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Corticotropin Releasing Factor (CRF) Modulates Fear-Induced Alterations in Sleep in Mice

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Abstract

Contextual fear significantly reduces rapid eye movement sleep (REM) during post-exposure sleep in mice and rats. Corticotropin releasing factor (CRF) plays a major role in CNS responses to stressors. We examined the influence of CRF and astressin (AST), a non-specific CRF antagonist, on sleep after contextual fear in BALB/c mice. Male mice were implanted with transmitters for recording sleep via telemetry and with a guide cannula aimed into the lateral ventricle. Recordings for vehicle and handling control were obtained after ICV microinjection of saline (SAL) followed by exposure to a novel chamber. Afterwards, the mice were subjected to shock training (20 trials, 0.5 mA, 0.5 s duration) for 2 sessions. After training, separate groups of mice received ICV microinjections of SAL (0.2 microl, n=9), CRF (0.4 microg, n=8), or AST (1.0 microg, n=8) prior to exposure to the shock context alone. Sleep was then recorded for 20 hours (8-hour light and 12-hour dark period). Compared to handling control, contextual fear significantly decreased REM during the 8-h light period in mice receiving SAL and in mice receiving CRF, but not in the mice receiving AST. Mice receiving CRF exhibited reductions in REM during the 12-h dark period after contextual fear, whereas mice receiving SAL or AST did not. CRF also reduced non-REM (NREM) delta (slow wave) amplitude in the EEG. Only mice receiving SAL prior to contextual fear exhibited significant reductions in NREM and total sleep. These findings demonstrate a role for the central CRF system in regulating alterations in sleep induced by contextual fear.

Keywords

corticotropin releasing factor; fear; mice; sleep; stress

Introduction

In contextual fear conditioning, initially neutral environments acquire the capacity to elicit fear responses through association with an unconditioned, fear-inducing stressor such as inescapable footshock (Liang et al., 1992). Subsequently, the fearful context alone elicits behavioral and physiological responses indicative of fear and anxiety. These responses include behavioral freezing (Blanchard and Blanchard, 1969; Phillips and LeDoux, 1992; Paylor et al., 1994), autonomic activation (Nijsen et al., 1998; Hode et al., 2000; Stiedl et al., 2004) and fear-

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potentiated startle (Liang et al., 1992). The footshock stressor and shock-associated fearful contexts also produce similar alterations in sleep, including a prominent reduction in rapid eye movement sleep (REM) that occurs in the first few hours after exposure (Sanford et al., 2003; Sanford et al., 2003; Sanford et al., 2003; Pawlyk et al., 2005) and that can occur without the REM rebound observed in various other stressors (Rampin et al., 1991; Gonzalez et al., 1995; Smith, 1995; Bonnet and Arand, 1997; Gonzalez and Valatx, 1997; Marinesco et al., 1999; Meerlo et al., 2001; Schiffelholz and Aldenhoff, 2002; Tang et al., 2004; Sanford et al., 2005; Tang et al., 2005; Tang et al., 2005).

Several lines of evidence indicate a major role for corticotropin releasing factor (CRF) in mediating central nervous system responses to stressors (Koob and Bloom, 1985 ; Heinrichs et al., 1995; Koob, 1999; Koob and Heinrichs, 1999; Bakshi and Kalin, 2000; Deussing and Wurst, 2005). Intracerebroventricular (ICV) administration of CRF in rats produces many of the signs associated with anxiety in humans, including increased wakefulness (Ehlers et al., 1986; Marrosu et al., 1990; Chang and Opp, 1998; Chang and Opp, 1999), altered locomotor activity, and an exaggerated startle response (Swerdlow et al., 1986 ; Heilig et al., 1994). By comparison, CRF antagonists attenuate behavioral responses to stress (e.g., (Aloisi et al., 1999; Basso et al., 1999; Deak et al., 1999; Spina et al., 2000)). Stress induces arousal (Chrousos, 1998) and CRF has been implicated in stress-induced alterations in sleep (Gonzalez and Valatx, 1998; Chang and Opp, 2002), particularly in the control of REM (Gonzalez and Valatx, 1997). For example, administration of CRF antagonists have been reported to decrease REM rebound after sleep deprivation (Gonzalez and Valatx, 1998) and to eliminate REM rebound after immobilization stress (Gonzalez and Valatx, 1997). This latter finding led to the suggestion that CRF is involved in the increased REM that occurs after immobilization stress (Gonzalez and Valatx, 1997); however, no one has administered CRF in conjunction with immobilization to confirm this hypothesis.

CRF also has been implicated in the effects of conditioned fear as evidenced by a role in the regulation of fear-potentiated acoustic startle. Both fear conditioning (Liang et al., 1992) and ICV administration of CRF (Swerdlow et al., 1986 ; Liang et al., 1992) can enhance the amplitude of acoustic startle. ICV administration of the non-selective CRF antagonist α HelCRF (α -helical CRF₉₋₄₁) blocks both CRF- and fear-potentiated startle, but does not alter lower baseline startle amplitude (Swerdlow et al., 1986). To our knowledge, the potential role of CRF in mediating fear-induced changes in EEG-determined arousal and sleep has not been examined.

In this study, we trained mice in a multi-trial contextual fear paradigm and administered CRF; astressin (AST), an antagonist at both CRF₁ and CRF₂ receptors (Gulyas et al., 1995); or vehicle (saline, SAL) alone, prior to exposure to the fearful context and recorded post-exposure sleep. The administration of CRF in an already stressful paradigm was prompted by our observations that contextual fear is not followed by recovery REM (Sanford et al., 2003; Sanford et al., 2003; Sanford et al., 2003) reported in other stress paradigms (Rampin et al., 1991; Gonzalez et al., 1995; Smith, 1995; Bonnet and Arand, 1997; Gonzalez and Valatx, 1997; Marinesco et al., 1999; Meerlo et al., 2001; Schiffelholz and Aldenhoff, 2002; Tang et al., 2004; Sanford et al., 2005; Tang et al., 2005; Tang et al., 2005) and suggestions that CRF is responsible for recovery REM after another stressor, restraint (Gonzalez and Valatx, 1997). That is, if CRF is responsible for recovery REM after stress, it should enhance post-stress REM after contextual fear. We conducted the study in “behaviorally reactive” BALB/cJ mice which show greater post-stress reductions in REM and alterations in sleep compared to C57BL/6J mice (Sanford et al., 2003; Sanford et al., 2003; Tang et al., 2004) and which also show greater alterations in sleep in response to CRF administered ICV (Sanford et al., 2008).

