Basolateral Amygdala and the Regulation of Fear-Conditioned Changes in Sleep: Role of Corticotropin-Releasing Factor

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Study Objective: To determine whether corticotropin-releasing factor (CRF) in the basolateral amygdala (BLA) modulated sleep and fear-conditioned alterations in sleep.

Design: After 2 days of habituation to recording procedures, baseline sleep recordings were obtained. The animals were then habituated to the handling procedure necessary for microinjections over 2 consecutive days. In experiment 1, rats received microinjections of 0.5 μL antalarmin (1.61 or 4.82 mM), a CRF receptor 1 antagonist, or distilled water once a week for 3 wk. In experiment 2, rats received a microinjection of either antalarmin or vehicle prior to inescapable shock training (ST; 20 shocks; 0.8 mA, 0.5 sec; 1 min interstimulus interval). The animals were placed back in the context 7 days later for 30 min without shock (CR; context re-exposure). Sleep was recorded for 8 h after each manipulation.

Setting: NA.

Subjects: Outbred Wistar rats.

Interventions: The rats were surgically implanted with electrodes for recording the electroencephalogram and electromyogram for determining arousal state and with bilateral guide cannulae directed at BLA.

Measurements and Results: Antalarmin microinjected into BLA did not significantly alter sleep under undisturbed conditions. However, antalarmin microinjected bilaterally into BLA prior to ST blocked reductions in rapid eye movement sleep that ST normally produces. Further, the single microinjection prior to ST blocked the reduction in rapid eye movement typically seen after subsequent CR. Behavioral freezing, an indicator of fear memory, was not altered.

Conclusions: CRF in BLA is involved in regulating stress-induced alterations in sleep and it plays a role in modulating how stressful memories influence sleep.

Keywords: Amygdala, corticotropin-releasing factor, fear conditioning, sleep

Citation: Wellman LL; Yang L; Ambrozewicz MA; Machida M; Sanford LD. Basolateral amygdala and the regulation of fear-conditioned changes in sleep: role of corticotropin-releasing factor. *SLEEP* 2013;36(4):471-480.

INTRODUCTION

Contextual fear conditioning is a variant of classic conditioning in which initially neutral environments come to elicit fear responses through association with an unconditioned, fear-inducing stressor (usually inescapable foot shock).[1] Subsequently, the fearful context alone elicits behavioral and physiologic responses indicative of fear and anxiety. Much of the work on contextual fear has focused on immediate responses to the fearful context including behavioral freezing, an indicator of fear memory,[2-4] autonomic activation,[5-7] and fear-potentiated startle.[1] Foot shock stress and fearful contexts also can produce alterations in sleep that can persist for several hours even after an animal has been returned to its home cage, a safe environment. Changes in sleep after training with inescapable foot shock and reexposure to related contexts include a prominent reduction in rapid eye movement (REM) sleep that occurs in the first few hours after exposure.[8-11] Nonrapid eye movement (NREM) sleep also can be altered.[12-14]

The basolateral amygdala (BLA) has an established, although not fully understood, role in the formation and consolidation of memories for emotional or stressful events. A variety of manipulations that damage or inactivate BLA prior to or after training[15-20] or prior to context re-exposure[21,22] attenuate freezing. BLA also has a role in the regulation of both NREM[23] and REM,[24] but its role in regulating the effects of fear on sleep has not been established.

Corticotropin-releasing factor (CRF) plays a significant role in mediating central nervous system responses to stressors[25-30] and has roles in anxiety and conditioned fear. Intracerebroventricular (ICV) administration of CRF in rats produces many of the signs associated with anxiety in humans, including increased wakefulness,[31-34] altered locomotor activity, and an exaggerated startle response.[35,36] Both fear conditioning[1] and ICV administration of CRF[1,36] can enhance the amplitude of acoustic startle whereas ICV administration of the nonselective CRF antagonist α-helical CRF (α-HelCRF)[9-41] blocks both CRF- and fear-potentiated startle, but does not alter lower baseline startle amplitude.[36]

CRF also plays a role in fear-conditioned alterations in sleep. In mice, ICV administration of CRF enhances the reduction in REM after fearful contexts associated with inescapable foot shock whereas ICV administration of the nonspecific CRF antagonist, astressin, attenuates fear-induced reductions in REM.[37] In addition, we have found that microinjections of the CRF1 receptor antagonist, antalarmin (ANT), into the central nucleus of the amygdala (CNA) in rats block fear-induced reductions in REM and attenuate Fos expression, a marker of neural activation, in regions important in stress and REM regulation includ-

ing the hypothalamic paraventricular nucleus, locus coeruleus, and dorsal raphe nucleus.[38] However, ANT microinjected into CNA does not alter freezing in the fearful context, a finding consistent with other work demonstrating that fear behavior in waking can be dissociated from post-fear changes in sleep.[39]

Most of the work on CRF regulation of the amygdala has focused on CNA; however, the BLA has greater densities of CRF1 receptors[40,41] and there are indications that they play a role in conditioned fear.[42] Their potential role in regulating sleep has not been examined. In this study, we examined the effects of microinjections of ANT into the BLA on spontaneous sleep. We also microinjected ANT into the BLA prior to inescapable foot shock training to determine potential roles of CRF in fear acquisition, and we tested for potential effects on contextual fear in a subsequent drug-free exposure to the training context alone. In this second experiment, we examined freezing as an index of fear and examined sleep after training and after context reexposure alone. Thus, we assessed the role of CRF in the BLA in regulating spontaneous sleep and we examined the potential role of the BLA in mediating the relationship between waking fear behavior and sleep.

METHODS

Study Subjects

The 25 study subjects were 90-day-old Wistar rats obtained from Harlan (Indianapolis, IN). Upon arrival, the rats were individually housed in polycarbonate cages and given *ad libitum* access to food and water. The rooms were kept on a 12:00:12:00 light:dark cycle with lights on from 07:00 to 19:00 h. Light intensity during the light period was 100–110 lux and less than 1 lux during the dark period. Ambient temperature was maintained at 24.5 ± 0.5°C.

Surgery

Beginning 1 week after arrival, the rats were anesthetized with isoflurane (5% induction; 2% maintenance) and implanted with skull screw electrodes for recording the electroencephalogram (EEG) and stainless-steel wire electrodes sutured to the dorsal neck musculature for recording the electromyogram (EMG). Leads from the recording electrodes were routed to a nine-pin miniature plug that mated to one attached to a recording cable. Bilateral guide cannulae (26 ga) for microinjections into the BLA were implanted with their tips aimed 1.0 mm above BLA (2.6 posterior, 4.8 lateral, and 8.0 ventral to bregma).[43] The recording plug and cannulae were affixed to the skull with dental acrylic and stainless-steel anchor screws. Ibuprofen (15 mg/kg) was made available in the water supply for relief of postoperative pain. 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 # 07-005).

