



Short communication

Corticotropin-releasing factor microinjection into the central nucleus of the amygdala alters REM sleep

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Abstract:

Psychological stressors have a prominent effect on rapid eye movement sleep (REMS) in humans and animals. We hypothesized that the stress-related neurochemical corticotropin-releasing factor (CRF), acting in the amygdala, could initiate neural events that lead to REMS alterations. Therefore, we made bilateral microinjections of three different doses of CRF into the central nucleus of the amygdala (CeA) in five rats. Only the lowest dose of CRF (1 ng) induced a change in sleep, specifically REMS, during the 4-h post-injection period. Thus, REMS alterations following psychological stress may depend, in part, on CRF release in the CeA.

Key words:

amygdala, corticotropin-releasing factor, stress, psychological, sleep, REM

Abbreviations: BLA – basolateral nucleus of the amygdala, CeA – central nucleus of the amygdala, CNS – central nervous system, CoA – cortical amygdala, CRF – corticotropin-releasing factor, MA – medial amygdala, REMS – rapid eye movement sleep

Introduction

Stressors have a prominent effect on rapid eye movement sleep (REMS) in humans and animals. However,

the precise changes and their mechanisms have not been determined. On the one hand, there is evidence that a recruitment of REMS mechanisms occurs; in animals, for example, a REMS increase following immobilization stress is well established [12, 19]. Conversely, REMS suppression following footshock, such as that utilized in fear conditioning protocols, is a response of both rats and mice [13, 14, 19].

Emotional influences on REMS likely are managed by the amygdala, a structure generally viewed as the hub of central nervous system (CNS) pathways in-

volved in “tagging” a sensory perception with emotional relevance and orchestrating the appropriate autonomic, hormonal, and behavioral responses [5, 9]. The amygdala has reciprocal connections with the basal forebrain and with pontine regions involved in the control of REMS and its component phasic events [3, 9]. The central nucleus of the amygdala (CeA) projects to the peribrachial area of the pons [3], where the ponto-geniculo-occipital waves that characterize REMS are thought to be generated [9, 16]. The CeA also sends a projection to the laterodorsal tegmental nucleus [3], which is important in REMS generation, and to the nucleus reticularis pontis oralis [3], the caudal part of which may be the REMS “trigger zone” [1].

Corticotropin-releasing factor (CRF) is known to play a major role in the CNS response to stressors [2, 3, 15]. Opp [10] has suggested that CRF may be uniquely involved in mediating certain stressor-induced alterations in sleep. The CeA contains a number of CRFergic neurons that project elsewhere in the brain [3, 15]. Studies on the distribution of CRF receptors in the CNS have shown that there are two major receptor subtypes, CRF-R1 and CRF-R2 α , and that CRF-R1 is expressed at low levels in the CeA while full-length CRF-R2 α is absent [15]. However, expression of a short isoform of CRF-R2 α , CRF-R2 α_{tr} , has been reported in the CeA [7]. Interestingly, it has been suggested that this receptor isoform plays a role in regulating CRF release from amygdalar neurons [7].

Amygdalar CRF receptors have been implicated in stress-related behavior in awake rats [17, 20]. CRF measured by microdialysis was differentially increased in the CeA by different stressors [4]. Microinjections of the CRF antagonist α -helical CRF into the CeA have been shown to attenuate stress-induced freezing [17], an indicator of increased anxiety in rodents, and there is a report that CRF microinjection into the CeA results in an increase in freezing; however, that report showed also that exploratory activity, an indicator of decreased anxiety in rodents, is increased [20]. Clearly, the role of CRF in the CeA in regulating stress-related behavior is complex.

Given the evidence for the importance of amygdalo-pontine circuitry in the control of REMS and the role of CRF in the amygdala in regulating behavioral responses to stress during wakefulness, it seemed likely that CRF acting in the CeA could underlie the effects of stress on REMS. In the current

study, we wondered whether rats that received an infusion of CRF into the CeA would show an alteration of REMS. Positive findings would suggest a possible neural substrate for the stress-induced changes in sleep that have been observed in humans and rodents.

Materials and Methods

Subjects were 7 male Sprague-Dawley rats weighing 250–350 g at the time of surgery. They were maintained on *ad libitum* food and water on a 12:12 light/dark cycle, with lights on at 07.00. All procedures were approved by the Institutional Animal Care and Use Committees of the Philadelphia VAMC and of the University of Pennsylvania in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals (NIH Publications No. 80–23).