Results

Effects of CRF and AST on Fear-induced Alterations in Sleep: Between Groups Comparisons

Statistical analyses were conducted on the 8 h light period total, 12 h dark period total and 20 h recording period and comparisons were made between groups. The three treatment groups (SAL, CRF and AST) did not significantly differ on baseline sleep or on sleep after handling control in any of the measures we examined (see Figure 1, A - F). They also did not differ in NREM or total sleep after exposure to the fearful context (Figure 1, H & I). The only significant differences among groups were found in REM.

A comparison of light period REM in the groups receiving SAL, AST or CRF prior to the fearful context is presented in Figure 1 G. The ANOVA comparing the three groups on light period REM was significant [$F(2,22) = 5.60, p < .01$]. Post hoc Tukey tests revealed that administration of CRF prior to exposure to the fearful context produced a significant reduction in REM compared to AST, but not to SAL. Comparison of the groups for dark period REM also resulted in a significant ANOVA [$F(2,22) = 4.52, p < .023$]. Post hoc tests found significant reductions in REM in the group receiving CRF prior to exposure to the fearful context compared to both the AST and SAL groups. The comparison of groups over the entire 20 hour recording period was also significant [$F(2,33) = 7.48, p < .003$]. Post hoc comparisons found significant reductions in REM in the CRF group compared to the AST and SAL groups. REM in the AST group did not significantly differ from that in the SAL group in any of the comparison periods.

The reductions in REM amounts were accompanied by significant reductions in the number of REM episodes. Significant ANOVAs were found for the number of REM episodes in the light period [$F(2,22) = 3.96, p < .04$], dark period [$F(2,22) = 3.97, p < .04$], and total 20 hour recording period [$F(2,22) = 5.30, p < .013$]. Post hoc Tukey tests for the light period found reduced REM episodes in the CRF group (8.0 ± 1.85) compared to the AST group ($15.87 \pm 2.08; p < .03$) but not SAL group (12.33 ± 1.89). The number of REM episodes during the dark period was reduced in the CRF group (19.75 ± 2.58) compared to the SAL group ($29.33 \pm 2.43; p < .04$) but not AST group (27.75 ± 2.70). However, when the entire 20 h recording period was considered, the number of REM episodes after CRF (27.75 ± 4.07) was significantly reduced compared to both AST ($43.63 \pm 4.17; p < .02$) and SAL ($41.66 \pm 2.92; p < .04$) groups. There was no significant difference between groups in the duration of REM episodes.

Comparisons across groups of the REM percent (total REM/total sleep time) was significant for the light period [$F(2,22) = 5.41, p < .012$], dark period [$F(2,22) = 6.91, p < .005$], and total recording period [$F(2,22) = 9.55, p < .002$]. In the light period, REM percent was reduced in the CRF group (4.5 ± 0.97) compared to the AST group ($8.7 \pm 0.86; p < .012$) whereas the comparison to the SAL group (7.6 ± 0.93) did not reach significance ($p < .062$). In the dark period, REM percent in the CRF group (9.16 ± 0.97) was reduced compared to both AST ($12.88 \pm 0.56; p < .01$) and SAL ($12.77 \pm 0.79; p < .01$) groups. In the total 20 recording period, REM percent in the CRF group (7.13 ± 0.90) was reduced compared to both AST ($10.73 \pm 0.57; p < .002$) and SAL ($11.03 \pm 0.59; p < .004$) groups. There were no other significant differences across conditions or groups for any other sleep parameters that we examined.

Effects of CRF and AST on Fear-induced Alterations in REM: Within Groups Comparisons

Hourly totals of sleep for baseline and after exposure to the handling control and to the fearful context are plotted in Figure 2 for the groups receiving SAL (Figure 2, A and B), CRF (Figure 2, C and D) and AST (Figure 2, E and F). Because comparisons between groups do not accurately reflect differences in the way individual animals respond to stressful stimuli, we also compared REM, NREM and total sleep after contextual fear to that in the handling control condition. Compared to handling control, contextual fear significantly decreased REM in mice

receiving SAL during the 8 hour light period and during the total 20 hour recording period (Figure 3A). Amounts of NREM (Figure 3B) and total sleep (Figure 3C) during the 8 hour light period and during the total 20 hour recording period were also significantly reduced. Dark period sleep was not significantly altered indicating that the major changes in all sleep states occurred in the light period after exposure to contextual fear. Compared to handling control, contextual fear resulted in a significant reduction in the number of REM episodes in the 8 hour light period (Table 1) and a reduction in the average duration of NREM episodes in all recording periods (Table 1). There were no other significant differences.

Compared to handling control, the mice receiving CRF prior to exposure to contextual fear showed significant reductions in REM amounts during the 8 hour light period, the 12 hour dark period and during the total 20 hour recording period (Figure 3D). The reduction in amount of REM was complemented by significant reductions in REM% and reductions in the number of REM episodes during all recording periods (Table 1). Amounts of NREM (Figure 3E) and total sleep (Figure 3F) and other sleep parameters that we analyzed were not significantly altered.

Mice receiving ICV microinjections of AST (Figure 3G, H, I and Table 1) did not significantly differ from handling control in any of the sleep parameters that we examined.

Effects of CRF and AST on Fear-induced Alterations in NREM EEG Spectra

Comparisons of NREM delta power (0.5 to 5.0 Hz) were made between baseline, the handling control and fearful context conditions within each drug treatment group (Figure 4). NREM delta power (0.5 to 5.0 Hz) did not significantly differ between baseline, handling control or contextual fear conditions in the mice receiving either SAL (Figure 4A and D) or AST (Figure 4C and F). By comparison, the analyses for the CRF group was characterized by significant ANOVAs for the light period [$F(2,14) = 6.49, p < .01$], dark period [$F(2,14) = 5.09, p < .02$], and total 20 hour recording period [$F(2,14) = 6.37, p < .01$]. NREM delta power was significantly decreased in the mice receiving CRF compared to their handling control, but not to baseline (Figure 4B and E). The decrease NREM delta power was significant in analyses of the 8-h light, 12-h dark and the total 20-h recording period.

