Increase of Extracellular Corticotropin-Releasing Factor-Like Immunoreactivity Levels in the Amygdala of Awake Rats during Restraint Stress and Ethanol Withdrawal as Measured by Microdialysis

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Previous research has suggested a role for corticotropin-releasing factor (CRF) in the anxiogenic effects of stressful stimuli and ethanol withdrawal. This hypothesis was explored in a series of experiments using intracranial microdialysis to monitor CRF-like immunoreactivity (CRF-IR) in the extracellular compartment of the rat amygdala. The synaptic origin of CRF-IR release in the amygdala was determined *in vitro* by assessing the Ca^{2+} dependency of 4-aminopyridine stimulated CRF-IR release from tissue preparations of rat amygdala. *In vivo* experiments were performed in awake rats after the placement of microdialysis probes in the amygdala. In the first experiment, transient restraint stress (20 min) produced an increase of CRF-IR release (basal levels, 1.19 ± 0.15 fmol/50 μl ; stress levels, 4.54 ± 1.33 fmol/50 μl ; $p < 0.05$) that returned to basal values within 1 hr. When 4-aminopyridine (5 mM) was added to the perfusion medium, it consistently increased CRF-IR release (4.83 ± 0.92 fmol/50 μl , $p < 0.05$). In the second experiment, CRF-IR release was measured during ethanol withdrawal in rats previously maintained for 2–3 weeks on a liquid diet containing ethanol (8.5%). Basal CRF-IR levels were 2.10 ± 0.43 fmol/50 μl in ethanol exposed rats and 1.30 ± 0.19 fmol/50 μl in control rats. During withdrawal, a progressive increase of CRF-IR levels over time was observed, reaching peak values at 10–12 hr after the onset of withdrawal (10.65 ± 0.49 fmol/50 μl vs 1.15 ± 0.30 fmol/50 μl of control rats, $p < 0.01$). Since the peak of CRF-IR release corresponded to the time of appearance of anxiogenic behavioral effects in rats, the present data lend further support to the hypothesis that the CRF-IR system in the amygdala participates in the media-

tion of the emotional component of the response to exogenous or endogenous stressful stimuli.

[Key words: corticotropin-releasing factor, intracranial microdialysis, 4-aminopyridine, central amygdaloid nucleus, stress, rat, ethanol withdrawal]

Converging lines of evidence suggest that corticotropin-releasing factor (CRF), a hypophysiotropic peptide of 41 amino acids isolated from bovine hypothalamus (Vale et al., 1981), participates in the mediation of behavioral responses to stress in mammals (Koob and Bloom, 1985; Dunn and Berridge, 1990; Nemeroff, 1991; Koob et al., 1993). High densities of CRF immunoreactive perikarya and terminals are present in limbic brain structures (Swanson et al., 1983; Sakanaka et al., 1986; Gray, 1990). CRF binding sites (De Souza et al., 1987; Hauger et al., 1987) and receptors (Perrin et al., 1993) are also widely distributed throughout the CNS. Although hypothalamic CRF is known to play a major role in neuroendocrine adaptation to stress (Rivier et al., 1982; Antoni, 1986; Plotsky, 1991), the role of CRF in specific extrahypothalamic regions of the brain is not clear yet. Changes of CRF-like immunoreactivity (CRF-IR) tissue levels (Chappel et al., 1986) and CRF gene expression (Imaki et al., 1991) are observed following transient or chronic stress in several hypothalamic and limbic structures, indirectly suggesting a response of CRF neurons to stress.