Under ketamine (85 mg/kg, *im*)/xylazine (15 mg/kg, *im*) anesthesia, stainless steel screw electrodes were implanted in the skull (coordinates ML 2.0, AP 2.0 and ML –2.0, AP –2.0) for recording the fronto-parietal electroencephalogram (EEG), and a pair of stainless steel wire electrodes was implanted in the neck muscles for recording the nuchal electromyogram (EMG). A grounding screw was placed in the skull just rostral to the braincase. Leads from these recording electrodes were routed to a miniature plug that mated to one attached to a recording cable. Guide cannulae (26 ga; Plastics One, Inc.) for making microinjections into the CeA were bilaterally implanted with their tips aimed 1.0 mm above the CeA (coordinates from bregma: ML 4.0, AP –2.3, DV –7.6). The recording plug and cannulae were affixed to the skull with dental acrylic and anchor screws. Postoperative pain and potential infection were controlled with butorphanol (0.35 mg/kg, *im*) and gentamicin (4.5 mg/kg, *im*), respectively. Animals were allowed to recover from surgery for a minimum of 1 week before being entered into pharmacological studies.

Following habituation to the recording set-up, studies were performed from 10 AM to 2 PM once per week over six weeks. In weeks two through five, four bilateral injections (0.2 μ l/side, 0.1 μ l/min) were administered with a Baby Bee microsyringe pump with a Worker Bee controller (BAS) through a temporary cannula (33 ga, Plastics One, Inc.) that extended

Tab. 1. Weekly pre-recording manipulations of rats used in this study

Rat	Week 1	Week 2	Week 3	Week 4	Week 5	Week 6
1	No injection	3 ng CRF	Vehicle	10 ng CRF	1 ng CRF	No injection
2	No injection	3 ng CRF	10 ng CRF	Vehicle	1 ng CRF	No injection
3	No injection	Vehicle	1 ng CRF	3 ng CRF	10 ng CRF	No injection
4	No injection	10 ng CRF	3 ng CRF	1 ng CRF	Vehicle	No injection
5	No injection	Vehicle	1 ng CRF	3 ng CRF	10 ng CRF	No injection

1 mm below the end of the guide cannula. Injections of artificial cerebrospinal fluid (aCSF; 128 mM NaCl, 3 mM KCl, 1.5 mM CaCl₂, 1 mM MgSO₄, 24 mM NaHCO₃, 0.5 mM NaH₂PO₄, 30 mM D-glucose, pH adjusted to 7.4 with 95% CO₂/5% O₂) alone and three doses of CRF (provided by Dr. Jean E. Rivier, The Salk Institute for Biological Sciences, San Diego, CA, USA) dissolved in aCSF (1 ng, 3 ng, and 10 ng CRF per side) were counterbalanced among the animals in weeks two through five. Animals were connected to the cable and sleep recordings were initiated immediately following the injections. To assure that tissue damage associated with repeated microinjections did not alter sleep, recordings were performed without lowering the injector cannulae on the first and sixth weeks. The manipulation performed each week to each rat prior to recording of sleep is shown in Table 1.

Rats were placed in individual cages and connected to cables that were counter-weighted and connected to a 12-channel freely rotating swivel (SL6C, Plastics One). Cables were designed to be as lightweight as possible to minimize discomfort to the animals. The recording cage was placed in a sound-dampened chamber (1 m³). Polygraphic studies were conducted for each experiment using a Grass Model 7 polygraph equipped with high gain, low noise amplifiers. EEG signals were filtered at 0.3 to 30 Hz, and EMG signals were filtered at 10 to 100 Hz. Paper recordings were scored in 10 s epochs for wakefulness, non-REMS (NREMS), and REMS according to standard laboratory guidelines.

Statistical analysis

The parameters examined were the hourly averages for the amount of time, number of episodes, and average episode length for wakefulness, NREMS, and REMS. The latencies to the first 30-s episodes of NREMS and REMS were also examined. To assess if the series of microinjections non-specifically affected

sleep the data from five animals were analyzed with 1 × 3 (Baseline1, Baseline2, Vehicle) repeated-measure ANOVA models. For determining the effects of CRF microinjection the data from the five animals were analyzed with 1 × 4 (Vehicle, 1 ng, 3ng, and 10 ng CRF) repeated-measure ANOVA models. Statistical analyses were performed using NCSS 2001 (Number Cruncher Statistical Systems, Kaysville, Utah, USA).

Results

Histological examination revealed that five of the seven implanted animals had bilateral cannulae placement in, or in one case on one side slightly dorsal to, the CeA. The positions of the tips of the injector cannulae are shown for these animals in Figure 1. Based on previous experience, we believe that substantial perfusion of the CeA, but not adjacent amygdalar nuclei, with the injected solutions occurred in these animals. Only data from these five animals are included in subsequent analyses.

