Corticotropin-Releasing Factor in the Basolateral Amygdala Enhances Memory Consolidation via an Interaction with the β -Adrenoceptor–cAMP Pathway: Dependence on Glucocorticoid Receptor Activation

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Extensive evidence indicates that stress hormone effects on the consolidation of emotionally influenced memory involve noradrenergic activation of the basolateral complex of the amygdala (BLA). The present experiments examined whether corticotropin-releasing factor (CRF) modulates memory consolidation via an interaction with the β -adrenoceptor–cAMP system in the BLA. In a first experiment, male Sprague Dawley rats received bilateral infusions of the CRF-binding protein ligand inhibitor CRF_{6–33} into the BLA either alone or together with the CRF receptor antagonist α -helical CRF_{9–41} immediately after inhibitory avoidance training. CRF_{6–33} induced dose-dependent enhancement of 48 h retention latencies, which was blocked by coadministration of α -helical CRF_{9–41}, suggesting that CRF_{6–33} enhances memory consolidation by displacing CRF from its binding protein, thereby increasing “free” endogenous CRF concentrations. In a second experiment, intra-BLA infusions of atenolol (β -adrenoceptor antagonist) and Rp-cAMPS (cAMP inhibitor), but not prazosin (α_1 -adrenoceptor antagonist), blocked CRF_{6–33}-induced retention enhancement. In a third experiment, the CRF receptor antagonist α -helical CRF_{9–41} administered into the BLA immediately after training attenuated the dose–response effects of concurrent intra-BLA infusions of clenbuterol (β -adrenoceptor agonist). In contrast, α -helical CRF_{9–41} did not alter retention enhancement induced by posttraining intra-BLA infusions of either cirazoline (α_1 -adrenoceptor agonist) or 8-br-cAMP (cAMP analog). These findings suggest that CRF facilitates the memory-modulatory effects of noradrenergic stimulation in the BLA via an interaction with the β -adrenoceptor–cAMP cascade, at a locus between the membrane-bound β -adrenoceptor and the intracellular cAMP formation site. Moreover, consistent with evidence that glucocorticoids enhance memory consolidation via a similar interaction with the β -adrenoceptor–cAMP cascade, a last experiment found that the CRF and glucocorticoid systems within the BLA interact in influencing β -adrenoceptor–cAMP effects on memory consolidation.

Key words: α -helical CRF_{9–41}; atenolol; CRF; CRF_{6–33}; CRF-binding protein; corticosterone; emotional arousal; norepinephrine; inhibitory avoidance

Introduction

Strong memories of emotionally significant experiences are critical for our adaptation and survival. Studies in both animals and humans provide extensive evidence that the enhancing effects of emotional arousal on memory consolidation depend on noradrenergic activation of the basolateral complex of the amygdala (BLA) (McGaugh, 2000; Strange and Dolan, 2004; Van Stegeren et al., 2005; Roozendaal, 2007). Such BLA activation, in turn, regulates the consolidation of different kinds of learning experi-

ences via its efferent projections to many other brain regions (McGaugh, 2004). Several hormones and neurotransmitters that are released by emotionally arousing training experiences influence memory consolidation via an interaction with the noradrenergic system of the BLA (McGaugh et al., 1988; Introini-Collison et al., 1989; Roozendaal et al., 2006a, 2007). There is extensive evidence that memory enhancement induced by administration of the adrenal hormones epinephrine and corticosterone requires training-induced increases in norepinephrine activity within the BLA (Quirarte et al., 1997; Roozendaal et al., 2002a, 2006b).

Corticotropin-releasing factor (CRF), a 41 residue neuropeptide, is also released during emotional states and known to act not only as a key mediator in the regulation of hypothalamic–pituitary–adrenocortical axis activity (Spiess et al., 1981; Vale et al., 1981), but also to modulate learning and memory (Sahgal et al., 1983; Radulovic et al., 1999), at least in part, by binding to cognate CRF receptors in the BLA (Liang and Lee 1988; Roozendaal

Received Sept. 11, 2007; revised April 28, 2008; accepted May 15, 2008.

This work was supported by National Science Foundation Grant IOB-0618211 (B.R.) and National Institute of Mental Health Grant MH12526 (J.L.M.). We thank Laura Stillman and Kathleen Perez for excellent technical assistance and Gabriel Hui for preparation of the figures.

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DOI:10.1523/JNEUROSCI.1336-08.2008

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et al., 2002b; Hubbard et al., 2007). Evidence from many studies indicates that there is an intimate relationship between CRF and the noradrenergic system, affecting anxiety, arousal, attention, and memory (Koob, 1999; Van Bockstaele et al., 1999; Liang et al., 2001; Sauvage and Steckler, 2001). CRF administration increases locus ceruleus neuronal activity (Valentino et al., 1983; Finlay et al., 1997; Jedema and Grace, 2004) and stimulates the release of norepinephrine in its terminal fields, including the amygdala (Chen et al., 1992; Lavicky and Dunn, 1993; Smagin et al., 1995; Isogawa et al., 2000). Moreover, a β -adrenoceptor antagonist administered intraventricularly blocks CRF effects on the consolidation of memory in a conditioning task (Cole and Koob, 1988).

The present study investigated whether CRF interacts with the norepinephrine-signaling cascade within the BLA in influencing memory consolidation. To increase CRF signaling in the BLA, we used immediate posttraining infusions of CRF_{6–33}, a CRF-binding protein (CRF-BP) ligand inhibitor that has been reported to displace endogenous CRF from its binding protein and enhance memory when administered systemically (Behan et al., 1995). Norepinephrine effects on memory consolidation involve activation of both postsynaptic β - and α_1 -adrenoceptors (Ferry et al., 1999a,b). The β -adrenoceptor is coupled to adenylate cyclase to stimulate the cAMP signaling pathway, whereas α_1 -adrenoceptor activation indirectly modulates the β -adrenoceptor response (Perkins and Moore, 1973; Schultz and Daly, 1973). Because CRF receptors are also G-protein-coupled receptors that stimulate adenylate cyclase activity (Bale and Vale, 2004), we were particularly interested in investigating whether the memory-enhancing effects of CRF, like that of corticosterone (Roozendaal et al., 2002a), depend on interactions with β -adrenoceptor–cAMP activity within the BLA. Moreover, because corticosterone is known to regulate CRF activity (Ma and Aguilera, 1999; Thompson et al., 2004), we further investigated whether the glucocorticoid receptor (GR) and CRF systems within the BLA interact in enhancing memory consolidation.