Drugs

The CRF1 receptor antagonist ANT was obtained from Sigma-Aldrich, St. Louis, MO, USA. All dosages were prepared in pyrogen-free distilled water (dW) and were sonicated for 20 min to ensure that the drug was dissolved completely. A fresh solution was prepared for each experimental day.

Procedures

All experimental manipulations were conducted during the fourth hour of the light period such that sleep recording would begin at the start of the fifth hour. This resulted in 8-h of light period recording on each experimental day.

Home cages were changed at least 3 days prior to injection day. The same room was used for animal housing and sleep recording. The microinjections and behavioral testing were conducted in a room separate from that used for recording.

Sleep Recording

For recording sleep, each animal, in its home cage, was placed on a rack outfitted for electrophysiologic recording and a lightweight, shielded cable was connected to the miniature plug on the rat's head. The cable was attached to a swivel that permitted free movement of the rat within its cage. EEG and EMG signals were processed by a Grass, Model 12 polygraph equipped with model 12A5 amplifiers (West Warwick, RI) and routed to an analog-to-digital board (Eagle PC30) housed in a Pentium class personal computer. The signals were digitized at 128 Hz and collected in 10-sec epochs using a custom sleep data collection program.

The rats were allowed a postsurgery recovery period of 14 days prior to beginning the experiment. Once recovered, the animals were randomly assigned to one of three groups: injection only (n = 8) for studies of effects of ANT on undisturbed sleep or to ANT prior to shock training (ANT-ST; n = 10) or dW prior to shock training (dW-ST; n = 7) for studies of its effects on shock training and fear. All rats were habituated to the recording cable and chamber over 3 consecutive days. Then the rats were habituated to the 5-min handling procedure necessary for microinjections over 2 consecutive days and a baseline following handling (BH) was recorded.

Microinjections

For microinjections, injection cannulae (33 ga) were secured in place within the guide cannulae, projecting 1.0 mm beyond the tip of the guide cannulae for delivery of drug into the target region. The injection cannulae were connected to one end of lengths of polyethylene tubing that had the other end connected to 5.0 μL Hamilton syringes. The injection cannulae and tubing were prefilled with the solution to be injected. Once the cannulae were in place, 0.5 μL of either drug or vehicle was bilaterally infused over 3-min. The cannulae were left in place 1-min preinjection and postinjection to allow for maximal absorption of the solution.

Fear Conditioning

Each ST session lasted 30-min. During this procedure, individual rats were placed in shock chambers (Coulbourn (Street Whitehall, PA) Habitest cages equipped with grid floors (Model E10-18RF) that were housed in Coulbourn Isolation Cubicles (Model H10-23)) and were allowed to freely explore for 5-min. Over the next 20 min, they were presented with 20 foot shocks (0.8 mA, 0.5-sec duration) at 1.0-min intervals. Shock was produced by Coulbourn Precision Regulated Animal Shockers (Model E13-14) and presented via the grid floor of the shock chamber. Five min after the last shock, the rats were returned to their home cages. On the CR day, the rats were returned to the shock chambers and allowed to explore freely for 30-min

(no shock presented) before being returned to their home cage. The shock chamber was thoroughly cleaned with diluted alcohol following each session. Each session was videotaped using mini video cameras (Weldex (Cypress, CA), WDH-2500BS, 3.6-mm lens) attached to the center of the ceiling of the shock chamber for subsequent visual scoring of freezing.

Experimental Design

Experiment 1

Experiment 1 assessed the effects of microinjections of ANT into the BLA on otherwise undisturbed sleep. The rats received an injection of either ANT (1.61 or 4.82 mM) or dW before being returned to the home cage for sleep recording. Injections were administered in a pseudo-randomized order at 7 day intervals over a period of 3 wk.

Experiment 2

Experiment 2 assessed the effects of microinjections of ANT into the BLA on changes in sleep induced by ST and on subsequent fear-induced alterations in sleep. On experimental day 1, animals in the ANT-ST and dW-ST groups received microinjections of drug (4.82 mM) or dW, respectively, prior to ST. Following ST, the animals were returned to their home cage for sleep recording. Seven days later, the animals underwent CR and were then returned to their home cage for sleep recording. The animals did not receive a microinjection on the CR day.

Data Analyses

Sleep

Computerized EEG and EMG records were visually scored by trained observers blind to drug condition in 10-sec epochs to determine wakefulness, NREM, and REM. Wakefulness was scored based on the presence of low-voltage, fast EEG and high amplitude, tonic EMG levels. NREM was characterized by the presence of spindles interspersed with slow waves, lower muscle tone, and no gross body movements. REM was scored continuously during the presence of low voltage, fast EEG, theta rhythm, and muscle atonia. Data were collapsed into 4-h blocks (Block 1 and Block 2) and total 8-h light period. The following sleep parameters were examined in the data analyses: total NREM (min), total REM (min); total sleep (REM + NREM), REM% (REM/total sleep*100) and number and average duration of NREM and REM episodes (defined as contiguous 10-sec epochs of a given state).

The sleep data for Experiment 1 were analyzed with repeated-measures analyses of variance (ANOVAs). The data in Experiment 2 was analyzed with two-way mixed factors (Group (dW-ST and ANT-ST) × Treatment (days)) ANOVAs with repeated measures on Treatment. *Post-hoc* Tukey tests were used to determine differences among means as appropriate.

Freezing

Videotapes of the ST and CR sessions were scoring for freezing, defined as the absence of body movement except for respiration.[44,45] Freezing was scored by a trained observer blind to condition in 5-sec intervals during 1.0-min observation periods over the course of the 30-min the rats were in the shock chamber. The percent-

age of time spent freezing was calculated (FT%: freezing time/ observed time × 100) for each animal for each observation period.

The freezing data for ST were analyzed in three periods: the 5-min preshock period, the following 20-min shock period (during which shock was experienced) and the 5-min postshock period. Freezing data for the CR day were analyzed in three 10-min blocks and compared with the preshock period on the ST day. The data for the preshock, shock, and postshock periods were analyzed with separate two-way mixed factors (Group (dW-ST and ANT-ST) × Period (Pre, Shock and Post for ST; Pre, Blocks1-3 for CR)) ANOVAs with repeated measures on Period. *Post-hoc* comparisons were conducted with Tukey tests. All reported sleep and freezing data were examined for and passed the normality test prior to conducting the relevant ANOVA.