Exogenous administration of CRF induces physiological and behavioral effects that resemble those produced by stress. Intracerebroventricular (i.c.v.) injections of CRF increase the firing of locus coeruleus neurons (Valentino and Foote, 1988), increase peripheral sympathetic outflow (Brown et al., 1982) and respiratory rate (Bohmer et al., 1990), and produce proconflict or “anxiogenic-like” effects in a variety of paradigms of emotionality in rodents (Britton et al., 1985; Dunn and File, 1987; Takahashi et al., 1989). Consistent with these observations, central injections of CRF antagonists block the behavioral effects of either exogenously administered CRF, stress, or conditioned fear (Britton et al., 1986; Kalin et al., 1988; Swerdlow et al., 1989; Liang et al., 1992a; Menzaghi et al., 1994).

Several of the stress-like responses associated with CRF administration can be mimicked by stimulating the central amygdaloid nucleus (Ce), a structure that plays a role in orchestrating various aspects of the emotional output (Swanson and Mogenson, 1981; Le Doux et al., 1988; Davis, 1992). Electrical stimulation of the Ce increases heart rate, blood pressure, peripheral sympathetic outflow, respiratory rate, and hypothalamo-pitui-

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tary-adrenal (HPA) axis activity, and produce behavioral effects similar of those seen in conditioned fear (Davis, 1986; Dunn and Whitener, 1986; Iwata et al., 1987; Le Doux et al., 1988). Consistent with these observations, lesions of the Ce attenuate the conditioned fear response (Sananes and Davis, 1992) and produce anti-conflict effects (Shibata et al., 1989). Since a rich plexus of CRF-containing terminals and neurons projecting to the brainstem is present in the Ce (Gray and Magnuson, 1987), it has been suggested that CRF in the amygdala participates in the mediation of the increased emotionality produced by exposure to stressors.

Prolonged increase in emotionality and HPA axis activation that resemble the effects of stress have been also described during ethanol withdrawal (Freund, 1969; Tabakoff et al., 1978; De Sota et al., 1985). In spite of the evidence suggesting a role of CRF in the ethanol withdrawal syndrome (Ehlers and Chaplin, 1987; Rivier et al., 1990; Baldwin et al., 1991), direct evidence on CRF release in the amygdala during exposure to stress or ethanol withdrawal is still lacking.

Recently, a microdialysis procedure to measure *in vivo* extracellular levels of CRF-IR in freely moving rats was developed (Merlo Pich et al., 1993). In the present work, this method was used to investigate the effects of restraint stress and ethanol withdrawal on CRF-IR release from the amygdala of awake rats.

Materials and Methods

Animals. Fifty-five male Wistar rats (Charles River), weighing 200–225 gm at the beginning of the experiments, were housed in groups of three in standard rat cages in a humidity and temperature (22°C) controlled vivarium with 12/12 hr L/D cycle (lights on at 1900 hr). Immediately after surgery or at the beginning of the experiments involving liquid diet consumption, the rats were caged individually. Food and water were provided *ad libitum* for the animals included in experiments 1 and 2, whereas nutritionally balanced liquid diets were used in experiment 3, as described below.

Dissection of amygdala for *in vitro* experiments. Rats were sacrificed by decapitation between 1000 hr and 1100 hr, their brain rapidly removed, and placed in a brain slicer (San Diego Instruments, San Diego, CA). A 3 mm thick coronal slice was cut, starting at 1.8 mm posterior to bregma (Paxinos and Watson, 1986). The amygdaloid regions were dissected out bilaterally from the slice under a low-magnification microscope by a vertical cut tangential to the external capsule and a diagonal cut along the medial border of the ipsilateral optic tract, as previously described (Raber et al., 1994). Amygdaloid regions were then placed on a Brinkmann tissue chopper to obtain 300 μ m slices. Minced amygdaloid regions were placed in tubes containing balanced Earle's salt solution (GIBCO, Gaithersburg, MD) supplemented with 0.1% bovine serum albumin (BSA, Pentex, Miles Inc., Kankakee, IL), 60 μ g/ml ascorbic acid (Aldrich, Milwaukee, WI) 0.54 mg/kg glucose, 20 mM bacitracin, 1 μ M phenylmethylsulfonyl fluoride (Sigma, St. Louis, MO), and 200 kIU/ml aprotinin (Boehringer-Mannheim, Indianapolis, IN). Slices of the whole amygdaloid regions were incubated at 37°C under O₂/CO₂ (95:5%) or 60–80 min before starting the experiment.