Materials and Methods

Subjects. Male adult Sprague Dawley rats (280–320 g at time of surgery) from Charles River Breeding Laboratories were kept individually in a temperature-controlled (22°C) colony room and maintained on a standard 12 h light/dark cycle (lights on from 7:00 A.M. to 7:00 P.M.) with *ad libitum* access to food and water. Training and testing were performed during the light phase of the cycle between 10:00 A.M. and 3:00 P.M. All procedures were performed in compliance with the National Institutes of Health guidelines and were approved by the Institutional Animal Care and Use Committee of the University of California, Irvine.

Surgery. Animals, adapted to the vivarium for at least 1 week, were anesthetized with sodium pentobarbital (50 mg/kg of body weight, i.p.), given atropine sulfate (0.4 mg/kg, i.p.) to maintain respiration, and were subsequently injected with 3.0 ml of saline to facilitate clearance of these drugs and prevent dehydration. The skull was positioned in a stereotaxic frame (Kopf Instruments), and two stainless-steel guide cannulas (15 mm; 23 gauge; Small Parts, Inc.) were implanted bilaterally with the cannula tips 2.0 mm above the BLA. The coordinates were based on the atlas of Paxinos and Watson (2005): anteroposterior, –2.8 mm from bregma; mediolateral, ± 5.0 mm from the midline; dorsoventral, –6.5 mm from skull surface; incisor bar, –3.3 mm from interaural. The cannulas were affixed to the skull with two anchoring screws and dental cement. Stylets (15-mm-long 00-insect dissection pins), inserted into each cannula to maintain patency, were removed only for the infusion of drugs. After surgery, the rats were retained in an incubator until recovered from anesthesia and were then returned to their home cages. The rats were allowed to recover for a minimum of 7 d before initiation of

training and were handled three times for 1 min each during this recovery period to accustom them to the infusion procedure.

Inhibitory avoidance apparatus and procedure. Rats were trained and tested in an inhibitory avoidance apparatus consisting of a trough-shaped alley (91 cm long, 15 cm deep, 20 cm wide at the top, and 6.4 cm wide at the bottom) divided into two compartments, separated by a sliding door that opened by retracting into the floor (McGaugh et al., 1988). The starting compartment (30 cm) was made of opaque white plastic and well lit; the shock compartment (60 cm) was made of dark, electrifiable metal plates and was not illuminated. Training and testing were conducted in a sound- and light-attenuated room.

For training, the rats were placed in the starting compartment of the apparatus, facing away from the door, and were allowed to enter the dark (shock) compartment. After the rat stepped completely into the dark compartment, the sliding door was closed and a single inescapable footshock (0.50 mA) was delivered for 1 s. Because the GR agonist and antagonist were dissolved in a vehicle containing 1% ethanol, which is slightly memory impairing, the experiment examining interactions between CRF and the GR system used a somewhat increased footshock intensity of 0.55 mA. Animals with entrance latencies of longer than 30 s (<2% of total number of rats) were eliminated from the study. The rats were removed from the shock compartment 15 s after termination of the footshock and, after drug treatment, returned to their home cages. On the 48 h retention test, as on the training session, the latency to reenter the shock compartment with all four paws (maximum latency of 600 s) was recorded and used as a measure of retention. Longer latencies were interpreted as indicating better retention. Shock was not administered on the retention test trial.

Drug and infusion procedures. For the first experiment, the CRF-BP ligand inhibitor human/rat CRF_{6–33} (0.01, 0.1, or 1 μ g in 0.2 μ l; Bachem; catalog #H-3456) was dissolved in saline and infused into the BLA immediately after inhibitory avoidance training either alone or together with the nonselective CRF receptor antagonist α -helical CRF_{9–41} (1 μ g; Bachem). For the second experiment, CRF_{6–33} (0.01, 0.1, or 1 μ g in 0.2 μ l) was infused into the BLA after inhibitory avoidance training either alone or together with the specific β_1 -adrenoceptor antagonist atenolol (0.5 μ g; Sigma-Aldrich), the specific α_1 -adrenoceptor antagonist prazosin (0.1 μ g; Sigma-Aldrich), or the cAMP inhibitor Rp-cAMPs (4 μ g; Sigma-Aldrich). For the third experiment, the nonselective CRF receptor antagonist α -helical CRF_{9–41} (1 μ g in 0.2 μ l) was dissolved in saline and infused into the BLA immediately after training together with the β -adrenoceptor agonist clenbuterol (1, 10, or 100 ng; Sigma-Aldrich), the α_1 -adrenoceptor agonist cirazoline (0.01, 0.1, or 1 μ g; Sigma-Aldrich), or the synthetic cAMP analog 8-br-cAMP (0.1, 0.3, or 1 μ g; Sigma-Aldrich). The drug doses were selected on the basis of previous experiments conducted in this laboratory (Ferry et al., 1999a,b; Roozendaal et al., 2002a,b). Bilateral infusions of drug, or an equivalent volume of saline, into the BLA were made by using 30 gauge injection needles connected to 10 μ l Hamilton microsyringes by polyethylene (PE-20) tubing. The injection needles protruded 2.0 mm beyond the cannula tips and a 0.2 μ l injection volume per hemisphere was infused over a period of 25 s by an automated syringe pump (Sage Instruments). The animals were gently restrained during the infusion procedure. The injection needles were retained within the cannulas for an additional 20 s after drug infusion to maximize diffusion and to prevent backflow of drug into the cannulas. The infusion volume was based on findings that this volume of an excitotoxin administered at identical injection sites induces selective lesions of the BLA (Roozendaal and McGaugh, 1996). Furthermore, drug infusions of this volume into the BLA, but not into the adjacent central nucleus of the amygdala, modulate memory consolidation (Parent and McGaugh, 1994; Roozendaal and McGaugh, 1997; Ma et al., 2005; Roozendaal et al., 2007). The use of posttraining infusions excluded the possibility that the different drug infusions altered retention by influencing anxiety, fear, locomotor activity, or attentional processes during acquisition (McGaugh, 1966), effects of CRF that have been ascribed to the amygdala (Liang et al., 1992; Swiergiel et al., 1993; Liebsch et al., 1995; Sajdyk et al., 1999; Merali et al., 2004). Furthermore, our previous finding that the CRF receptor antagonist α -helical CRF_{9–41} dose-dependently impaired inhibitory avoidance retention when infused into