RESULTS

Experiment 1

Histology

In the injection-only group, one animal had to be excluded in the middle of the study due to signal loss and another was excluded due to improperly placed cannulae. Therefore, statistical analysis was completed on six animals whose cannulae were bilaterally located in the BLA (Figure 1A).

Sleep

There were no significant differences between dW and either the 1.62 mM or 4.82 mM concentrations of ANT of any of the sleep measures (REM, NREM, or total sleep) we examined over the 8-h recording period (Table 1).
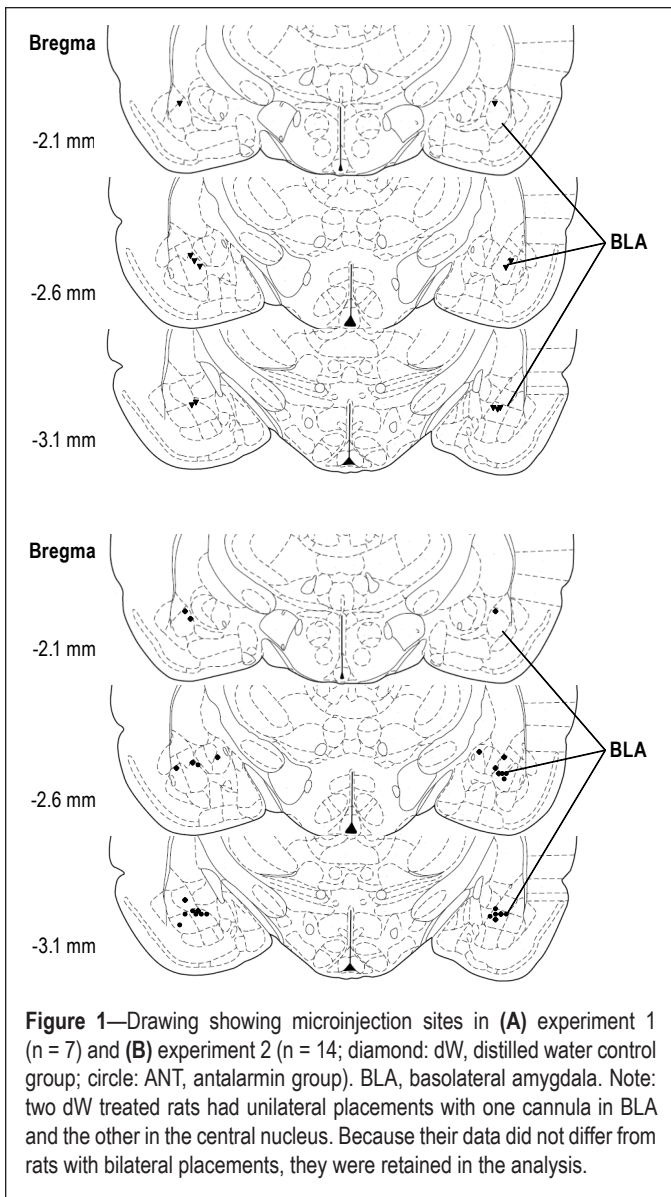
Experiment 2

Histology

In the dW-ST group, three of seven animals had one cannula located in the area between the BLA and the CNA. We ran two-way ANOVA and *t*-tests comparing the sleep parameters between the animals with bilateral BLA cannulae and the animals with unilateral BLA cannulae and did not find any significant differences. Therefore, all animals in the dW-ST group were used for further statistical analyses (Figure 1B).

In the ANT-ST group, three animals had one cannula located in the area between the BLA and CNA. Analyses using two-way ANOVA and *t*-tests showed significant differences between animals with bilateral BLA cannulae and the animals with unilateral BLA cannula. These data indicated that bilateral administration of ANT into the BLA was required to produce significant effects on REM. Therefore, data for the seven animals with bilateral BLA cannulae were used in the full statistical analyses (Figure 1B).

Freezing

On the ST day, there was a main effect of period ($F_{2,24}$ = 30.8, P < 0.001). FT% was significantly increased during the shock (P < 0.001) and postshock (P < 0.001) periods compared with the preshock period (Figure 2A). However, the two groups did not differ in FT%.

On the CR day, there was a main effect of block for period ($F_{3,36}$ = 7.1, P = < 0.001). FT% was significantly increased during

Figure 1—Drawing showing microinjection sites in **(A)** experiment 1 (n = 7) and **(B)** experiment 2 (n = 14; diamond: dW, distilled water control group; circle: ANT, antalarmin group). BLA, basolateral amygdala. Note: two dW treated rats had unilateral placements with one cannula in BLA and the other in the central nucleus. Because their data did not differ from rats with bilateral placements, they were retained in the analysis.

Table 1

	Block 1	Block 2	Total
REM Duration			
dW	5.89 (1.6)	17.9 (1.3)	23.8 (2.7)
ANT-L	11.5 (2.9)	18.2 (1.8)	29.7 (4.1)
ANT-H	6.8 (2.4)	12.8 (2.8)	19.6 (4.6)
REM Episodes			
dW	3.7 (1.1)	10.3 (0.7)	14.0 (1.5)
ANT-L	3.5 (1.2)	7.3 (1.7)	10.8 (2.6)
ANT-H	7.3 (1.7)	10.3 (1.2)	17.7 (2.1)
Avg REM Duration			
dW	1.7 (0.1)	1.7 (0.1)	1.7 (0.9)
ANT-L	1.9 (0.2)	1.5 (0.4)	1.8 (0.2)
ANT-H	1.6 (0.1)	1.9 (0.3)	1.7 (0.1)
NREM Duration			
dW	143 (6.5)	127 (5.1)	270 (10.0)
ANT-L	141 (3.1)	128 (5.9)	268 (7.1)
ANT-H	135 (8.6)	137 (2.9)	272 (8.3)
NREM Episodes			
dW	31.0 (3.0)	37.5 (3.1)	68.5 (4.6)
ANT-L	29.7 (4.9)	39.3 (2.0)	69.0 (4.3)
ANT-H	31.5 (3.7)	37.0 (2.5)	68.5 (5.8)
Avg NREM Duration			
dW	4.9 (0.5)	3.5 (0.4)	4.1 (0.4)
ANT-L	5.1 (0.8)	3.5 (0.1)	4.0 (0.3)
ANT-H	4.9 (0.8)	3.6 (0.4)	4.1 (0.5)

Time spent (min) in REM/NREM, REM/NREM episode number and REM/NREM average duration (mean (SEM)) during 4-h blocks (Block 1 & Block 2) and total 8-h light period after microinjection of vehicle (dW, distilled water) or ANT (L - 1.61mM or H - 4.82 mM; 0.5µL) into BLA.

each 10-min block compared with preshock values (Figure 2B). There were no significant differences in FT% between groups or across blocks on the CR day.