Surgery for *in vivo* experiments with microdialysis. Seven days before the microdialysis experiments, rats were anesthetized (1% halothane in O₂/CO₂, 95:5%) and placed in a stereotaxic apparatus (David Kopf Instruments, Tujunga, CA). A guide cannula (CMA/10, Bioanalytical Systems, West Lafayette, IN) was unilaterally implanted 7 mm above the final location of the microdialysis probe tip, and secured with stainless steel skull screws and dental cement. Final stereotaxic coordinates with respect to the microdialysis probe tip were 2.6 mm posterior to bregma, 4.2 mm lateral to the midline, –8.7 mm below dura (Paxinos and Watson, 1986).

Microdialysis apparatus and perfusion media. Experiments were performed using commercially available microdialysis probes (CMA/10, external diameter of 0.5 mm, 2 mm dialysis membrane tip, molecular cutoff of 20,000 Da). Perfusion medium was loaded into Hamilton syringes operated by a pulseless microinfusion pump (CMA/100). A liquid switch (CMA/110) was used to permit rapid changes of the perfusion medium. Perfusate was collected in polyethylene tubes kept in ice

during the perfusion interval (20 min). Perfusion fractions were frozen on dry ice immediately after collection and stored at –70°C until radioimmunoassay. Before each experiment, microdialysis probes were perfused at room temperature with artificial cerebrospinal fluid containing 1% BSA to saturate nonspecific binding sites for peptides within the perfusion system.

The basic perfusion medium consisted of artificial cerebrospinal fluid (ACSF) containing NaCl (149.0 mM), KCl (3.7 mM), MgCl₂ (0.9 mM), CaCl₂ (1.2 mM), NaH₂PO₄ (1.2 mM), and BSA (1%). The ACSF (pH 7.4) was filtered sterile with nylon filters (Gelman Acrodisc 4192, Ann Arbor, MI). This medium was used for rinsing the probe, and perfusion of probes implanted into the animals during periods when CRF levels were not monitored. The perfusion medium used for measuring CRF was prepared by adding specific anti-CRF serum (rC70 or rC69, kindly provided by W. Vale) to ACSF at a dilution of 1:80,000. Anti-CRF serum was added because previous observations have shown that the addition of a specific, well-characterized anti-CRF rabbit serum to the perfusion medium can improve the recovery of CRF-IR (Merlo Pich et al., 1993). At the end of each experiment, an aliquot of remaining perfusion medium containing anti-CRF serum was frozen together with the collected fractions, and stored at –70°C. This medium was then used in the construction of the standard curve of the radioimmunoassay.

CRF radioimmunoassays. Detection and quantification of CRF was adapted from Vale et al. (1983). This procedure of CRF measurement is based on competitive binding radioimmunoassay, and was validated with independent HPLC studies. However, in our study, no data verifying the identity of the immunoreactivity are presented, due to the low amounts recovered by the experimental techniques we used. Therefore, the measurements are reported as CRF-like immunoreactivity (CRF-IR).

The CRF radioimmunoassay standard method was employed to measure the concentrations of CRF-IR released from amygdaloid region tissue *in vitro*, as previously described (Smith et al., 1986; Calogero et al., 1989).