the BLA immediately, but not several hours, after training (Roozendaal et al., 2002b) provides strong evidence that CRF enhances retention by modulating time-dependent processes underlying long-term memory consolidation.

For the fourth experiment investigating whether CRF interacts with the glucocorticoid system in the BLA in influencing memory consolidation, the CRF-BP ligand inhibitor CRF_{6–33} (0.01, 0.1, or 1 μ g in 0.2 μ l) was infused into the BLA immediately after the inhibitory avoidance training trial either alone or together with the GR antagonist 17 β -hydroxy-11 β -(4-dimethylaminophenyl)-17 α -(1-propynyl)-oestra-4,9-dien-3-one (RU 38486) (1 ng; kindly provided by sanofi-aventis). In the second part of this experiment, the GR agonist 11 β ,17 β -dihydroxy-6,21-dimethyl-17 α -pregna-4,6-trien-20-yn-3-one (RU 28362) (1, 3, or 10 ng in 0.2 μ l; kindly provided by sanofi-aventis) was administered into the BLA alone or together with α -helical CRF_{9–41} (1 μ g). Receptor binding studies have shown that RU 28362 has selective and high affinity for GRs (Teutsch et al., 1981) but that RU 38486 has also known antiprogesterone activities (Philibert et al., 1991). Because RU 28362 and RU 38486 are lipophilic, the drug combinations were first dissolved in 100% ethanol and subsequently diluted in saline to reach a final ethanol concentration of 1%. The vehicle solution used for this experiment contained 1% ethanol in saline only. Drug doses of RU 28362 and RU 38486 were selected on the basis of our previous studies (Roozendaal and McGaugh, 1997; Roozendaal et al., 2002a).

Histology. Rats were anesthetized with an overdose of sodium pentobarbital ($\approx$ 100 mg/kg, i.p.; Sigma-Aldrich) and perfused intracardially with 0.9% saline (w/v) solution followed by 4% formaldehyde (w/v) dissolved in water. After decapitation, the brains were removed and immersed in fresh 4% formaldehyde. At least 24 h before sectioning, the brains were submerged in a 20% sucrose (w/v) solution in saline for cryoprotection. Coronal sections of 40 μ m were cut on a freezing microtome, mounted on gelatin-coated slides, and stained with cresyl violet. The sections were examined under a light microscope and determination of the location of injection needle tips in the BLA was made according to the standardized atlas plates of Paxinos and Watson (2005) by an observer blind to drug treatment condition. Rats with injection needle placements outside the BLA or with extensive tissue damage at the injection needle tips, were excluded from analysis.

Statistics. In each experiment, training and retention test latencies were analyzed using two-way ANOVAs with immediate posttraining infusions of different doses of the agonist or control and antagonist or control as between-subject variables. Additional analysis used Fisher's *post hoc* tests to determine the source of the detected significances. To determine whether learning had occurred, paired *t* tests were used to compare the training and retention latencies. For all comparisons, a probability level of <0.05 was accepted as statistical significance. The number of rats per group is indicated in the figure legends.

Results

Intra-BLA infusions of the CRF-BP ligand inhibitor CRF_{6–33} enhance memory consolidation of inhibitory avoidance training

This experiment investigated whether immediate posttraining infusions of the CRF-BP ligand inhibitor CRF_{6–33} into the BLA would enhance 48 h retention performance of inhibitory avoidance training. Previous findings have indicated that CRF_{6–33}, which has a high affinity for the CRF-BP and is devoid of any intrinsic activity at the CRF receptor (Sutton et al., 1995), displaces CRF from the CRF-BP complex and increases the “free” concentration of endogenous CRF (Behan et al., 1995). To further investigate whether CRF_{6–33} might enhance memory consolidation by increasing the availability of endogenous CRF to bind to its cognate receptor, we also examined whether coadministration of the nonselective CRF receptor antagonist α -helical CRF_{9–41} would block the dose–response effects of CRF_{6–33}.

Average step-through latencies for all groups during training (i.e., before footshock or drug treatment) were 11.4 ± 0.5 s

Figure 1. Step-through latencies (mean $\pm$ SEM) in seconds on the 48 h inhibitory avoidance retention test of rats given immediate posttraining infusions into the basolateral amygdala of the CRF-binding protein ligand inhibitor CRF_{6–33} (0.01, 0.1, or 1 μ g in 0.2 μ l) either alone or together with the nonselective CRF receptor antagonist α -helical CRF_{9–41} (1 μ g). ** $p < 0.01$ compared with the saline group; ♦♦ $p < 0.01$ compared with the corresponding CRF_{6–33} group ($n = 10$ –12 per group).