REM Sleep

The analysis of total REM during Block 1 was characterized by a significant Group × Treatment interaction ($F_{2,24} = 17.8$, $P < 0.001$). *Post-hoc* analyses for each recording day found no difference in sleep on BH, but REM was reduced in the dW-ST group compared with the ANT-ST group (Figure 3) on the ST day ($P < 0.001$) and on the CR day ($P < 0.001$). Comparisons within groups for the dW-ST rats (Figure 4) found significantly reduced REM during ST ($P < 0.001$) and CR ($P < 0.002$) compared with BH. REM was also significantly reduced on ST compared with CR ($P < 0.032$). Total REM in the ANT-ST rats did not significantly differ across recording days (Figure 5).

The analysis of total REM during Block 2 was characterized by a significant Group Main Effect ($F_{1,12} = 5.4$, $P < 0.04$). Total REM was greater in the ANT-ST rats, but the comparison across treatment days was not significant.

The analysis of total REM for the 8-h recording period revealed a significant Group × Treatment interaction ($F_{2,24} = 5.2$, $P < 0.015$). *Post-hoc* analyses for each recording day found no difference in sleep on BH, but REM was reduced in the dW-ST group compared with the ANT-ST group on the ST day ($P < 0.005$) and on the CR day ($P < 0.005$). Comparisons within groups for the dW-ST rats found significantly reduced REM during ST ($P < 0.001$) and CR ($P < 0.02$) compared with BH. REM on ST and CR did not significantly differ. The ANT-ST rats did not significantly differ across recording days.

The analysis of REM% during Block 1 revealed significant Group ($F_{1,12} = 14.5$, $P < 0.003$) and Treatment ($F_{2,24} = 10.7$, $P < 0.001$) main effects. REM% was greater in the ANT-ST rats, but did not differ across treatment days. There was no difference in Block 2. However, when the entire 8-h recording period was considered, there were significant differences between groups and within days. The analysis for total 8-h REM% reveal a significant Group × Treatment interaction ($F_{2,24} = 4.4$, $P < 0.025$). REM% did not differ between groups on BH (dW = 10.3% ± 1.1%; ANT = 13.1% ± 1.1%), but was significantly greater in the ANT-ST rats after ST ($P = 0.005$; 11.1% ± 1.5%) and after CR ($P = 0.015$; 12.2% ± 0.5%) than the dW rats (ST = 4.3% ± 1.4% and CR = 7.1% ± 1.1%). REM% was decreased in the dW-ST rats on ST ($P < 0.001$)

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and CR (P < 0.008) compared with BH, whereas the ANT-ST rats did not differ across days.

The analysis of REM episodes (Table 2) during Block 1 was characterized by a significant Group × Treatment interaction ($F_{2,24}$ = 3.628, P = 0.042). *Post-hoc* analyses for each recording day found no difference in sleep on BH, but REM episodes were reduced in the dW-ST group compared with the ANT-ST group on the ST day (P = 0.001) and on the CR day (P = 0.048). Comparisons within groups for the dW-ST rats found significantly reduced REM episodes during ST (P = 0.002) compared with BH. The ANT-ST rats did not significantly differ across recording days. Comparisons for Block 2 were not significant.

The analysis of REM episodes for the 8-h recording period revealed a significant Group × Treatment interaction ($F_{2,24}$ = 3.911, P = 0.034). *Post-hoc* analyses for each recording day found no difference in sleep on BH, but REM episodes were reduced in the dW-ST group compared with the ANT-ST group on the ST day (P = 0.008) and there was a trend toward a reduction on the CR day (P = 0.054). Comparisons within groups for the dW-ST rats found significantly reduced REM episodes during ST (P = 0.018) compared with BH. The ANT-ST rats did not significantly differ across recording days.

Total Sleep and NREM Sleep

The analyses for total sleep amount revealed a significant Treatment Main Effect for Block 1 ($F_{2,24}$ = 4.2, P = 0.03). Total sleep was decreased during Block 1 after ST compared with BH (P = 0.04). The difference between BH and CR did not reach significance (P = 0.08). There was no significant difference between groups. The analysis of total sleep in Block 2 or the total 8-h recording period did not find differences for either groups or treatments.

The analysis did not reveal any significant differences in total NREM or NREM episode duration for either 4-h block or total 8-h period.

DISCUSSION

The current study demonstrates that microinjecting ANT into the BLA does not significantly alter sleep under baseline, undisturbed conditions, but can significantly attenuate stress- and fear-induced reductions in REM. Consistent with previous work on fear conditioning in rats,[8] training with inescapable foot shock and reexposure to the shock context alone reduced REM in animals receiving vehicle microinjections. By comparison, bilateral (but not unilateral) microinjections of ANT into BLA before training blocked reductions in REM on both ST and CR. Moreover, the effect of ANT on sleep was observed even though it did not significantly alter freezing compared with a control group on either the ST or CR day. These results demonstrate a significant role for CRF1 receptors in the BLA in regulating stress- and fear-induced changes in REM and provide further evidence that fear memory, as indicated by freezing, can be dissociated from the effects of stress and fear on sleep.