Repeated injection does not non-specifically alter sleep. One concern was that multiple injections at the

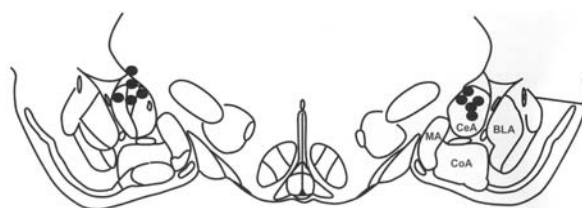


Fig. 1. Schematic diagram of the rat brain 2.3 mm posterior to bregma showing the tips of the cannulae for the five animals with histologically verified bilateral CeA placement. Placement was determined by cresyl violet staining of 45 μm serial sections from rat brains fixed by intracardial perfusion of saline followed by 10% formalin. CeA – central nucleus of the amygdala, BLA – basolateral nucleus of the amygdala, MA – medial amygdala, CoA – cortical amygdala

Tab. 2. Effect of CRF injections on sleep architecture of rats^a

	aCSF	1 ng CRF	3 ng CRF	10 ng CRF
Wakefulness				
Minutes per hour	34.7 ± 3.1	39.3 ± 2.7	36.5 ± 2.9	38.2 ± 2.9
Episodes per hour	14.8 ± 1.6	13.1 ± 1.1	14.8 ± 1.6	13.1 ± 1.5
Episode length (min/ep)	4.5 ± 1.5	7.0 ± 3.1	6.6 ± 3.1	7.0 ± 2.9
NREMS				
Minutes per hour	20.4 ± 2.6	17.4 ± 2.2	19.0 ± 2.2	17.2 ± 2.2
Episodes per hour	15.0 ± 1.6	12.9 ± 1.2	14.4 ± 1.6	13.2 ± 1.5
Episode length (min/ep)	1.4 ± 0.2	1.2 ± 0.1	1.3 ± 0.1	1.2 ± 0.1
Latency (min)	38.3 ± 7.0	37.2 ± 8.7	39.4 ± 8.1	34.4 ± 6.6
REMS				
Minutes per hour ^b	4.9 ± 0.9	3.3 ± 0.8 ^c	4.6 ± 0.9	4.7 ± 0.9
Episodes per hour	2.3 ± 0.4	2.2 ± 0.5	2.5 ± 0.4	2.4 ± 0.5
Episode length (min/ep)	1.6 ± 0.3	1.1 ± 0.2	1.3 ± 0.2	1.4 ± 0.2
Latency (min)	99.9 ± 9.4	115.1 ± 24.7	98.5 ± 9.5	119.6 ± 15.0

^a Latencies are the mean number of minutes from the initiation of the sleep study ± standard error of measurement. All other values are mean per hour ± standard error of measurement; ^b significant main effect at $p < 0.05$ using repeated measure ANOVA models; ^c different from vehicle alone according to Tukey-Kramer *post-hoc* test

same site in the same animal could result in sufficient tissue damage to affect sleep. We took two measures to control for this. First, the order of vehicle and CRF injections was counterbalanced. This assured that neuronal damage would not be systematically associated with any particular CRF dosage. Second, we performed polysomnographic studies both the week before and the week after the series of injections. There were no obvious or statistically significant differences between the parameters from these two baseline sessions and the vehicle alone session (data not shown), indicating that neither mechanical nor neurotoxic damage altered sleep across the duration of the study.

Parameters for wakefulness, NREMS, and REMS following vehicle and CRF injections are reported in Table 2. Data for all injection groups were analyzed by a 1×4 (treatment) repeated measures ANOVA model, and Tukey-Kramer *post-hoc* tests were performed when main effects were observed. There was no effect of CRF on any of the wakefulness or NREMS parameters examined in this study. Of the REMS parameters, average hourly time spent in REMS displayed a main effect of treatment, and Tukey-Kramer *post-hoc* analyses indicated that the low dose of CRF produced an effect significantly different from that of the vehicle injection (Tab. 2). No main effect of treatment was observed for either the

number or duration of REMS episodes, and therefore the decrease in time spent in REMS could not be attributed to a decrease in either alone.

Thus, a low dose of CRF injected bilaterally into the CeA induced a specific decrease in REMS for the 4-h period following the injection. This effect was not observed for the medium and high dosages of CRF used in this study.

