during the 3rd [t(8)3.4, P < 0.05], 4th [t(8)5.6, P < 0.001], and 6th [t(8)2.7, P < 0.05] h after cage exchange. Mean duration of REM sleep episodes was significantly decreased in CRF-treated vs. control rats during the 3rd [t(8)2.5, P < 0.05], 4th [t(8)5.9, P < 0.001], 5th [t(8)5.4, P < 0.001], and 6th [t(8)3.7, P < 0.01] h following the exposure to cage exchange. Error bars represent means ± SEs. *P < 0.001, **P < 0.01, ***P < 0.05.

Disturbances identified in experiment 2.1. Specifically, rats were perfused in 5.5 h following the cage exchange. The numbers of single c-Fos+ cells in the MnPN and VLPO of CRF-treated rats were significantly higher compared with saline-injected rats (Fig. 6A). Single GAD+ cell counts did not differ between the groups, but the numbers of dual Fos+GAD+ neurons in the MnPN/VLPO were lower in CRF-treated than the saline-treated rats (Fig. 6, B and C, respectively). The comparison (unpaired t-test) of wake-sleep characteristics for the last 90-min period prior to the perfusion between these two groups of rats revealed no significant differences in the percentages of total time and the mean durations of wakefulness and NREM sleep, but the differences in REM sleep characteristics were statistically significant (see Table 3).
pression of sleep-regulatory GABAergic neuronal activity in the preoptic hypothalamus.

Administration of CRF (0.1 nmol icv) in baseline conditions (experiment 1.1) led to a transient increase in the percentage of time spent in wakefulness, and a decrease in the percentage of NREM sleep time (see Fig. 2, A and B, respectively), significantly delayed the onset of both NREM and REM sleep, and shortened NREM sleep episodes (see Table 1). Time spent in wakefulness and NREM sleep differed significantly for the first 2 h postinjection, whereas the following 3 h were mainly characterized by moderate NREM and REM sleep fragmentation. There was a tendency for REM sleep suppression in CRF- vs. saline-injected rats, but the differences did not achieve statistical significance (see Fig. 2C). EEG SWA during NREM sleep was not significantly altered by CRF administration (see Table 1). Our findings on the effect of intracerebroventricular CRF administration of CRF (0.1 nmol icv) in baseline conditions (experiment 1.1) led to a transient increase in the percentage of time spent in wakefulness, and a decrease in the percentage of NREM sleep time (see Fig. 2, A and B, respectively), significantly delayed the onset of both NREM and REM sleep, and shortened NREM sleep episodes (see Table 1). Time spent in wakefulness and NREM sleep differed significantly for the first 2 h postinjection, whereas the following 3 h were mainly characterized by moderate NREM and REM sleep fragmentation. There was a tendency for REM sleep suppression in CRF- vs. saline-injected rats, but the differences did not achieve statistical significance (see Fig. 2C). EEG SWA during NREM sleep was not significantly altered by CRF administration (see Table 1). Our findings on the effect of intracerebroventricular CRF on spontaneous wake-sleep cycle in rats are largely in agreement with the results of earlier studies (16, 29, 30).

Intracerebroventricular CRF negatively impacted recovery sleep following acute SD (experiment 1.2). We have previously shown that 2 h of enforced wakefulness by gentle handling at ZT1-3 results in increased sleep amount, sleep depth, and sleep consolidation during subsequent recovery sleep (1, 2, 23, 24). We have also shown that compensatory sleep rebound following acute SD in the light phase is accompanied by increases in sleep-related neuronal activity within the MnPN and VLPO, measured by c-Fos expression (23, 24) or by single-unit activity (1). In the present experiment, rats were subjected to a previously used protocol of gentle handling-induced SD at ZT1-3. During a 90-min sleep opportunity following SD, rats treated with CRF exhibited increased time awake, reduced NREM and REM sleep times, delayed onset and fragmentation of sleep states, and reduced EEG SWA compared with saline-treated rats (see Table 2). CRF-treated rats had higher numbers of single c-Fos-immunopositive cells in the MnPN and VLPO than saline-treated rats (see Fig. 3A), but dual Fos+/GAD+ cell counts were lower in CRF-treated rats (see Fig. 3C). MnPN and VLPO single GAD+ cell counts did not differ between the groups (see Fig. 3B). This is the first demonstration that CRF-induced sleep disturbance is associated with altered c-Fos expression in sleep regulatory preoptic nuclei. It has been previously reported that exogenous CRF disrupts the homeostatic response to REM sleep deprivation in rats (27).