CRF and Spontaneous Sleep

We found no effect of microinjections of ANT into the BLA on spontaneous light period sleep. This result is generally con-
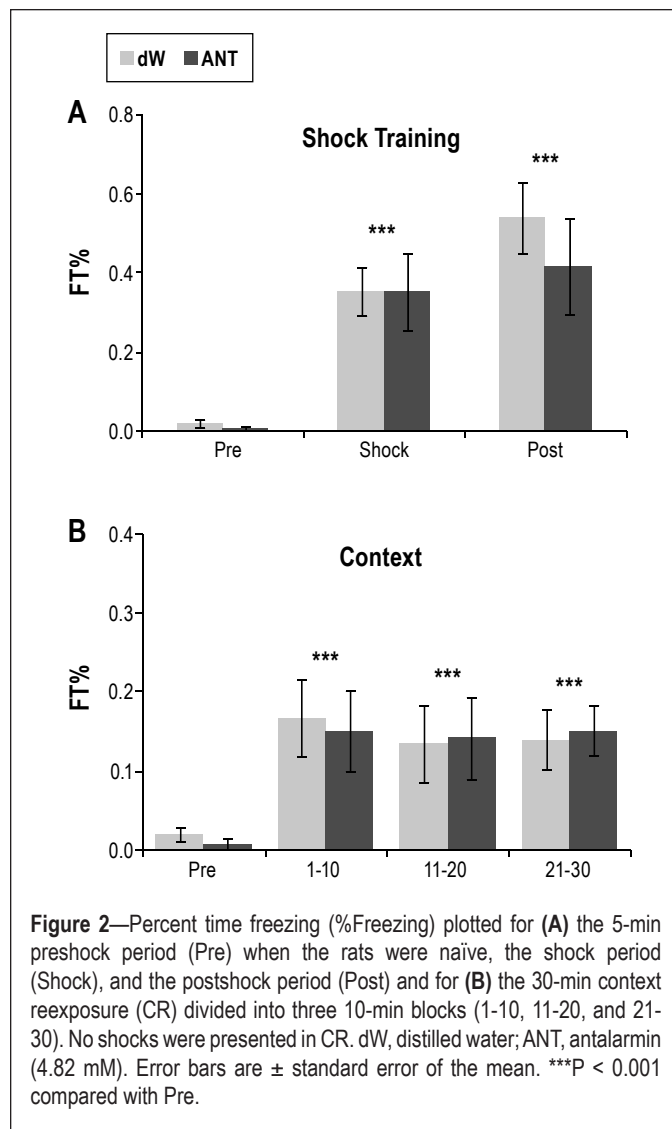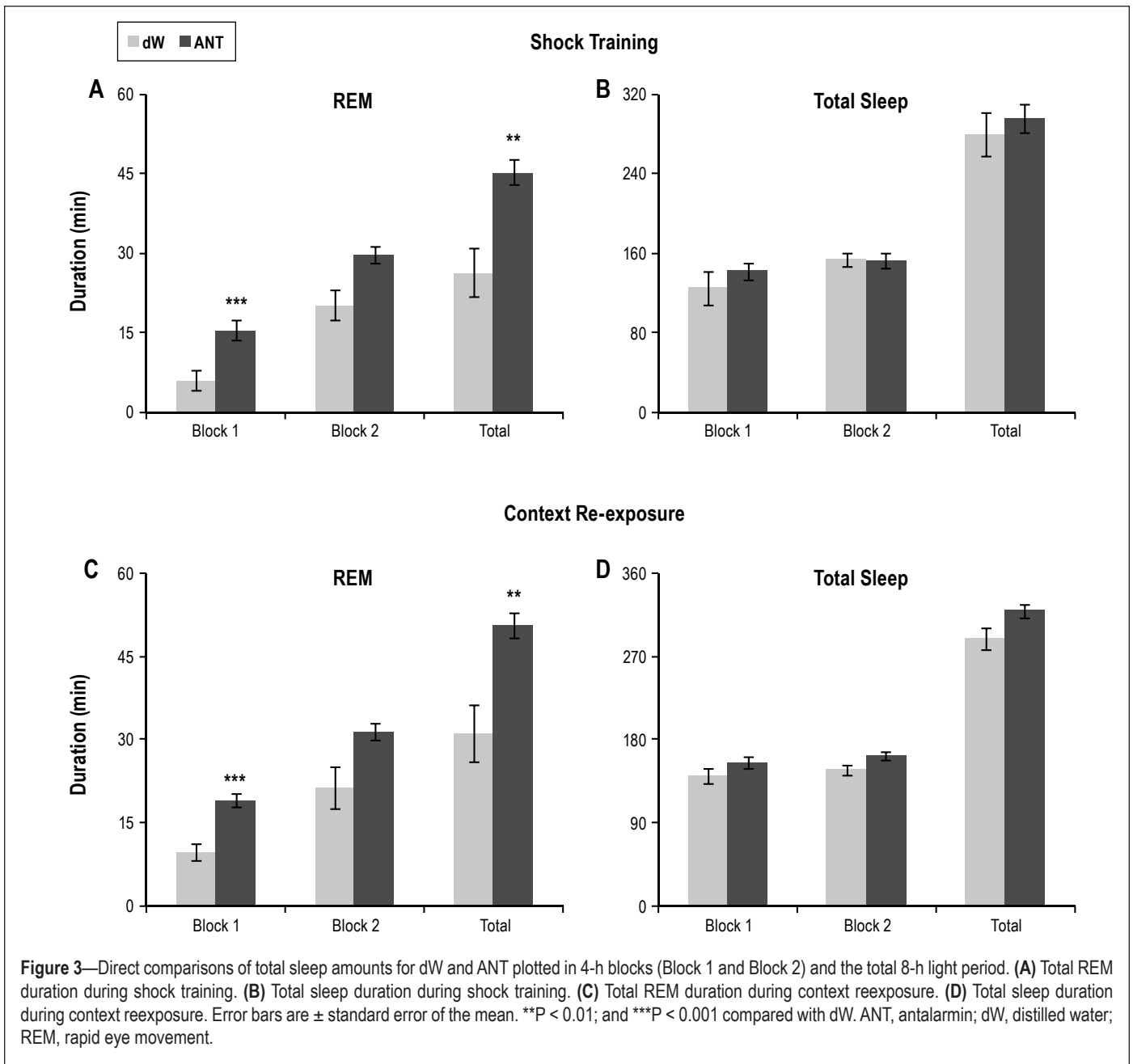

Figure 2—Percent time freezing (%Freezing) plotted for **(A)** the 5-min preshock period (Pre) when the rats were naïve, the shock period (Shock), and the postshock period (Post) and for **(B)** the 30-min context reexposure (CR) divided into three 10-min blocks (1-10, 11-20, and 21-30). No shocks were presented in CR. dW, distilled water; ANT, antalarmin (4.82 mM). Error bars are ± standard error of the mean. ***P < 0.001 compared with Pre.

sistent with the findings of other studies administering CRF antagonists during the light period in nonstressed conditions. For example, ICV administration into rats with two specific CRF-receptor antagonists, αHelCRF and astressin, did not alter waking when administered before the light period.[33] However, the specific effects of CRF antagonists on sleep may vary with the time of administration. For instance, Chang and Opp[33] found that ICV administration of CRF antagonists reduced wakefulness when administered before the dark period, although others have reported no effect of αHelCRF-administered ICV before the dark period on sleep and waking.[46] Strains with genetic differences in the CRF system may also respond differently to CRF antagonists. For example, differences in the CRF system have been reported for C57BL/6J mice and BALB/cJ mice[47] and we found that astressin-administered ICV did not significantly alter wakefulness or sleep in C57BL/6J mice but decreased active wakefulness and significantly increased REM at some dosages in BALB/cJ mice,[48] who show increased activity and less spontaneous sleep during the light period. Thus, most data suggest that antagonizing CRF could increase sleep during periods of high spontaneous arousal, as suggested by Chang and Opp,[33] but would have minimal effects when sleep was already high.