(mean $\pm$ SEM). A two-way ANOVA for training latencies showed no significant differences between groups (for all comparisons, $p > 0.16$) (data not shown). The 48 h retention latencies of rats infused with saline into the BLA immediately after training were significantly longer than their response latencies on the training trial (31.0 ± 5.7 s; paired *t* test, $p < 0.05$), indicating that the rats retained significant memory of the shock experience. Two-way ANOVA for retention latencies revealed a significant CRF_{6–33} effect ($F_{(3,81)} = 3.37$; $p < 0.05$), a significant α -helical CRF_{9–41} effect ($F_{(1,81)} = 12.03$; $p < 0.001$), as well as a significant interaction between both factors ($F_{(3,81)} = 3.39$; $p < 0.05$). As is shown in Figure 1, CRF_{6–33} infusions alone induced a dose-dependent enhancement of retention performance. The 0.1 μ g dose of CRF_{6–33} significantly enhanced retention ($p < 0.01$), whereas retention latencies of animals given the lower (0.01 μ g) or higher (1 μ g) doses failed to reach significance ($p > 0.06$). The nonspecific CRF receptor antagonist α -helical CRF_{9–41} (1 μ g) infused alone into the BLA immediately after the training trial did not impair retention latencies in the dose used, but blocked the retention enhancement induced by concurrently administered CRF_{6–33}. Retention latencies of rats treated with α -helical CRF_{9–41} together with the intermediate dose of CRF_{6–33} were significantly lower than those of rats given CRF_{6–33} alone ($p < 0.01$).

Intra-BLA infusions of a β -adrenoceptor antagonist or cAMP inhibitor, but not an α_1 -adrenoceptor antagonist, block CRF_{6–33}-induced enhancement of inhibitory avoidance memory consolidation

This experiment examined whether immediate posttraining bilateral infusions of a β -adrenoceptor or α_1 -adrenoceptor antagonist or cAMP inhibitor into the BLA would block 48 h retention enhancement induced by concurrently administered CRF_{6–33} (0.01, 0.1, or 1 μ g). As is shown in Figure 2, the β -adrenoceptor antagonist atenolol (0.5 μ g) blocked CRF_{6–33}-induced retention enhancement. Two-way ANOVA for retention latencies revealed a significant CRF_{6–33} effect ($F_{(3,77)} = 3.06$; $p < 0.05$), a significant atenolol effect ($F_{(1,77)} = 20.59$; $p < 0.0001$), as well as a significant interaction between both factors ($F_{(3,77)} = 2.77$; $p < 0.05$). CRF_{6–33} infusions alone induced a dose-dependent enhancement of retention performance. The 0.1 μ g dose of CRF_{6–33} significantly enhanced retention ($p < 0.01$), whereas retention latencies of animals given the lower (0.01 μ g) or higher (1 μ g)

Figure 2. Step-through latencies (mean ± SEM) in seconds on the 48 h inhibitory avoidance retention test of rats given immediate posttraining infusions into the basolateral amygdala of the CRF-binding protein ligand inhibitor CRF₆₋₃₃ (0.01, 0.1, or 1 μg in 0.2 μl) either alone or together with the β-adrenoceptor antagonist atenolol (0.5 μg), the α₁-adrenoceptor antagonist prazosin (0.1 μg), or the cAMP inhibitor Rp-cAMPS (4 μg). * $p < 0.05$, ** $p < 0.01$ compared with the corresponding saline group; ♦ $p < 0.05$, ♦♦ $p < 0.01$ compared with the corresponding CRF₆₋₃₃ group ($n = 9$ –13 per group).

doses failed to reach significance ($p > 0.07$). The β-adrenoceptor antagonist atenolol infused alone into the BLA immediately after the training trial did not impair retention latencies, but blocked the retention enhancement induced by concurrently administered CRF₆₋₃₃. Retention latencies of rats treated with atenolol together with either of the two lower doses of CRF₆₋₃₃ were significantly lower than those of rats given corresponding doses of CRF₆₋₃₃ alone (0.01 μg, $p < 0.05$; 0.1 μg, $p < 0.01$). In contrast, the α₁-adrenoceptor antagonist prazosin infused into the BLA immediately posttraining did not block CRF₆₋₃₃-induced retention enhancement ($p > 0.26$, compared with CRF₆₋₃₃ alone). Two-way ANOVA for retention latencies revealed a significant CRF₆₋₃₃ effect ($F_{(3,80)} = 5.21$; $p < 0.005$), but no significant prazosin effect ($F_{(1,80)} = 1.60$; $p = 0.21$) or interaction between both factors ($F_{(3,80)} = 0.47$; $p = 0.70$). As with CRF₆₋₃₃ administered alone, the 0.1 μg dose of CRF₆₋₃₃ infused together with prazosin induced significant enhancement of retention latencies ($p < 0.05$). The pattern of results for the cAMP inhibitor Rp-cAMPS infused into the BLA was similar to that of atenolol. Two-way ANOVA for retention latencies revealed a significant CRF₆₋₃₃ effect ($F_{(3,75)} = 2.78$; $p < 0.05$), a significant Rp-cAMPS effect ($F_{(1,75)} = 19.22$; $p < 0.0001$), as well as a significant interaction between both factors ($F_{(3,75)} = 3.07$; $p < 0.05$). With the dose used, Rp-cAMPS did not induce memory impairment when administered alone, but blocked the retention enhancement induced by concurrently administered CRF₆₋₃₃. Retention latencies of rats given any of the doses of CRF₆₋₃₃ together with Rp-cAMPS were significantly lower than those of rats given these doses of CRF₆₋₃₃ alone (0.01 or 1 μg, $p < 0.05$; 0.1 μg, $p < 0.01$).