Cano et al. (4) validated a model of transient stress-induced insomnia in rats that involves exposing male rats to the soiled cage of a male conspecific. Dirty-cage exposure resulted in reduced time asleep, increased sleep fragmentation, and increased high-frequency EEG activity in the sleep EEG for a period of 4–6 h (4). We found (experiment 2.1) that exogenous CRF potentiated the wake-sleep response to dirty-cage exposure compared with saline-treated rats. CRF- and saline-treated rats were mostly awake during the 1st h after cage exchange. During the following 3 h after cage exchange, CRF-treated rats

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Table 3. Wake-sleep characteristics for the 90-min period prior to the perfusion of saline- and CRF-injected cage exchange rats, Experiment 2.2

<table>
<thead>
<tr>
<th>ZT6.5–8</th>
<th>Saline-Injected Cage Exchange Rats</th>
<th>CRF-Injected Cage Exchange Rats</th>
</tr>
</thead>
<tbody>
<tr>
<td>% Wakefulness</td>
<td>26.5 ± 2.3</td>
<td>40 ± 12.8</td>
</tr>
<tr>
<td>% NREM sleep</td>
<td>61.2 ± 2.7</td>
<td>56.6 ± 10.1</td>
</tr>
<tr>
<td>% REM sleep</td>
<td>17.8 ± 1.8</td>
<td>7.3 ± 1.8</td>
</tr>
<tr>
<td>Mean duration of wake episodes, min</td>
<td>1 ± 0.1</td>
<td>2.1 ± 1.4</td>
</tr>
<tr>
<td>Mean duration of NREM sleep episodes, min</td>
<td>3 ± 0.2</td>
<td>2.4 ± 0.5</td>
</tr>
<tr>
<td>Mean duration of REM sleep episodes, min</td>
<td>1.8 ± 0.1</td>
<td>0.7 ± 0.2*</td>
</tr>
</tbody>
</table>

Values are expressed as means ± SE; n = 6 in each group. CRF-injected vs. saline-injected rats tended to exhibit a higher % of time spent in wakefulness (t = 10-1.7, P = 0.13), lower % of time spent in NREM sleep (t = 10-4.4, P = 0.067), longer episodes of wakefulness (t = 10-1.5, P = 0.015), and shorter episodes of NREM sleep (t = 10-2.2, P = 0.02), but the differences did not achieve statistical significance. The % of REM sleep time (t = 10-4.1, P < 0.001) and the mean duration of REM sleep episodes (t = 10-5.4, P < 0.001) in CRF-treated rats was significantly lower compared to the control animals. *P < 0.001.
exhibited a higher percentage of time spent in wakefulness and reduced percentage of NREM sleep time, compared with saline controls (see Fig. 4, A and B). CRF also caused significant decreases in REM sleep, although this effect emerged an hour later than the NREM sleep insomnia (see Fig. 4C). CRF-induced reduction in the amounts of sleep states was accompanied by fragmentation of both NREM sleep and REM sleep episodes (see Fig. 5, B and C).

The sleep-disruptive effect of intracerebroventricular CRF was potentiated under conditions of acute psychological stress (experiment 2.1) compared with baseline, home cage conditions (experiment 1.1). Specifically, intracerebroventricular CRF in dirty cage vs. home cage condition led to longer suppression of NREM sleep (see Figs. 2B, 4B, and 5B). Also, exogenous CRF had no significant effects on spontaneous REM sleep measures recorded in the home cage (see Fig. 2C and Table 1) but resulted in significant REM sleep disruption compared with vehicle-treated rats exposed to acute psychological stress (see Figs. 4C and 5C). Contextual fear and inescapable shock cause REM sleep suppression that is potentiated by CRF and prevented by CRF antagonists (reviewed in Ref. 38). CRF actions in the central nucleus of the amygdala and the basolateral amygdala are implicated in the REM-suppressing effects of contextual fear and inescapable stress (47, 50), and activation of amygdala circuits could underlie the enhanced REM sleep suppression evoked by CRF that we observed during dirty-cage exposure.