Discussion

The current results demonstrate a role for the central CRF system in regulating alterations in sleep that occur in the aftermath of contextual fear. Prior administration of CRF exacerbated the decrease in REM produced by contextual fear compared to that seen in mice receiving either SAL or AST. CRF also enhanced the reduction in REM after contextual compared to handling control whereas AST attenuated the fear-induced reduction in REM to levels seen in the handling control condition.

While there is considerable evidence that CRF is a major regulator of the stress response (Koob and Bloom, 1985; Heinrichs et al., 1995; Koob, 1999; Koob and Heinrichs, 1999; Bakshi and Kalin, 2000; Deussing and Wurst, 2005) and has a role in regulating spontaneous arousal (Opp, 1995; Opp, 1997; Chang and Opp, 1998; Chang and Opp, 2001), its role in regulating electrophysiologically defined sleep in the aftermath of stress has received minimal attention. A few earlier studies used immobilization or restraint as the stressor and immobilization stress applied at the onset of the dark period was reported to induce a subsequent increase in sleep, in particular, REM (Rampin et al., 1991). The ICV administration of α HelCRF in rats prior to immobilization prevented this increase, but did not alter spontaneous REM, NREM or wakefulness in non-stressed animals (Gonzalez and Valatx, 1997). However, other investigators reported no effect of restraint stress applied at the beginning of the dark period on subsequent sleep, and also no effect of AST on post-stress sleep (Chang and Opp, 2002). By comparison, restraint administered at the onset of the light period increased wakefulness

and decreased both NREM and REM, and ICV administration of AST attenuated the increase in wakefulness only in the five hour period immediately after restraint was removed (Chang and Opp, 2002). Differences in the procedures used for restraint (e.g., whether or not it was conducted in the home cage (Chang and Opp, 2002)) potentially could have accounted for different results in these studies.

Our study found that decreases in REM after contextual fear-induced were exacerbated with administration of CRF and attenuated with administration of AST (compared to handling control). The results of studies with the two types of stressors (restraint and contextual fear) may appear to be in conflict. However, in both studies, the antagonist was administered prior to exposure to the stressor and simply may have prevented or attenuated the CRF response to the stressor. Our data indicate that exogenous CRF increases stress-induced reductions in REM as well as reduces spontaneous REM (Sanford et al., 2008). Unfortunately, the studies involving immobilization stress did not examine the effects of ICV administration of CRF on post-immobilization sleep. Thus, the role of CRF in regulating post-stress sleep in the immobilization/restraint paradigm remains unclear; however, it is unlikely that stress-induced increases in CRF are directly responsible for enhanced REM that occurs after a number of stressors.

The brief manual restraint necessary for conducting microinjections is itself a stressor that can significantly alter subsequent sleep and that can influence the alterations in sleep produced by other stressors (Tang et al., 2007). For example, in rats, five minutes of manual restraint administered prior to 20 inescapable footshocks prevented the decrease in light period REM observed when the footshock was presented without prior restraint. The effects were limited to the light period suggesting that manual restraint significantly altered the initial changes in sleep induced by footshock stress. These findings suggest that our results should be considered in the context of the effects of CRF and AST on two interacting stressors, and not just their effects on behavior and sleep in response to contextual fear.

Several studies have indicated that, in the absence of stressors, CRF contributes to the regulation of spontaneous waking (Opp, 1995; Opp, 1997; Chang and Opp, 1998; Chang and Opp, 2001). CRF also has been implicated in the control of sleep. For example, in rats, ICV administration of the CRF antagonist, α HelCRF, every two h during sleep deprivation via the water-tank method reduced REM during the recovery period, but did not significantly alter NREM. (Gonzalez and Valatx, 1998). In a more recent paper in humans subjected to 40 h of sleep deprivation, multiple systemic administrations of CRF during recovery sleep decreased REM rebound after sleep deprivation (Schussler et al., 2006). Both studies are consistent with the hypothesis that CRF can reduce REM. The latter study in humans also reported an enhancement of NREM during the recovery period in people administered CRF compared to those receiving only placebo (Schussler et al., 2006).

In a recent study, we examined the effects of ICV administered three dosages of CRF (0.04 μ g, 0.2 μ g, 0.4 μ g) and three dosages of AST (0.1 μ g, 0.4 μ g, 1.0 μ g) on wakefulness and sleep in BALB/cJ mice and less reactive C57BL/6J mice (Sanford et al., 2008). In C57BL/6J mice, REM was significantly decreased after microinjections of the two higher dosages of CRF (0.2 μ g and 0.4 μ g), and NREM and total sleep were decreased after microinjection of the high dosage of CRF (0.4 μ g). None of the dosages of AST significantly changed wakefulness or sleep. By comparison, in BALB/cJ mice, all three concentration of CRF increased wakefulness and decreased NREM sleep and the middle and high dosage of CRF decreased REM. AST decreased active wakefulness and significantly increased REM at the low and high dosages, but did not significantly alter NREM. These strain differences in the effects of CRF and AST may be linked to the relative responsiveness of C57BL/6J and BALB/cJ mice to stressors and

to underlying differences in the CRF system. Differences in the CRF system have been reported for C57BL/6J mice and BALB/cJ mice (Blank et al., 2003), but have not been fully described.

Stress can be a significant factor in the etiology of insomnia (Healey et al., 1981; Basta et al., 2007) which has been hypothesized to be a disorder of hyperarousal in the central nervous system and not actual sleep loss (Bonnet and Arand, 1997; Vgontzas et al., 2001; Basta et al., 2007). This hypothesis is based on data that the hypothalamic-pituitary-adrenal (HPA) axis is more active in insomniacs who show higher levels of ACTH and cortisol than do individuals without insomnia (Vgontzas et al., 1998; Vgontzas et al., 2001; Basta et al., 2007). Based on elevated HPA axis activity and the fact that elevated levels of nocturnal cortisol found in insomnia are markers of elevated CRF, it has been suggested that treating the underlying disturbance in CRF could be a potential treatment for insomnia (Foa et al., 1992). The current work also suggests that CRF likely plays a significant role in the neural pathways by which stress induces arousal and reduces sleep.