Intra-BLA infusions of α-helical CRF₉₋₄₁ differentially affect inhibitory avoidance retention enhancement induced by activation of β-adrenoceptors, α₁-adrenoceptors, or cAMP

The finding that noradrenergic blockade in the BLA prevented CRF₆₋₃₃-induced memory enhancement indicates that CRF effects on memory consolidation require activation of the β-adrenoceptor–cAMP signaling pathway in the BLA. However, these findings do not allow any conclusion concerning whether CRF acts at a postsynaptic level in BLA neurons to actively modulate β-adrenoceptor–cAMP activity. If CRF effects on memory consolidation are mediated by altering the β-adrenoceptor response,

then a blockade of CRF receptors in the BLA should reduce memory enhancement induced by noradrenergic activation. To investigate this issue, this series of experiments examined whether a blockade of CRF receptors in the BLA alters the dose–response effects on retention enhancement induced by posttraining activation of several postsynaptic components of the noradrenergic system. Figure 3, A–C, shows 48 h retention latencies of rats given immediate posttraining intra-BLA infusions of the nonselective CRF receptor antagonist α-helical CRF₉₋₄₁ together with clenbuterol (a β-adrenoceptor agonist), cirazoline (an α₁-adrenoceptor agonist), or 8-br-cAMP (a synthetic cAMP analog). The training latencies for these groups, before footshock or drug treatment, did not differ significantly (for all comparisons, $p > 0.06$), and 48 h retention latencies of saline groups were significantly longer than their training latencies (paired t test, $p < 0.01$), indicating memory of the shock experience.

Clenbuterol

The CRF receptor antagonist α-helical CRF₉₋₄₁ (1 μg), administered bilaterally into the BLA immediately after training, shifted the dose–response effects of concurrently administered clenbuterol (1, 10, or 100 ng) on 48 h retention performance (Fig. 3A). A two-way ANOVA of retention latencies showed nonsignificant clenbuterol ($F_{(3,77)} = 2.18$; $p = 0.10$) or α-helical CRF₉₋₄₁ effects ($F_{(1,77)} = 0.003$; $p = 0.96$), but a significant interaction between conditions ($F_{(3,77)} = 4.82$; $p < 0.005$). Relative to saline controls, immediate posttraining infusions of clenbuterol into the BLA induced dose-dependent enhancement of retention performance. The lowest dose (1 ng) enhanced retention ($p < 0.05$), whereas higher doses of clenbuterol (10 or 100 ng) were ineffective. α-Helical CRF₉₋₄₁ administered alone did not affect retention latencies but shifted the dose–response effects of clenbuterol. In rats also given α-helical CRF₉₋₄₁, the lowest dose of clenbuterol (1 ng) failed to enhance retention, and a much higher dose of clenbuterol (100 ng) was necessary to induce significant retention enhancement ($p < 0.01$).

Cirazoline

α-Helical CRF₉₋₄₁ infused into the BLA immediately posttraining did not affect retention enhancement induced by concurrent administration of the α₁-adrenoceptor agonist cirazoline (Fig. 3B). Two-way ANOVA for retention latencies showed a significant cirazoline effect ($F_{(3,79)} = 4.38$; $p < 0.01$), but no significant α-helical CRF₉₋₄₁ effect ($F_{(1,79)} = 0.32$; $p = 0.58$) or interaction between these factors ($F_{(3,79)} = 0.13$; $p = 0.94$). Relative to saline controls, cirazoline administered into the BLA immediately posttraining induced dose-dependent retention enhancement. The intermediate dose of cirazoline (0.1 μg) enhanced retention performance ($p < 0.05$), whereas lower (0.01 μg) or higher (1 μg) doses were ineffective. Cirazoline infused into the BLA together with α-helical CRF₉₋₄₁ (1 μg) induced similar dose–response effects on retention performance. As with cirazoline alone, the 0.1 μg dose of cirazoline administered together with α-helical CRF₉₋₄₁ induced significant enhancement of retention performance ($p < 0.05$).

It should be noted that, although cirazoline is reportedly one of the most selective α₁-adrenoceptor agonists currently available (Pigini et al., 2000), it also shows high affinity for the functionally related nonadrenergic imidazoline I₂ binding site (Bricca et al., 1989; Pineda et al., 1993). To determine whether the above-described cirazoline effect is mediated by an activation of α₁-adrenoceptors, we investigated whether prazosin (0.1 μg), an α₁-adrenoceptor antagonist without known affinity for imidazo-

line receptors (Docherty, 1998), blocked the retention enhancement induced by concurrently administered cirazoline (0.1 μ g). Immediate posttraining intra-BLA infusions of cirazoline significantly increased retention performance (mean $\pm$ SEM, 114 ± 23.4 s; $n = 11$) compared with saline controls (30.3 ± 6.2 s; $n = 8$; $p < 0.01$), an effect that was completely and selectively blocked by coadministration of prazosin (33.2 ± 8.1 s; $n = 11$; $p < 0.01$) (data not shown). These findings thus indicate that the enhancement in memory consolidation induced by cirazoline, at least at the doses studied, likely involves an α_1 -adrenoceptor mechanism.

8-br-cAMP

Intra-BLA infusions of α -helical-CRF_{9–41} (1 μ g) did not alter retention enhancement induced by concurrent infusions of the synthetic cAMP analog 8-br-cAMP (Fig. 3C). Two-way ANOVA for retention latencies showed a significant 8-br-cAMP effect ($F_{(3,83)} = 3.55$; $p < 0.05$), a nonsignificant α -helical CRF_{9–41} effect ($F_{(1,83)} = 0.28$; $p = 0.60$) and a nonsignificant interaction between these factors ($F_{(3,83)} = 0.05$; $p = 0.98$). Immediate posttraining intra-BLA infusions of 8-br-cAMP (0.1, 0.3, or 1 μ g) induced dose-dependent retention enhancement, both when administered alone or together with α -helical CRF_{9–41}. In both conditions, the lowest dose (0.1 μ g) induced significant enhancement of retention performance ($p < 0.05$).