Discussion

The results of this study show that REMS, but not NREMS, is suppressed when CRF is administered bilaterally into the CeA at the lowest dosage examined (1 ng). This dosage of CRF reduced REMS to 67% of that seen after vehicle alone, a percentage reduction strikingly similar to the 65% decrease in REMS we have reported following footshock in the rat [14]. Interestingly, higher doses of CRF (3 ng and 10 ng) compared to vehicle alone failed to induce notable changes in REMS. This dose-responsive suppression of REMS after CRF microinjection into the CeA may suggest a neuropharmacological mechanism by which stress influences sleep.

The CeA is well positioned to modulate REMS via a CRFergic mechanism. CRF-containing terminals of neurons with cell bodies in the lateral hypothalamus, the dorsal raphe nucleus, and the CeA itself form synaptic contacts with CRFergic neurons of the CeA [3]. Thus, CRF appears to naturally modulate the activity of CRFergic output neurons of the CeA. CRF-containing neurons of the CeA contact locus coeruleus (LC) dendrites in the rostralateral pericoerulear region [3]. These connections likely produce LC excitation predominantly [18], and LC cell firing correlates negatively with REMS occurrence [16]. Therefore, facilitation of an excitatory amygdalar input to the LC could inhibit REMS.

A possible explanation of the different effects of the 1, 3, and 10 ng CRF dosages may lie in the differential properties of the two major CRF receptor subtypes, CRF-R1 and CRF-R2 [2, 15]. CRF-R1 is a high affinity CRF receptor expressed at low levels in the CeA, the cortical amygdala (CoA), and the medial amygdala (MA), and at high levels in the basolateral nucleus of the amygdala (BLA) [15]. The centrally relevant CRF-R2 isoform, CRF-R2 α , is a lower affinity CRF receptor for which urocortin and/or sauvagine, and not CRF, may be natural ligands [15]. CRF-R2 α is expressed at high levels in the CoA and MA; there is no detectable expression in the CeA or BLA [15]. While the differential central roles of CRF-R1 and CRF-R2 α in stress and anxiety are quite complex and involve numerous neural substrates [2, 15], it appears that CRF-R1 mediates anxiogenic responses while CRF-R2 α exerts an opposing anxiolytic response [2].

Recently, the transcript for a truncated form of CRF-R2 α , CRF-R2 α_{tr} , has been isolated from the rat amygdala [8]. This isoform is the major form of CRF-R2 in the rat amygdala, and it binds CRF with an affinity similar to that of the full length receptor although it does not bind urocortin or sauvagine [7, 8], suggesting that CRF is its natural ligand. CRF-R2 α_{tr} is expressed at high levels in the CoA and at moderate levels in the CeA; its expression was not detected in the MA or BLA [7]. It has been suggested that CRF-R2 α_{tr} functions to directly regulate the release of endogenous CRF from amygdalar neurons [7]. Consistent with that report, Lee et al. [6] observed, in the mouse cerebellum, that protein for both the full-length and truncated forms of CRF-R2 α could be detected and that CRF-R2 α_{tr} was located presynaptically while CRF-R2 α was located postsynaptically. Furthermore, CRF application has been shown to in-

hibit rat CeA neurons *in vitro*, and the authors suggested that this occurs via an action on inhibitory somatodendritic autoreceptors [11].

Based on the above evidence, we hypothesize that activation of CeA CRFergic output neurons, and consequently REMS suppression, occurs at low doses of CRF and is mediated via CRF-R1, while at higher CRF doses inhibition of these same output neurons occurs via a CRF-R2 α_{tr} mechanism so that REMS is no longer suppressed. This hypothetical inhibition of CRFergic output neurons at higher CRF doses could occur via somatodendritic CRF-R2 α_{tr} on CRFergic output neurons or interneurons, or via CRF-R2 α_{tr} on axon terminals of CRFergic interneurons.

While we have focused on the effects of CRF on CRFergic systems within the CeA, it is likely that CRF affects neurons of different types and that the natural response of sleep to stress involved also other neural substrates and circuitry [9].

We have shown that the administration of a low dose, but not two higher doses, of CRF into the CeA induces a decrease in the average hourly time spent in REMS during the following 4 h that is remarkably similar in specificity and magnitude to the REMS decrease following footshock [14]. We hypothesize that the REMS decrease is mediated by CRF-R1 at low doses and is attenuated at higher doses *via* CRF-R2 α_{tr} . Future work utilizing ultrastructural and double-immunolabeling techniques as well as a broader range of doses as well as receptor subtype-specific agonists and antagonists will be necessary to fully elucidate the role of CRF in the CeA in modulating the REMS response to stress.

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