The CRF system (Bremner et al., 1997; Baker et al., 1999; Sautter et al., 2003; Neylan et al., 2006; de Kloet et al., 2007) and fear conditioning (Foa et al., 1992; Shalev et al., 1992; Grillon et al., 1996) figure prominently in current ideas regarding the development of anxiety disorders including post-traumatic stress disorder (PTSD). Altered sleep in the aftermath of a traumatic event also is thought to play a role in PTSD and continuing difficulties in sleep appear to be predictive of future development of emotional and physical disorders (Lavie, 2001; Koren et al., 2002). PTSD is characterized by disturbed REM, hypervigilance to unfamiliar stimuli and stereotypical anxiety dreams (Ross et al., 1989; Ross et al., 1994) as well as insomnia (Harvey et al., 2003). Although the emphasis has been on REM, changes in NREM also occur (Schlosberg and Benjamin, 1978). Specifically, both visually scored delta sleep and EEG delta amplitude are reduced in PTSD patients (Neylan et al., 2006). The changes in NREM delta have been suggested to involve persistent increases in CRF activity coupled with either enhanced negative feedback or downregulated CRF receptors (Neylan et al., 2006). Our finding of reduced NREM delta, though not NREM amounts, in mice that received microinjections of CRF and psychological stress from exposure to a fearful context is consistent with a putative role of CRF in regulating NREM delta in stressful conditions.

The finding of reduced delta without a decrease in NREM after contextual fear in the CRF treated group may appear surprising as NREM delta has been reported to be increased in rats after other stressors including social stress (Meerlo et al., 2001) and stress induced by a simulated predator (Lesku et al., 2008). However, across studies using inescapable shock training similar to that reported here, changes in NREM have been more variable with some strains showing increases and some showing overall decreases compared to handling controls even though the shock parameters were identical. Unfortunately, the EEG spectra across studies and strains have not been fully characterized though there is some evidence that delta amplitude may vary as well. Indeed, we have found that NREM delta may be relatively less in mice that show greater reductions in REM (Tang et al., 2006). In addition, at least one study in rats using a relatively intense shock paradigm (Adrien et al., 1991) reported changes in sleep comparable to those we observed in mice that were treated with CRF prior to contextual fear. The rats were presented with 60 inescapable footshocks of relatively high intensity (0.8 mA) and duration (15 s) over the course of one hour. Compared to handling control rats that experienced the shock chamber without receiving footshock, rats trained with inescapable shock showed greater REM latency, and during the first three h after training, shock trained rats also showed reduced REM and increased light (non-delta) NREM compared to the control group and their own baseline sleep. Afterwards, REM returned to control amounts, but no REM rebound was observed in recordings that night or the following day. Light NREM also was enhanced over the dark period recordings. Thus, the changes we observed in mice treated with CRF, which

would be expected to enhance the stress response, may be similar to those that occur after a relatively more intense inescapable shock stressor.

In summary, the role of CRF in regulating spontaneous arousal and stress-induced alterations in arousal has been minimally studied. The current data demonstrate a role for CRF in modulating sleep that occurs after exposure to a psychological stressor. Elevated CRF can produce both significant reductions in REM and reductions in NREM delta. Work is now needed to determine the neural sites at which CRF act to alter arousal and sleep.

Experimental Procedure

Subjects

The subjects were 25 male BALB/c J mice weighing 20 to 25 gm. All animals were obtained from The Jackson Laboratory, Bar Harbor, Maine. The animals were individually housed after arrival, and food and water were available ad libitum. The recording room was kept on a 12:12 light:dark cycle with lights on from 7:00 AM to 7:00 PM. Ambient temperature was maintained at $24.5^{\circ}\text{C} \pm 0.5^{\circ}$.

Surgery

All mice were implanted intraperitoneally with telemetry transmitters (DataSciences ETA10-F20) for recording EEG and activity as previously described (Tang and Sanford, 2002). EEG leads from the transmitter body were led subcutaneously to the head, and the free ends were placed into holes drilled in the dorsal skull to allow recording cortical EEG. In the same surgery, the mice were stereotactically implanted with a cannula to allow ICV microinjections. A 1-mm hole in the skull drilled 1.00 mm lateral and 0.5-mm posterior to the Bregma (-0.5) and the tip of a 26-gauge stainless steel infusion cannula was placed 2.00 mm below the skull surface into the right ventricle. The cannula was secured to the skull with dental cement and a stylus was inserted to maintain patency. All surgery was conducted with the mouse under isoflurane (as inhalant: 5% induction; 2% maintenance) anesthesia. Ibuprofen (30 mg/kg, oral) was continuously available in each animal's drinking water for 24 to 48 h preoperatively and for a minimum of 72 h post operatively. All procedures were conducted in accordance with the National Institutes of Health *Guide for the Care and Use of Experimental Animals* and were approved by Eastern Virginia Medical School's Animal Care and Use Committee (Protocol # 05-017).

Drugs and Microinjections

After recovery from surgery, ICV location of the cannula was verified with administration of angiotensin (200 ng in 0.2 μl ICV) and observation for drinking (Walker and Romsos, 1992). Only mice that showed a clear angiotensin-induced drinking response were used in the study.

CRF and AST (cyclo(33)[D-Phe¹²,Nle^{21,38},Glu³⁰,Lys³³] h/rCRF₍₁₂₋₄₁₎) were obtained in powder form from Sigma-Aldrich (St. Louis, MO) and were diluted to the desired concentrations in pyrogen-free SAL. Concentrations (CRF 0.4 μg (0.42 mM); AST: 1.0 μg (1.4 mM)) used in this experiment were based on dosages effective in altering sleep in a previous study (Sanford et al., 2008). Separate groups of animals received ICV microinjections (0.2 μl) of CRF (n=8), AST (n=8) or SAL (n=9) prior to exposure to the fearful context.

For microinjections, injection cannulae (33 ga.), which projected 1.0 mm beyond the tip of the guide cannulae, were secured in place within the guide cannulae. The injection cannulae were connected to lengths of polyethylene tubing that in turn were connected to 5.0 μl Hamilton syringes. The injection cannulae and tubing had been pre-filled with the solution to be injected. The solutions in a volume of 0.2 μl were slowly infused over one min.