Interactions between CRF and glucocorticoids in the BLA in memory enhancement for inhibitory avoidance training

The above-mentioned findings that CRF_{6–33}, by increasing free CRF concentrations, enhances memory consolidation via a facilitation of β -adrenoceptor–cAMP activity within the BLA are remarkably similar to those of a previous study investigating interactions between glucocorticoids and the noradrenergic system within the BLA on memory consolidation for inhibitory avoidance training (Roosendaal et al., 2002a). Because extensive evidence indicates that the CRF and glucocorticoid systems are intimately linked and cooperate in many brain regions to regulate a variety of behavioral and physiological functions (Owens et al., 1990; Thompson et al., 2004), a last series of experiments investigated whether CRF and glucocorticoids might interact within the BLA in influencing memory consolidation of inhibitory avoidance training. Glucocorticoid-induced enhancement of memory consolidation depends predominantly on an activation of the low-affinity GR (Oitzl and de Kloet, 1992; Roosendaal et al., 1996; Conrad et al., 1999). A first experiment investigated whether a blockade of GRs with posttraining infusions of the receptor antagonist RU 38486 into the BLA altered the dose-response effects on retention enhancement induced by concurrently administered CRF_{6–33} and a second experiment used an opposite approach and examined whether posttraining blockade of CRF receptors in the BLA with infusions of α -helical CRF_{9–41} shifted the dose-response effects of a concurrently administered GR agonist (Fig. 4A,B).

The GR antagonist RU 38486 (1 ng) infused into the BLA immediately after the training trial completely blocked retention enhancement induced by concurrent infusions of the CRF-BP ligand inhibitor CRF_{6–33} (0.01, 0.1, or 1 μ g) (Fig. 4A). Two-way

Figure 3. Step-through latencies (mean $\pm$ SEM) in seconds on the 48 h inhibitory avoidance retention test of rats given immediate posttraining infusions into the basolateral amygdala of the CRF receptor antagonist α -helical CRF_{9–41} (1 μ g in 0.2 μ l) together with the β -adrenoceptor agonist clenbuterol (1, 10, or 100 ng) (A), the α_1 -adrenoceptor agonist cirazoline (0.01, 0.1, or 1 μ g) (B), or the synthetic cAMP analog 8-br-cAMP (0.1, 0.3, or 1 μ g) (C). * $p < 0.05$, ** $p < 0.01$ compared with the corresponding saline group; $\blacklozenge p < 0.05$, $\blacklozenge p < 0.01$ compared with the corresponding clenbuterol-alone group ($n = 9$ –13 per group).

ANOVA for retention latencies revealed significant CRF_{6–33} ($F_{(3,85)} = 3.11$; $p < 0.05$) and RU 38486 effects ($F_{(1,85)} = 5.67$; $p < 0.05$) as well as a significant interaction between conditions ($F_{(3,85)} = 3.68$; $p < 0.05$). In agreement with the above-mentioned findings, posttraining infusions of CRF_{6–33} into the BLA induced dose-dependent enhancement of retention performance. The intermediate dose of CRF_{6–33} (0.1 μ g) enhanced retention latencies ($p < 0.05$), whereas lower (0.01 μ g) or higher (1 μ g) doses were ineffective. This low dose of RU 38486 infused alone into the BLA immediately after the training trial did not impair retention performance, but blocked the retention enhancement induced by concurrently administered CRF_{6–33}. Retention latencies of rats treated with RU 38486 together with the intermediate dose of CRF_{6–33} were significantly lower than those of rats given the corresponding dose of CRF_{6–33} alone ($p < 0.05$).

In contrast, the CRF receptor antagonist α -helical CRF_{9–41} administered into the BLA shifted the dose-response effects of the GR agonist RU 28362 administered concurrently on 48 h inhibitory avoidance retention performance (Fig. 4B). Relative to vehicle controls, immediate posttraining infusions of RU 28362 (1, 3, or 10 ng) into the BLA induced dose-dependent enhancement of retention performance. The lowest dose (1 ng) enhanced retention ($p < 0.01$), whereas higher doses of RU 28362 (3 or 10 ng) were ineffective. Two-way ANOVA for retention latencies showed nonsignificant RU 28362 ($F_{(3,69)} = 2.57$; $p = 0.06$) or α -helical CRF_{9–41} effects ($F_{(1,69)} = 0.05$; $p = 0.83$), but a significant interaction between conditions ($F_{(3,69)} = 5.36$; $p < 0.005$). α -Helical CRF_{9–41} (1 μ g) administered alone did not affect retention latencies, but shifted the dose-response effects of RU 28362. When α -helical CRF_{9–41} was coinfused with RU 28362, the lowest dose of RU 28362 (1 ng) failed to enhance retention performance, and a 10 times higher dose of RU 28362 was necessary to induce significant retention enhancement ($p < 0.01$).

Histology

A representative photomicrograph of a needle track terminating within the BLA is shown in Figure 5. Only rats with needle tips within the boundaries of the BLA were included in the data analysis. Approximately 19% of the animals were excluded from analysis because of either cannula misplacement or damage to the targeted tissue.

Discussion

The present findings indicate that posttraining infusions of the CRF-BP ligand inhibitor CRF_{6–33} into the BLA induce dose-

Figure 4. Step-through latencies (mean $\pm$ SEM) in seconds on the 48 h inhibitory avoidance retention test. **A**, Rats were given immediate posttraining infusions into the basolateral amygdala of the CRF-binding protein ligand inhibitor CRF₆₋₃₃ (0.01, 0.1, or 1 μ g in 0.2 μ l) either alone or together with the glucocorticoid receptor antagonist RU 38486 (1 ng). **B**, Rats were given immediate posttraining infusions into the basolateral amygdala of the GR agonist RU 28362 (1, 3, or 10 ng in 0.2 μ l) either alone or together with the CRF receptor antagonist α -helical CRF₉₋₄₁ (1 μ g). * p < 0.05, ** p < 0.01 compared with the corresponding vehicle group; $\diamond$ p < 0.05, $\diamond\diamond$ p < 0.01 compared with the corresponding CRF₆₋₃₃- or RU 28362-alone group (n = 8–11 per group).