Figure 3—Direct comparisons of total sleep amounts for dW and ANT plotted in 4-h blocks (Block 1 and Block 2) and the total 8-h light period. **(A)** Total REM duration during shock training. **(B)** Total sleep duration during shock training. **(C)** Total REM duration during context reexposure. **(D)** Total sleep duration during context reexposure. Error bars are ± standard error of the mean. **P < 0.01; and ***P < 0.001 compared with dW. ANT, antalarmin; dW, distilled water; REM, rapid eye movement.

CRF and Stress-Induced Alterations in Sleep

Microinjections of ANT into the BLA blocked the reduction in REM normally produced by inescapable foot shock stress. CRF has been implicated in stress-induced alterations in sleep,[49,50] particularly in the control of REM.[46] Our results indicate that CRF in the BLA plays a role in inhibiting REM during stress; however, there are hypotheses that CRF promotes REM after stress. These hypotheses are primarily based on studies that applied CRF antagonists in association with a stress paradigm. For example, findings that ICV administration of αHelCRF before restraint stress prevented the increase in REM[46] that can follow restraint administered at dark onset[51] led to the suggestion that CRF mediates the increase. However, other investigators found no increase in REM after dark-onset restraint and no effect of the CRF antagonist, astressin, on sleep after restraint,[49] but did find that astressin attenuated the increase in wakefulness over a 5-h period immediately after light period restraint.[49]

A recent study[52] examined baseline and recovery sleep after sleep deprivation in conditional mouse mutants that overexpress CRF in the entire central nervous system or only in the forebrain. In baseline recordings, homozygous mice with either global or forebrain overexpression of CRF showed increased REM compared with control mice and both homozygous and heterozygous mice with global overexpression of CRF showed enhanced recovery REM after sleep deprivation. Enhanced REM recovery, but not NREM recovery, was blocked by oral administration of the CRF1 receptor antagonist, DMP696, 1-hour before the end of sleep deprivation. Peripheral stress hormone levels were not elevated during baseline and did not differ across genotypes after sleep deprivation. The authors concluded that enhanced REM in these mice was most likely induced through the activation of CRF1 receptors. Consistent with this conclusion is a report that repeated administration of αHelCRF in rats during sleep deprivation also reduced the amount of REM recovery.[50] However,
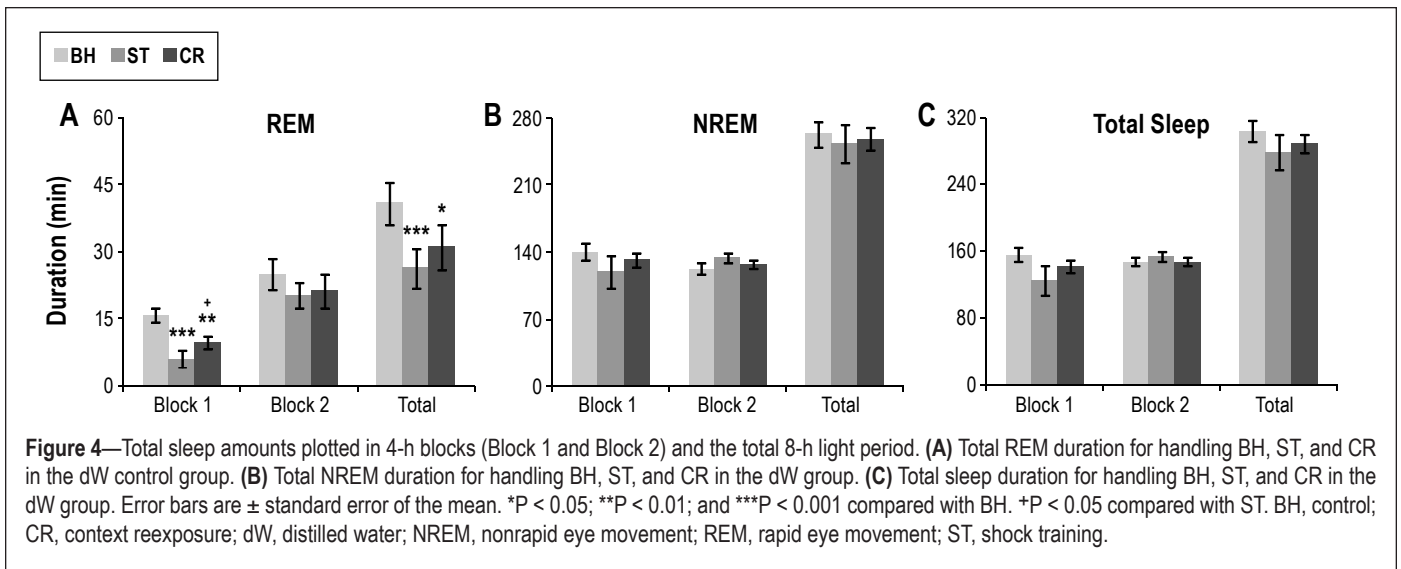
Figure 4—Total sleep amounts plotted in 4-h blocks (Block 1 and Block 2) and the total 8-h light period. **(A)** Total REM duration for handling BH, ST, and CR in the dW control group. **(B)** Total NREM duration for handling BH, ST, and CR in the dW group. **(C)** Total sleep duration for handling BH, ST, and CR in the dW group. Error bars are ± standard error of the mean. *P < 0.05; **P < 0.01; and ***P < 0.001 compared with BH. +P < 0.05 compared with ST. BH, control; CR, context reexposure; dW, distilled water; NREM, nonrapid eye movement; REM, rapid eye movement; ST, shock training.
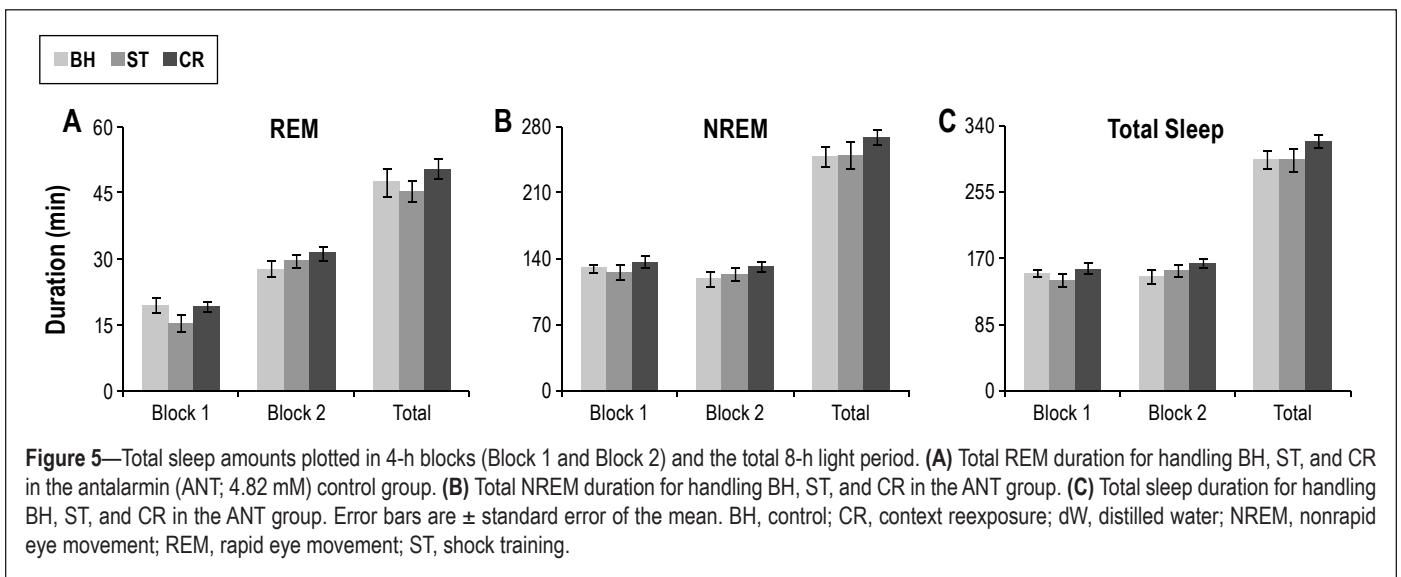