Determination of Behavioral State

Telemetry signals (EEG and activity) were processed by a DataSciences analog converter (ART Analog-8 CM) and routed to an A/D board (Eagle PC30) housed in a Pentium class PC. The signals were digitized at 256 Hertz (Hz) and collected in 10-second epochs using a commercial sleep data-collection program SleepWave™ (Biosoft Studio, Hershey, PA). The epochs were visually scored from computer records as wakefulness, NREM, or REM based on EEG and gross whole-body activity. A full description of the procedures for using telemetry to record sleep in mice can be found in (Tang and Sanford, 2002).

An on-line fast Fourier transformation (FFT) was performed on EEG data in consecutive 2 sec epochs of data (256 samples) after a Hanning window treatment. The FFT analyses generated the power density values from 0.0 to 63.5 Hz at a resolution of 0.5 Hz. The FFT data were further averaged in the range of 0 to 40 Hz in 10 sec epochs. The sleep data and FFT results were saved to the hard disk every 10 sec for additional off-line analyses.

Procedure for Fear Conditioning and Sleep Recording

The mice were housed and studied in the same room. For recording, individual home cages were placed on a DataSciences telemetry receiver (RPC-1) and the transmitter activated with a magnetic switch. When the animals were not on study, the transmitter was inactivated. Cages and bedding were changed 2 days prior to recording onset for each phase of the experiment and then not disturbed until that phase was complete.

The mice were habituated to three daily sessions of the handling procedures needed for administering microinjections. Afterwards, all mice received an ICV SAL microinjection followed by exposure to a Plexiglas® chamber of approximately the same size as chambers used for shock training and allowed to explore for 30 min. These were located outside the recording room. Afterwards, the mice were returned to their home cages and EEG and activity data were collected for 20 h. This latter procedure was designed to control for handling including transportation to another room and exposure to a novel environment, such as the mice would experience in the shock training portion of fear conditioning.

One week after experiencing the handling control, the mice were subjected to contextual fear conditioning. Fear conditioning was also conducted in chambers located outside the recording room. Coulbourn Precision Regulated Animal Shockers (Model E13-14) were used to produce shock, and Coulbourn Habitest cages (H10-11M-TC) equipped with grid floors were used to deliver the footshock. The cages were housed in Coulbourn Isolation Cubicles (Model H10-23). The chambers were thoroughly cleaned with diluted alcohol prior to each session.

Training was conducted during the fourth hour of the light period. The animals were placed in the shock chamber and allowed to freely explore for 5 minutes prior to beginning shock training. For shock training, the mice were presented with 20 footshocks (0.5 mA, 0.5-seconds duration) at equal intervals over the course of 20 minutes on 2 consecutive days. Five minutes after the last shock, the mice were returned to their home cages and EEG and activity data were collected for 20 h.

Five to 6 days following the last shock training trial, the shock-trained mice were given ICV microinjections of SAL, CRF or AST, placed in the shock chamber and allowed to explore freely for 30 minutes with no shock presented (FC). Afterward, the mice were returned to their home cages and sleep again was recorded for 20 hours.

Data Analyses

The study focused on the impact of CRF and AST on sleep and EEG delta power after exposure to an environment made fearful by previous administration of footshock. Baseline sleep (Tang and Sanford, 2002) and sleep after footshock training (Sanford et al., 2003; Sanford et al., 2003) in BALB/cJ mice have been described in a number of previous papers and these data are not included here.

Sleep parameters for the 20 h total sleep recording time and for the 8 h light and 12 h dark periods were examined. The following parameters were evaluated: total REM, NREM and sleep (as percentage of total recording time); REM percentage ($[\text{total REM} / \text{total sleep}] * 100$); number of REM and NREM episodes; average REM and NREM duration (in minutes). Comparisons were made between sleep after a microinjection of SAL followed by handling control exposure and sleep after an injection of SAL, CRF or AST followed by exposure to the fearful context. Therefore, each mouse served as its own control.

The absolute EEG power during wakefulness, NREM, and REM were calculated in 0.5 Hz bins from 0.5 to 30 Hz for the entire 20-h records of each recording day. These data were used to analyze EEG power in the delta frequency band (0.5 to 5.0 Hz) during NREM across conditions in each group.

All statistical analyses were conducted using SigmaStat software (SPSS, Inc., Chicago, Illinois). Separate between subjects ANOVAs were conducted across groups for comparisons of baseline, handling control and fearful context. Post hoc comparisons among means were conducted using Tukey tests when the ANOVAs indicated significant differences. In addition, we examined NREM delta power within groups and across treatment conditions with repeated measures ANOVAs followed by Tukey post hoc tests when appropriate. Comparisons of only two conditions within groups were conducted with paired t-tests.

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References

- Adrien J, Dugovic C, et al. Sleep-wakefulness patterns in the helpless rat. *Physiol Behav* 1991;49:257–262. [PubMed: 2062895]
- Aloisi AM, Bianchi M, et al. Neuroendocrine and behavioral effects of CRH blockade and stress in male rats. *Physiol Behav* 1999;66(3):523–8. [PubMed: 10357444]
- Baker DG, West SA, et al. Serial CSF corticotropin-releasing hormone levels and adrenocortical activity in combat veterans with posttraumatic stress disorder. *Am J Psychiatry* 1999;156(4):585–8. [PubMed: 10200738]
- Bakshi VP, Kalin NH. Corticotropin-releasing hormone and animal models of anxiety: gene-environment interactions. *Biol Psychiatry* 2000;48(12):1175–98. [PubMed: 11137059]
- Basso AM, Spina M, et al. Corticotropin-releasing factor antagonist attenuates the “anxiogenic-like” effect in the defensive burying paradigm but not in the elevated plus-maze following chronic cocaine in rats. *Psychopharmacology (Berl)* 1999;145(1):21–30. [PubMed: 10445369]
- Basta M, Chrousos GP, et al. Chronic Insomnia and Stress System. *Sleep Med Clin* 2007;2(2):279–291. [PubMed: 18071579]
- Blanchard RJ, Blanchard DC. Crouching as an index of fear. *J Comp Physiol Psychol* 1969;67(3):370–5. [PubMed: 5787388]
- Blank T, Nijholt I, et al. Corticotropin-releasing factor receptors couple to multiple G-proteins to activate diverse intracellular signaling pathways in mouse hippocampus: role in neuronal excitability and associative learning. *J Neurosci* 2003;23(2):700–7. [PubMed: 12533630]