Figure 5. Representative photomicrograph illustrating placement of a cannula and needle tip in the basolateral amygdala. The arrow points to the needle tip. The gray area in the diagram represents the different nuclei of the basolateral complex of the amygdala: the lateral nucleus (L), basal nucleus (B), and accessory basal nucleus (AB). CEA, Central nucleus of the amygdala.

dependent enhancement of 48 h inhibitory avoidance retention performance and that the effects depend on interactions with the noradrenergic system of the BLA and require concurrent GR activation. Immediate posttraining infusions of CRF₆₋₃₃ administered into the BLA, which contains a high level of CRF-BP (Herrington et al., 2004; Roseboom et al., 2007), dose-dependently enhanced inhibitory avoidance retention latencies. Consistent with the findings of many previous pharmacological studies of memory, whereas moderate doses of CRF₆₋₃₃ enhanced retention performance, lower and higher doses were ineffective. The mechanism underlying this dose–response effect is unknown but the generality of such a bell-shaped curve across drug systems (Rooszendaal et al., 2007) strongly suggests that the ineffectiveness of higher doses in the present study is not caused by any specific characteristics of CRF₆₋₃₃. Such CRF₆₋₃₃-induced memory enhancement is consistent with previous evidence of dose-dependent enhancement of inhibitory avoidance retention latencies after posttraining intraventricular administration of CRF₆₋₃₃ (Heinrichs et al., 1997). Moreover, systemic or intraventricular administration of CRF₆₋₃₃ improves cognitive functioning on several other learning tasks (Behan et al., 1995; Heinrichs et al., 1997). Because CRF₆₋₃₃ has high affinity for the CRF/CRF-BP complex but is devoid of any intrinsic activity at the CRF receptor (Sutton et al., 1995), such findings suggest that CRF₆₋₃₃ enhances memory consolidation by displacing CRF from its binding pro-

tein and increasing free levels of CRF (Behan et al., 1995). Although we did not measure free CRF levels in the BLA, in support of such a mechanism we found that the nonspecific CRF receptor antagonist α -helical CRF₉₋₄₁ blocked the CRF₆₋₃₃ effect. These findings are consistent with extensive evidence that exogenous administration of CRF enhances memory consolidation of both aversively and appetitively motivated learning tasks when administered intraventricularly (Sahgal et al., 1983; Cole and Koob, 1988), directly into the amygdala complex (Liang and Lee, 1988; Lee and Sung, 1989) or into the bed nucleus of the stria terminalis after training (Liang et al., 2001). Furthermore, electrophysiological studies in hippocampal slices have shown that exogenous CRF application facilitates long-term potentiation, a synaptic correlate of memory (Aldenhoff et al., 1983; Hollrigel et al., 1998; Blank et al., 2002) and that a CRF receptor antagonist blocks stress-induced facilitation of hippocampal long-term potentiation (Blank et al., 2002).

The present finding that immediate posttraining intra-BLA infusions of either the β -adrenoceptor antagonist atenolol or the cAMP inhibitor Rp-cAMPS prevented CRF₆₋₃₃-induced retention enhancement indicates that CRF influences memory consolidation via an interaction with the noradrenergic system at its terminal field within the BLA. These findings are thus consistent with, but significantly extend, previous evidence that CRF administration enhances memory consolidation by activating noradrenergic neurons in the locus ceruleus (Valentino et al., 1983; Smagin et al., 1995; Finlay et al., 1997; Jedema and Grace, 2004) and stimulating the release of norepinephrine in different forebrain regions, including the BLA (Chen et al., 1992; Lavicky and Dunn, 1993; Isogawa et al., 2000; Asbach et al., 2001). It is presently unknown whether CRF administered into the BLA is capable of locally increasing the availability of norepinephrine. Such an action would imply that CRF has to act on presynaptic noradrenergic terminals to stimulate its release. Alternatively, because CRF is known to increase the excitability of BLA pyramidal cells via an inhibition of GABA-mediated afterhyperpolarizing potentials (Rainnie et al., 1992) and GABA administration reduces the release of norepinephrine in the BLA (Hatfield et al., 1999), CRF might increase norepinephrine levels in the BLA via a suppression of GABAergic mechanisms. It is unlikely that the CRF-induced memory enhancement is attributable to a diffusion of CRF from the BLA to the neighboring central nucleus of the amygdala, which contains a large population of CRF-expressing neurons that project to the locus ceruleus region (Van Bockstaele et al., 1999), because we previously found that α -helical CRF₉₋₄₁ infused posttraining into the central nucleus did not affect memory consolidation (Rooszendaal et al., 2002b). Such a selective involvement of the BLA in modulating memory consolidation is consistent with extensive previous evidence obtained with intraamygdala nuclei infusions of many other hormones and neurotransmitters as well as with both anatomical and functional evidence indicating that neuromodulatory influences within the BLA regulate memory consolidation processes via an extensive network of BLA projections to other brain regions, including the hippocampus and several cortical regions (McGaugh, 2004).

Because CRF receptors are G-protein-coupled membrane receptors (Perrin et al., 1993) and, like the β -adrenoceptor, influence adenylate cyclase activity and cAMP accumulation (Schultz and Daly, 1973; Bale and Vale, 2004), it is likely that these two systems, at least in part, interact postsynaptically at the level of adenylate cyclase. In support of the view that CRF enhances

memory consolidation by activating cAMP in the BLA, a recent study reported that blockade of CRF1 receptors with DMP696 reduces pCREB (phosphorylated cAMP response element-binding protein) levels in the BLA after contextual fear conditioning (Hubbard et al., 2007). If CRF enhances memory consolidation by potentiating β -adrenoceptor-mediated cAMP activity in the BLA, then a blockade of CRF receptors in the BLA should attenuate the effect of β -adrenoceptor activation on memory consolidation. In support of this interpretation, we found that the CRF receptor antagonist α -helical CRF_{9–41} infused into the BLA shifted the dose–response effects of clenbuterol such that a 100 times higher dose of clenbuterol was required to induce memory enhancement. In contrast, α -helical CRF_{9–41} did not modify the dose–response effects of 8-br-cAMP, indicating that cAMP acts downstream from the locus of interaction of CRF with the β -adrenoceptor–cAMP pathway in the BLA. Such a selective influence of CRF receptor blockade on β -adrenoceptor-, but not 8-br-cAMP-induced memory enhancement is very similar to that obtained previously with α_1 -adrenoceptor blockade in the BLA (Ferry et al., 1999b). Blockade of α_1 -adrenoceptors within the BLA induced a similar shift in the dose–response effects of clenbuterol, but did not modify those of 8-br-cAMP. Such findings suggest that these two neuromodulatory systems might act at a common site to influence β -adrenoceptor–cAMP activity in the BLA. However, the present finding that a blockade of either CRF receptors or α_1 -adrenoceptors did not modify the memory-enhancing effects of each other's agonist suggests that CRF receptor and α_1 -adrenoceptor activation facilitate β -adrenoceptor-mediated adenylylase activity via two independent mechanisms.