Figure 5—Total sleep amounts plotted in 4-h blocks (Block 1 and Block 2) and the total 8-h light period. **(A)** Total REM duration for handling BH, ST, and CR in the antalarmin (ANT; 4.82 mM) control group. **(B)** Total NREM duration for handling BH, ST, and CR in the ANT group. **(C)** Total sleep duration for handling BH, ST, and CR in the ANT group. Error bars are ± standard error of the mean. BH, control; CR, context reexposure; dW, distilled water; NREM, nonrapid eye movement; REM, rapid eye movement; ST, shock training.

conflicting evidence is found in a study in sleep-deprived humans reporting that repeated administration of CRF during the actual recovery period blocked the increase in REM.[53] Additionally, in rats, administration of CRF and αHelCRF during extended REM deprivation produced similar effects on initial recovery REM, primarily reduced REM episode duration.[54] Thus, the timing of the treatment appears to be a significant factor in whether antagonizing CRF blocks enhanced REM.

Other work consistent with CRF playing an inhibitory role in regulating REM comes from studies of escapable shock. Training with escapable shock and reminders of escapable shock can produce significant enhancements in REM.[55] Microinjections of either saline or astressin prior to training produce similar, significant enhancements in post-stress REM relative to a non-shocked handling control condition whereas the increases in REM are blocked by pretreatment with CRF.[56] The effect of CRF was relatively specific for REM as changes in NREM and wakefulness were minimal.

Conditioned Fear and Sleep

Behavioral freezing has been used to evaluate fear and fear memory, with greater FT% being interpreted as indicating

stronger fear reactions.[2-4,44,45] Minimal if any freezing occurred in either group during the preshock period. ST resulted in similar significant increases in FT% compared with the preshock period in control and ANT-treated rats. Compared with the pre-shock period, the control and ANT-treated rats also showed similar FT% during context re-exposure (no shock presented). Thus, based on freezing, microinjections of ANT into the BLA did not alter behavioral fear responses.

We and others have demonstrated that training with inescapable foot shock and subsequent presentation of fearful cues and contexts associated with inescapable foot shock produce very similar alterations in post-stress sleep, primarily a reduction in REM. We also have demonstrated that continued freezing in a fearful context is associated with reduced REM, whereas extinguished fear is associated with normalized REM.[14] Thus, given this relationship between waking fear behavior and REM, the apparent disassociation of freezing and later REM amounts after microinjections of ANT into the BLA may appear surprising. However, mice trained with controllable and uncontrollable stress, modeled by escapable and inescapable foot shock, show statistically equivalent amounts of freezing when reexposed to the chamber used for training, but directionally differ-

Table 2

		Block 1	Block 2	Total
REM Episodes				
BH	dW-ST	9.3 (0.9)	13.6 (2.1)	22.9 (2.8)
	ANT-ST	10.3 (0.9)	13.9 (1.1)	24.1 (1.1)
ST	dW-ST	3.6 (0.8)	11.3 (1.0)	14.9 (1.7)
	ANT-ST	8.1 (0.9)*	15.6 (1.2)	23.7 (2.0)*
CR	dW-ST	6.6 (1.0)	12.3 (1.8)	18.9 (2.6)
	ANT-ST	9.1 (0.6)*	15.9 (1.7)	25.0 (2.2)
Avg REM Duration				
BH	dW-ST	1.7 (0.1)	1.9 (0.1)	1.8 (0.0)
	ANT-ST	2.0 (0.2)	2.1 (0.2)	2.0 (0.2)
ST	dW-ST	1.3 (0.3)	1.8 (0.2)	1.7 (0.1)
	ANT-ST	1.9 (0.1)	2.0 (0.2)	2.0 (0.1)
CR	dW-ST	1.6 (0.1)	1.7 (0.2)	1.6 (0.1)
	ANT-ST	2.1 (0.2)	2.1 (0.2)	2.1 (0.2)
NREM Episodes				
BH	dW-ST	31.8 (1.4)	38.1 (0.8)	70.0 (1.4)
	ANT-ST	30.4 (3.8)	41.3 (3.0)	71.7 (5.9)
ST	dW-ST	25.1 (3.2)	36.6 (2.7)	61.7 (4.3)
	ANT-ST	24.6 (1.8)	33.7 (2.6)	58.3 (3.8)
CR	dW-ST	29.7 (2.5)	41.6 (3.1)	71.3 (3.9)
	ANT-ST	25.3 (1.3)	35.0 (2.0)	60.3 (2.6)
Avg NREM Duration				
BH	dW-ST	4.5 (0.4)	3.2 (0.2)	3.8 (0.3)
	ANT-ST	4.7 (0.6)	3.0 (0.4)	3.7 (0.4)
ST	dW-ST	4.5 (0.5)	3.8 (0.4)	4.1 (0,3)
	ANT-ST	5.4 (0.6)	3.8 (0.4)	4.1 (0.4)
CR	dW-ST	4.6 (0.4)	3.2 (0.3)	3.7 (0.3)
	ANT-ST	5.5 (0.3)	3.9 (0.3)	4.5 (0.3)