- Bonnet MH, Arand DL. Hyperarousal and insomnia. *Sleep Med Rev* 1997;1(2):97–108. [PubMed: 15310517]
- Bremner J, Licinio J, et al. Elevated CSF corticotropin-releasing factor concentrations in posttraumatic stress disorder. *Am J Psychiatry* 1997;154:624–629. [PubMed: 9137116]
- Chang FC, Opp MR. Blockade of corticotropin-releasing hormone receptors reduces spontaneous waking in the rat. *Am J Physiol* 1998;275(3 Pt 2):R793–802. [PubMed: 9728077]
- Chang FC, Opp MR. Pituitary CRH receptor blockade reduces waking in the rat. *Physiology & behavior* 1999;67(5):691–6. [PubMed: 10604839]
- Chang FC, Opp MR. Corticotropin-releasing hormone (CRH) as a regulator of waking. *Neurosci Biobehav Rev* 2001;25(5):445–53. [PubMed: 11566481]
- Chang FC, Opp MR. Role of corticotropin-releasing hormone in stressor-induced alterations of sleep in rat. *Am J Physiol Regul Integr Comp Physiol* 2002;283(2):R400–7. [PubMed: 12121853]
- Chrousos GP. Stressors, stress, and neuroendocrine integration of the adaptive response. *Ann N Y Acad Sci* 1998;851:311–35. [PubMed: 9668623]
- de Kloet CS, Vermetten E, et al. Elevated plasma corticotrophin-releasing hormone levels in veterans with posttraumatic stress disorder. *Prog Brain Res* 2007;167:287–91. [PubMed: 18037027]
- Deak T, Nguyen KT, et al. The impact of the nonpeptide corticotropin-releasing hormone antagonist antalarmin on behavioral and endocrine responses to stress. *Endocrinology* 1999;140(1):79–86. [PubMed: 9886810]
- Deussing JM, Wurst W. Dissecting the genetic effect of the CRH system on anxiety and stress-related behaviour. *C R Biol* 2005;328(2):199–212. [PubMed: 15771006]
- Ehlers CL, Reed TK, et al. Effects of corticotropin-releasing factor and growth hormone-releasing factor on sleep and activity in rats. *Neuroendocrinology* 1986;42(6):467–74. [PubMed: 3084988]
- Foa EB, Zinbarg R, et al. Uncontrollability and unpredictability in post-traumatic stress disorder: an animal model. *Psychol Bull* 1992;112(2):218–38. [PubMed: 1454893]
- Gonzalez MM, Debilly G, et al. Sleep increase after immobilization stress: role of the noradrenergic locus coeruleus system in the rat. *Neurosci Lett* 1995;202(12):5–8. [PubMed: 8787817]
- Gonzalez MM, Valatx JL. Effect of intracerebroventricular administration of alpha-helical CRH (9-41) on the sleep/waking cycle in rats under normal conditions or after subjection to an acute stressful stimulus. *J Sleep Res* 1997;6(3):164–70. [PubMed: 9358394]
- Gonzalez MM, Valatx JL. Involvement of stress in the sleep rebound mechanism induced by sleep deprivation in the rat: use of alpha-helical CRH (9-41). *Behav Pharmacol* 1998;9(8):655–62. [PubMed: 9890255]
- Grillon C, Southwick SM, et al. The psychobiological basis of posttraumatic stress disorder. *Mol Psychiatry* 1996;1(4):278–97. [PubMed: 9118351]
- Gulyas J, Rivier C, et al. Potent, structurally constrained agonists and competitive antagonists of corticotropin-releasing factor. *Proc Natl Acad Sci U S A* 1995;92(23):10575–9. [PubMed: 7479843]
- Harvey AG, Jones C, et al. Sleep and posttraumatic stress disorder: a review. *Clin Psychol Rev* 2003;23(3):377–407. [PubMed: 12729678]
- Healey ES, Kales A, et al. Onset of insomnia: role of life-stress events. *Psychosom Med* 1981;43(5):439–51. [PubMed: 7313035]
- Heilig M, Koob G, et al. Corticotropin-releasing factor and neuropeptide Y: role in emotional integration. *TINS* 1994;17:80–85. [PubMed: 7512773]
- Heinrichs SC, Menzaghi F, et al. The role of CRF in behavioral aspects of stress. *Ann N Y Acad Sci* 1995;771:92–104. [PubMed: 8597448]
- Hode Y, Ratomponirina C, et al. Hypoexpression of benzodiazepine receptors in the amygdala of neophobic BALB/c mice compared to C57BL/6 mice. *Pharmacol Biochem Behav* 2000;65(1):35–8. [PubMed: 10638633]
- Koob G, Bloom F. Corticotropin-releasing factor and behavior. *Fed Proc* 1985;44:259–263. [PubMed: 3871412]
- Koob GF. Corticotropin-releasing factor, norepinephrine, and stress. *Biol Psychiatry* 1999;46(9):1167–80. [PubMed: 10560023]