Our finding that the GR antagonist RU 38486 infused into the BLA blocked the enhancing effect of concurrent CRF_{6–33} administration on the consolidation of memory of inhibitory avoidance training and that the CRF antagonist attenuated the dose–response effects of GR activation is consistent with previous evidence indicating close interactions between these two systems. Elevated corticosterone levels, in conjunction with other factors (Shepard et al., 2005), are well known to limit the sensitivity and magnitude of stress-induced CRF transcription and expression in the paraventricular nucleus (Sawchenko, 1987; Ma and Aguilera, 1999; Helmreich et al., 2001), but to increase CRF synthesis in the central amygdala (Thompson et al., 2004). Most relevantly, glucocorticoids alter postsynaptic sensitivity of locus ceruleus neurons to CRF (Pavcovich and Valentino, 1997). We previously reported that intra-BLA infusions of the GR antagonist RU 38486, as with the CRF antagonist, induced a shift in the dose–response effects of the β -adrenoceptor agonist clenbuterol on inhibitory avoidance memory (Roozendaal et al., 2002a). However, an important difference from the effects of CRF receptor blockade is that the GR antagonist also prevented the memory-enhancing effect of α_1 -adrenoceptor activation. In agreement with neurochemi-

Figure 6. Schematic summarizing CRF and glucocorticoid effects on the β -adrenoceptor–cAMP signaling pathway in the basolateral amygdala in influencing memory consolidation. Norepinephrine (NE) is released after training in emotionally arousing tasks and binds to both β -adrenoceptors and α_1 -adrenoceptors at postsynaptic sites. The β -adrenoceptor is coupled directly to adenylylase to stimulate cAMP formation. The α_1 -adrenoceptor is known to modulate the response induced by β -adrenoceptor stimulation. CRF may also facilitate the β -adrenoceptor–cAMP response, but independently from the α_1 -adrenoceptor-induced modulation. Glucocorticoids enhance memory consolidation via a synergistic interaction with both the CRF and α_1 -adrenoceptor systems in potentiation training-induced β -adrenoceptor–cAMP activation. Other studies have demonstrated that cAMP may initiate a cascade of intracellular events involving the activation of cAMP-dependent protein kinase (PKA). Our findings suggest that these effects in the basolateral amygdala are required for regulating memory consolidation in other brain regions. α_1 , α_1 -Adrenoceptor; α_2 , α_2 -adrenoceptor; AC, adenylylase; β , β -adrenoceptor; CRF-R, corticotropin-releasing factor receptor.

cal evidence (Stone et al., 1987; Duman et al., 1989), we previously concluded that GR stimulation in the BLA enhances memory consolidation via a modulation of α_1 -adrenoceptor-mediated facilitation of β -adrenoceptor–cAMP activity. However, the present findings indicating that glucocorticoids also interact with CRF in influencing memory consolidation suggest that glucocorticoids influence β -adrenoceptor–cAMP activity in the BLA via interactions with both the CRF and α_1 -adrenoceptor systems. Because norepinephrine administration rapidly induces increases in cAMP levels (Stone et al., 1987), such a potentiation of this intracellular response seems incompatible with the classic view of glucocorticoids affecting gene transcription through an activation of nuclear GRs. Rather, this time frame suggests that glucocorticoid effects on the CRF receptor and α_1 -adrenoceptor systems in influencing β -adrenoceptor–cAMP activity might involve rapid, non-genomic actions via membrane-associated GRs (Johnson et al., 2005). Figure 6 shows a diagram summarizing the proposed interaction of CRF and glucocorticoids with the noradrenergic system of the BLA, as suggested by our current and previous findings.

The finding that CRF interacts with noradrenergic mechanisms in the BLA in influencing memory consolidation is consistent with extensive evidence indicating that the memory-modulatory effects of drugs affecting many other neuromodulatory and hormonal systems rely on noradrenergic activation within the BLA or (as assessed in early studies) within the amygdaloid complex (Dias et al., 1979; Liang et al., 1986; Introini-Collison et al., 1989; McGaugh et al., 1996; LaLumiere et al., 2004; Roozendaal et al., 2007). Although CRF also interacts with other neurotransmitter systems in influencing learning and memory (Radulovic et al., 2000), such an interaction with the noradrenergic system may have significant consequences for its

role in regulating memory consolidation. Recent findings of animal and human studies investigating the effects of adrenocortical hormones on memory consolidation indicated that glucocorticoids enhance memory of emotionally arousing, and not emotionally neutral, experiences (Buchanan and Lovall, 2001; Okuda et al., 2004; Abercrombie et al., 2006; Kuhlmann and Wolf, 2006; Van Stegeren et al., 2007) because of such a critical dependence on emotional arousal-induced noradrenergic activation within the BLA (Roosendaal et al., 2006b). The present findings that CRF enhances memory consolidation via an interaction with β -adrenoceptor–cAMP activity in the BLA suggests that CRF might also play a selective role in ensuring long-lasting strong memories of emotionally arousing experiences. Furthermore, because both aversive and appetitive stimulation is known to induce the release of CRF in the amygdala (Merali et al., 2003), such findings are in accordance with the general view that amygdala-induced memory enhancement depends on the salience, and not valence, of the training experience (McGaugh, 2004).

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