REM/NREM episode number and REM/NREM average duration (mean (SEM)) during 4-h blocks (Block 1 & Block 2) and total 8-h light period after baseline with handling (BH) shock training (ST) or context reexposure (CR) for either the dW-ST or ANT-ST group. *P < 0.05 compared to dW-ST of same condition.

ent amounts of REM.[39] Mice trained with escapable shock can show increased REM whereas mice trained with inescapable shock show decreased REM. We recently showed that injections of ANT into the CNA prior to context reexposure attenuates subsequent REM reductions normally observed without affecting freezing.[38] Thus, these evolving lines of evidence indicate that fear behavior in wakefulness (as indicated by freezing) is not predictive of subsequent REM, and that CRF in the amygdala plays a significant role in regulating the relationship between fear and sleep. Indices of acute stress (corticosterone[39] and stress-induced hyperthermia[57]) are also similar for animals trained with escapable and inescapable stress, though post-stress alterations in REM may be directionally different.

One of the more puzzling results in this study was the finding that antagonizing CRF1 receptors in the BLA prior to ST blocked fear-induced reductions in REM after a subsequent exposure to the ST context alone, but did not alter fear memory, as indicated by freezing. That is, microinjections of ANT into the BLA blocked the reduction in REM that is normally produced

by both the initial stressor and contextual reminders of the stressors, but did not block waking fear behavior. This finding is consistent with the potential for dissociation between waking fear behavior and sleep, but also suggests that specific aspects of the stressful experience and stressful memory responsible for the reductions in REM were altered by antagonizing CRF1 receptors in the BLA. Support for the specificity of CRF1 receptors in our results is based on *in vitro* findings that ANT displaced ^{125}I-oCRF binding in rat pituitary, frontal cortex, and cerebellum, which have greater amounts of CRF1 receptors, but not in the heart, which has greater amounts of CRF2 receptors.[58]

Amygdala, Conditioned Fear, and the Regulation of Sleep

To our knowledge, there has been limited examination of the potential role of CRF in the BLA in regulating contextual fear. A previous study reported that bilateral microinjections of DMP696, a nonpeptide CRF1 receptor antagonist, into the BLA did not significantly alter the acquisition of conditioned fear but did reduce freezing on reexposure to the fearful context.[42] We also found no effect of microinjections of ANT into the BLA on fear acquisition, but our results for context reexposure are dramatically different as we saw equivalent FT% in the control and drug groups. One potential reason for the discrepancy is differences in the training paradigms. The study reporting an effect of antagonizing CRF1 receptors in the BLA on freezing used five foot shocks (1 mA, 1-sec duration) delivered at 2.0-min intervals whereas we used 20 foot shocks (0.8 mA, 0.5-sec duration) at 1.0-min intervals. Overtraining can overcome the effects of BLA lesions on contextual fear,[59-61] indicating that circuitry outside the BLA can be involved in contextual fear under some circumstances.[59] Thus, it is possible that the more extensive training paradigm (with respect to number of trials) we used may have engaged other regions involved with memory of contextual fear, though we must note that pretraining lesions of the BLA can impair acquisition with up to 25 training trials.[59]

The involvement of the BLA in the control of sleep is indicated by reports that bilateral electrolytic and chemical lesions of the BLA increase NREM and total sleep time in rats[23] and that bilateral chemical lesions of the amygdala produce more consolidated sleep in chair-restrained Rhesus monkeys.[62] Electrical and chemical stimulation of the BLA also increase low-voltage, high-frequency activity in the cortical EEG and decrease NREM and total sleep time, respectively.[23,63] Recently we found that microinjections into the BLA of the Group II metabotropic glutamate (mGlu) receptor agonist, LY379268, selectively reduced REM without significantly altering wakefulness or NREM.[24] Thus, though limited, current data demonstrate roles for BLA in regulating both NREM and REM. The influence on REM is likely enacted via influences on the CNA, which has projections to brainstem REM regulatory/generator regions and an established role in regulating REM[64] and possibly through the bed nucleus of the stria terminals, which has brainstem targets similar to those of CNA.[65]

CRF, Conditioned Fear, Sleep, and Psychopathology

The CRF system,[66-70] fear conditioning,[71-75] and stress-induced alterations in sleep[76-79] are implicated in the etiology of anxiety disorders, including posttraumatic stress disorder (PTSD). The amygdala has a critical role in the acquisition and expression of conditioned fear[59-61,74,75,80] and recent evidence suggests that

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it is a strong regulator of conditioned alterations in sleep.[38,81] It also plays a significant role in anxiety and PTSD.[82] However, fear memory, as indicated by freezing, can be acquired with very few training trials whereas conditioned alterations in sleep require relatively extensive training procedures.[11] As discussed previously, several lines of evidence now indicate that behavioral fear and subsequent sleep also can dissociate even with relatively extensive fear training and that the amygdala is important for regulating the relationship between waking fear behavioral and subsequent sleep. The current work demonstrates that the neural processes underlying conditioned behavioral fear and sleep can be separated and indicates that a full understanding of fear conditioning and its relevance for disease will require determining its effects on sleep and well as fear behavior in wakefulness. The importance of understanding the neural substrate underlying fear-conditioned changes in sleep is found in the association of significant sleep disturbances with anxiety, PTSD, and virtually all emotional disorders.

CONCLUSION

ANT microinjected into the BLA prior to fear conditioning attenuated reductions in REM that occur following foot shock training as well those following reexposure to the fearful context. This attenuation occurred without any change to fear-dependent freezing. Several lines of work now indicate that freezing can dissociate from stress- and fear-induced alterations in sleep. The current data suggest that CRF1 receptors within the BLA are important for regulating stress-induced alterations in REM, and that they play a role in modulating how stressful memories influence sleep. However, additional work is needed to establish the specific role of the BLA in mediating the effects of fear memory on sleep to determine whether CRF alters fear learning or actual memory formation. Given the role of CRF in the stress response, delineating the processes by which it regulates how stressful memories affect sleep could have significance for understanding the mechanisms underlying the sleep disturbances associated with emotional disorders. The effects we observed with manipulations in the BLA suggest that these mechanisms may be located in a very circumscribed region in the brain.

ACKNOWLEDGMENTS

DISCLOSURE STATEMENT

This was not an industry supported study. The authors have indicated no financial conflicts of interest.

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