- Koob GF, Heinrichs SC. A role for corticotropin releasing factor and urocortin in behavioral responses to stressors. *Brain Res* 1999;848(12):141–52. [PubMed: 10612706]
- Koren D, Arnon I, et al. Sleep complaints as early predictors of posttraumatic stress disorder: a 1-year prospective study of injured survivors of motor vehicle accidents. *Am J Psychiatry* 2002;159:855–857. [PubMed: 11986142]
- Lavie P. Sleep disturbances in the wake of traumatic events. *N Engl J Med* 2001;345(25):1825–32. [PubMed: 11752360]
- Lesku JA, Bark RJ, et al. Predator-induced plasticity in sleep architecture in wild-caught Norway rats (*Rattus norvegicus*). *Behavioural Brain Research*. 2008In Press
- Liang KC, Melia KR, et al. Corticotropin-releasing factor: long-lasting facilitation of the acoustic startle reflex. *J Neurosci* 1992;12(6):2303–12. [PubMed: 1351540]
- Marinesco S, Bonnet C, et al. Influence of stress duration on the sleep rebound induced by immobilization in the rat: a possible role for corticosterone. *Neuroscience* 1999;92(3)
- Marrosu F, Gessa GL, et al. Corticotropin-releasing factor (CRF) increases paradoxical sleep (PS) rebound in PS-deprived rats. *Brain Res* 1990;515(12):315–8. [PubMed: 2357570]
- Meerlo P, Easton A, et al. Restraint increases prolactin and REM sleep in C57BL/6J mice but not in BALB/cJ mice. *Am J Physiol Regul Integr Comp Physiol* 2001;281(3):R846–54. [PubMed: 11507000]
- Neylan TC, Otte C, et al. Neuroendocrine regulation of sleep disturbances in PTSD. *Ann N Y Acad Sci* 2006;1071:203–15. [PubMed: 16891571]
- Nijssen M, Croiset G, et al. Conditioned fear-induced tachycardia in the rat: vagal involvement. *Eur J Pharmacol* 1998;350:211–222. [PubMed: 9696410]
- Opp MR. Corticotropin-releasing hormone involvement in stressor-induced alterations in sleep and in the regulation of waking. *Adv Neuroimmunol* 1995;5(2):127–43. [PubMed: 7496608]
- Opp MR. Rat strain differences suggest a role for corticotropin-releasing hormone in modulating sleep. *Physiology & behavior* 1997;63(1):67–74. [PubMed: 9402617]
- Pawlyk AC, Jha S. k. et al. A rodent model of sleep disturbances in posttraumatic stress disorder: The role of context after fear conditioning. *Biol Psychiatry* 2005;57(3):268–77. [PubMed: 15691528]
- Paylor R, Tracy R, et al. DBA/2 and C57BL/6 mice differ in contextual fear but not auditory fear conditioning. *Behav Neurosci* 1994;108:810–817. [PubMed: 7986374]
- Phillips RG, LeDoux JE. Differential contribution of amygdala and hippocampus to cued and contextual fear conditioning. *Behav Neurosci* 1992;106(2):274–85. [PubMed: 1590953]
- Rampin C, Cespuglio R, et al. Immobilisation stress induces a paradoxical sleep rebound in rat. *Neurosci Lett* 1991;126(2):113–8. [PubMed: 1922920]
- Ross R, Ball W, et al. Sleep disturbance as the hallmark of posttraumatic stress disorder. *Am J Psychiatry* 1989;146:697–707. [PubMed: 2658624]
- Ross RJ, Ball WA, et al. Rapid eye movement sleep disturbance in posttraumatic stress disorder. *Biol Psychiatry* 1994;35(3):195–202. [PubMed: 8173020]
- Sanford LD, Fang J, et al. Sleep after differing amounts of conditioned fear training in BALB/cJ mice. *Behav Brain Res* 2003;147(12):193–202. [PubMed: 14659585]
- Sanford LD, Tang X, et al. Influence of shock training and explicit fear-conditioned cues on sleep architecture in mice: strain comparison. *Behav Genet* 2003;33(1):43–58. [PubMed: 12645821]
- Sanford LD, Xiao J, et al. Influence of avoidance training (AT) and AT cues on sleep in C57BL/6J (B6) and BALB/cJ (C) mice. *Sleep (Abstract Supplement)* 2005;28:A6.
- Sanford LD, Yang L, et al. Influence of contextual fear on sleep in mice: a strain comparison. *Sleep* 2003;26(5):527–40. [PubMed: 12938805]
- Sanford LD, Yang L, et al. Mouse strain differences in the effects of corticotropin releasing hormone (CRH) on sleep and wakefulness. *Brain Res* 2008;1190:94–104. [PubMed: 18053970]
- Sautter FJ, Bissette G, et al. Corticotropin-releasing factor in posttraumatic stress disorder (PTSD) with secondary psychotic symptoms, nonpsychotic PTSD, and healthy control subjects. *Biol Psychiatry* 2003;54(12):1382–8. [PubMed: 14675802]
- Schiffelholz T, Aldenhoff JB. Novel object presentation affects sleep-wake behavior in rats. *Neurosci Lett* 2002;328(1):41–4. [PubMed: 12123855]

- Schlosberg A, Benjamin M. Sleep patterns in three acute combat fatigue cases. *J Clin Psychiatry* 1978;39(6):546–9. [PubMed: 207680]
- Schussler P, Yassouridis A, et al. Growth hormone-releasing hormone and corticotropin-releasing hormone enhance non-rapid-eye-movement sleep after sleep deprivation. *Am J Physiol Endocrinol Metab* 2006;291(3):E549–56. [PubMed: 16912060]
- Shalev AY, Ragel-Fuchs Y, et al. Conditioned fear and psychological trauma. *Biol Psychiatry* 1992;31(9):863–5. [PubMed: 1637927]
- Smith C. Sleep states and memory processes. *Behav Brain Res* 1995;69(12):137–45. [PubMed: 7546305]
- Spina MG, Basso AM, et al. Behavioral effects of central administration of the novel CRF antagonist astressin in rats. *Neuropsychopharmacology* 2000;22(3):230–9. [PubMed: 10693150]
- Stiedl O, Tovote P, et al. Behavioral and autonomic dynamics during contextual fear conditioning in mice. *Auton Neurosci* 2004;115(12):15–27. [PubMed: 15507402]
- Swerdlow N, Geyer M, et al. Corticotropin-releasing factor potentiates acoustic startle in rats: Blockade by chlordiazepoxide *Psychopharmacology* 1986;88:147–152.
- Tang X, Liu X, et al. Rat strain differences in sleep after acute mild stressors and short-term sleep loss. *Behav Brain Res* 2005;160(1):60–71. [PubMed: 15836901]
- Tang X, Sanford LD. Telemetric recording of sleep and home cage activity in mice. *Sleep* 2002;25(6):691–9. [PubMed: 12224849]
- Tang X, Xiao J, et al. Strain differences in the influence of open field exposure on sleep in mice. *Behav Brain Res* 2004;154(1):137–47. [PubMed: 15302119]
- Tang X, Xiao J, et al. Differential effects of two types of environmental novelty on activity and sleep in BALB/cJ and C57BL/J mice. *Physiol Behav* 2005;85:419–429. [PubMed: 16019041]
- Tang X, Yang L, et al. Spectral EEG Power after Uncontrollable Shock (US) and Fearful Context (FC): Variability amongst Mouse Strains. *Sleep (Abstract Supplement)* 2006;29:A11.
- Tang X, Yang L, et al. Interactions between brief restraint, novelty and footshock stress on subsequent sleep and EEG power in rats. *Brain Res* 2007;1142:110–8. [PubMed: 17300767]
- Vgontzas AN, Bixler EO, et al. Chronic insomnia is associated with nyctohemeral activation of the hypothalamic-pituitary-adrenal axis: clinical implications. *J Clin Endocrinol Metab* 2001;86(8):3787–94. [PubMed: 11502812]
- Vgontzas AN, Tsigos C, et al. Chronic insomnia and activity of the stress system: a preliminary study. *J Psychosom Res* 1998;45(1 Spec No):21–31. [PubMed: 9720852]
- Walker HC, Romsos DR. Glucocorticoids in the CNS regulate BAT metabolism and plasma insulin in ob/ob mice. *Am J Physiol* 1992;262(1 Pt 1):E110–7. [PubMed: 1733241]