In experiment 2.2, groups of CRF- and saline-treated rats were killed 5.5 h after dirty-cage exposure and c-Fos+, and GAD+ cell counts were performed in the MnPN and VLPO. Total sleep times did not differ between the control and experimental groups during the 90 min preceding death, although REM sleep suppression was present in CRF-treated rats (see Table 3). In both nuclei, single c-Fos IR cell counts were higher in CRF- vs. saline-treated rats, but the numbers of dual c-Fos/GAD IR neurons were reduced in CRF-treated animals (see Fig. 6, A and C, respectively). Total numbers of single GAD IR cells did not differ between the groups. This was similar to the effect of intracerebroventricular CRF on preoptic c-Fos expression during post-SD recovery sleep in experiment 1.2. c-Fos expression in nonGABAergic MnPN and VLPO neurons is typically low during spontaneous and enforced wakefulness during the light phase, and sleep disruption induced by exogenous CRF appears to be associated with a reorganization of state-dependent neuronal activity in preoptic sleep-regulatory nuclei. We have previously shown that intracerebroventricular administration of ANG II, a treatment that evokes arousal and a strong drinking response, increases c-Fos expression in nonGABAergic MnPN neurons (22). Sleep disturbance occurring in response to physiological and psychological stressors may be associated with both suppression of GABAergic sleep-related neuronal activity and activation of nonGABAergic neuronal groups in the MnPN and VLPO that is mediated, in part, by CRF. Because of the limitations of our approach, we cannot draw any direct causal inferences between the observed changes in preoptic c-Fos expression and CRF-induced changes in sleep-wake.

Cano et al. (4) conducted an extensive evaluation of c-Fos expression in multiple brain regions in animals killed 5.5 h after cage exchange. They found increased c-Fos in the cerebral cortex, limbic system, and parts of the arousal and autonomic systems in rats subjected to the stress of cage exchange vs. controls (4). They also reported increased c-Fos expression in the VLPO, suggesting simultaneous activation of arousal, autonomic, and sleep regulatory circuits in response to acute social stressor (4). Our analysis of c-Fos expression was confined to the preoptic area. We compared CRF- and saline-treated groups that were both exposed to the stress of cage exchange and demonstrated increased c-Fos IR in nonGABAergic neurons in response to CRF. Direct comparison between our findings and those of Cano et al. is difficult because of differences in the experimental approach. It remains to be determined how changes in endogenous CRF signaling that occur in response to acute stress impact the activity of preoptic GABAergic, sleep regulatory neurons.

A limitation of our approach is the inability to determine the extent to which CRF exerts direct actions on sleep-regulatory neurons in the MnPN/VLPO independent of actions on other neuronal systems, including HCT and NA neurons and neurons in the amygdala. Anatomical studies have demonstrated the presence of CRF receptors in the rat preoptic area (5, 11, 17), but the specific neuronal targets have not been identified. Studies of localized infusions of CRF agonists and antagonists into the preoptic area on behavioral state and sleep-related neuronal activity could more directly address this issue, as could optogenetic or pharmacogenetic interrogation of CRF-expressing neuronal populations that project to the preoptic hypothalamus.

Perspectives and Significance

The present study demonstrated that acute elevation of CRF in rats results in disruption of homeostatic responses to sleep loss and potentiation of stress-induced sleep disturbance. A sleep-disruptive effect of exogenous CRF in both conditions was associated with reduced c-Fos-immunoreactivity in sleep-regulatory GABAergic neurons in the preoptic hypothalamus. These findings support the hypothesis that increased CRF signaling in the brain negatively impacts brain systems that regulate sleep. Further studies are needed to determine the direct effects of CRF on functional activity of sleep-promoting neuronal systems. This is important for understanding mechanisms of stress-induced sleep disturbance.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS

Author contributions: I.G. and R.S. conception and design of research; I.G. and S.K. performed experiments; I.G. and N.S. analyzed data; I.G. interpreted results of experiments; I.G. drafted manuscript; I.G., D.M., and R.S. edited and revised manuscript; I.G., D.M., and R.S. approved final version of manuscript; N.S. prepared figures.

REFERENCES