Figure 1.

Between group comparisons of the time spent in REM, NREM and total sleep during 8-h light, 12-h dark and total 20-h recording periods for baseline (A, B, C), after exposure to handling control (D, E, F) and fearful context (G, H, I). The data are plotted as percentage total time for light, dark and total recording periods. Significant differences for comparisons to SAL (S) and AST (A) are indicated, Tukey test, $p < .05$.

Figure 2.

Hourly plots of time spent in REM (left panels) and NREM (right panels) for uninterrupted baseline (Base), after exposure to handling control (Ctrl) and after fearful context (FC) in the mice receiving ICV microinjections of SAL (A, B), CRF (C, D) and AST (E, F). The heavy line on the X-axis indicates the dark period.

Figure 3.

Time spent in REM, NREM and total sleep during 8-h light, 12-h dark and total 20-h recording periods after exposure to handling control (Ctrl) and fearful context (FC) in the mice receiving ICV microinjections of SAL (A, B, C), CRF (D, E, F) and AST (G, H, I). The data are plotted as percentage total time for light, dark and total recording periods. Differences between treatments: * $p < 0.05$, ** $p < 0.01$, paired t-test.

Figure 4.

The upper panels show NREM delta power (0.5 to 5.0 Hz) plotted hourly across the 20 h of recording for SAL (A), CRF (B) and AST (C). The lower panels show total NREM delta power during the 8-h light, 12-h dark and total 20-h recording periods after exposure to handling control (Ctrl) and fearful context (FC) in mice receiving ICV microinjections of SAL (D), CRF (E) and AST (F). The heavy line on the X-axis indicates the dark period. Significant differences for comparisons to Ctrl (c) are indicated, Tukey test, $p < .05$.

Selected sleep parameters (Means $\pm$ SEM) after exposure to novel and fearful environments. All mice received ICV microinjections of SAL prior to exposure to the handling control and separate groups of mice received SAL (n=9), CRF (n=8) or AST (n=8) prior to exposure to the fearful context. All comparisons were made between the novel and fearful environments within groups.

Table 1

	<u>SAL</u>		<u>CRF</u>		<u>AST</u>	
	<u>Handling Control</u>	<u>Fearful Context</u>	<u>Handling Control</u>	<u>Fearful Context</u>	<u>Handling Control</u>	<u>Fearful Context</u>
REM% (REM/TST)						
Light 8-h	8.7 (0.86)	7.6 (0.93)	7.3 (0.54)	4.5 (0.91) *	8.7 (0.76)	8.7 (0.86)
Dark 12-h	13.1 (0.62)	12.8 (0.79)	12.7 (0.57)	9.2 (0.91) **	12.3 (0.36)	12.9 (0.56)
Total 20-h	11.2 (0.53)	10.7 (0.57)	10.3 (0.53)	7.1 (0.85) *	10.6 (0.44)	11.0 (0.59)
Number of REM Episodes						
Light 8-h	15.6 (2.22)	12.3 (1.89) *	13.8 (1.0)	8.0 (1.75) *	15.0 (1.25)	15.9 (2.08)
Dark 12-h	30.8 (1.88)	29.3 (2.43)	30.0 (1.76)	19.7 (2.43) ***	26.5 (1.97)	27.8 (2.70)
Total 20-h	46.3 (3.50)	41.7 (2.92)	43.8 (1.90)	27.3 (3.84) **	41.5 (2.01)	43.6 (4.17)
Average Duration of REM Episodes						
Light 8-h	1.5 (0.07)	1.5 (0.06)	1.4 (0.11)	1.6 (0.25)	1.6 (0.1)	1.5 (0.09)
Dark 12-h	1.5 (0.05)	1.4 (0.08)	1.4 (0.05)	1.3 (0.07)	1.5 (0.05)	1.5 (0.06)
Total 20-h	1.5 (0.05)	1.4 (0.06)	1.4 (0.04)	1.4 (0.07)	1.5 (0.05)	1.5 (0.07)
Number of NREM Episodes						
Light 8-h	56.0 (5.93)	63.6 (6.92)	60.0 (5.27)	55.6 (6.21)	62.0 (6.4)	59.3 (5.78)
Dark 12-h	72.8 (4.98)	85.0 (5.89)	72.6 (5.21)	73.6 (5.43)	75.1 (5.73)	72.4 (5.77)
Total 20-h	128.8 (6.54)	148.6 (10.49)	132.6 (5.94)	129.3 (10.87)	137.1 (10.71)	131.6 (9.07)
Average Duration of NREM Episodes						
Light 8-h	4.6 (0.47)	3.7 (0.42) *	4.3 (0.36)	4.4 (0.74)	4.3 (0.5)	4.3 (0.55)
Dark 12-h	4.2 (0.17)	3.4 (0.22) **	3.9 (0.15)	3.6 (0.40)	3.8 (0.27)	4.0 (0.33)
Total 20-h	4.3 (0.25)	3.5 (0.27) **	4.0 (0.21)	3.9 (0.52)	4.0 (0.36)	4.1 (0.35)

* p < 0.05,

** p < 0.01,

p < 0.001, paired t-tests.