This procedure has been extended to microdialysis applications in a series of *in vitro* and *in vivo* validations (Merlo Pich et al., 1993). Briefly, the concentration of specific anti-CRF serum within the perfusion medium was calculated in order to optimize the radioimmunoassay for CRF. For each experiment standard curves were prepared in duplicate using 50 μ l/tube of the same perfusion medium containing anti-CRF serum used for microdialysis probe perfusion and fraction collection. Final anti-CRF serum dilution was 1:800,000, and the final incubation volume was 500 μ l/tube. Standards and samples were incubated in borosilicate glass tubes for 24 hr at +4°C before addition of 16,000 cpm/tube of the tracer, [¹²⁵I-Tyr⁶]rCRF (DuPont/NEN, Boston, MA) diluted in normal rabbit serum (Peninsula, Belmont, CA). A second incubation of 24 hr was followed by precipitation of the antigen-antiserum complex with pretitrated goat anti-rabbit antiserum (Peninsula), polyethylene glycol 8000, and 30 min centrifugation at 3000 $\times$ g. After decanting the supernatant, residual radioactivity was counted in each tube using a gamma-counter (APEX10, ICN Biomedicals, Costamesa, CA). A logistic four-parameter model was used for interpolation of standard curves. Sensitivity of the assay was 0.35 fmol/tube or less.

Verification of microdialysis probe placement. The day after the experiment, rats were deeply anesthetized with halothane, and microdialysis probes were gently removed and inspected for viability. The animals were then decapitated, the brain dissected out, and immediately frozen on dry ice. Unfixed brains were cut on a cryostat.

The brain of one group of rats was cut in thin sections (20 μ m), postfixed in 4% paraformaldehyde for 1 min, and stained with cresyl violet for rostrocaudal analysis of probe trace location within the amygdala. Since the anatomical plane of cutting was different from the plane used for stereotaxic guide cannula implantation, the localization of the active zone of microdialysis probe within the amygdala was obtained by inspecting various adjacent sections. The analysis started from the more caudal level that displayed the deepest lesion trace produced by the microdialysis probe, corresponding to the location of the probe tip. Rostrocaudal and dorsoventral location of probes active zone in each rat brain was identified by selecting the section with a trace positioned at approximately 1 mm distance from the tip. The rostrocaudal levels of this section was attributed using a stereotaxic atlas (Paxinos and Watson, 1986), and represented as a 2 mm segment on a semianatomical scheme of the same stereotaxic level.

In a second group of rats, an alternative approach was used to evaluate the anatomical probe location in the rat brains. Thick sections (200

μm) were collected and postfixed in 4% paraformaldehyde for at least 1 hr. After rinsing with PBS, wet sections were mounted on slides, put on a negative carrier of a photographic enlarger, and projected onto the easel to obtain "negative" photographic prints. Adjacent sections were also cut and stained with cresyl violet to provide additional information about the anatomical localization of the microdialysis probe tip. The location of the active zone of the microdialysis probes was then identified as described above.

Diet, ethanol administration, and blood alcohol level determination. Seventeen rats selected for experiment 3 were maintained on a nutritionally balanced liquid diet consisting of a Sustacal (Mead Johnson, Evansville, IN) base supplemented with vitamins (ICN Nutritional Biochemicals, Aurora; 0.3 g/100 ml) and minerals (ICN Nutritional Biochemicals; 0.5 g/100 ml) as previously described (Baldwin et al., 1991). Ethanol (8.5% v/v) was added to the liquid diet of experimental rats. Control diet was made equicaloric by adding sucrose (Sigma). Both control and ethanol liquid diets were given for 2–3 weeks, and the diets were available *ad libitum*. The volume of diet consumed was measured each morning. A "pair-feeding" procedure was instituted for control rats by keeping the volume of available control diet equal to the volume of ethanol-containing diet consumed by the rats of the ethanol group on the previous day. The effect of this diet regimen on body weight was monitored regularly.

Blood alcohol levels (BAL) were measured in each rat 10 d after the beginning of the ethanol diet exposure, and 3 d before the experiment. Blood (0.5 ml) was taken from the tail, collected into 4 μl heparin (1000 USP units/ml), and centrifuged for 5 min at 10,000 rpm. The plasma was acid extracted and alcohol content was determined using the NAD/ADH method (Sigma). Rats were included into the experimental group when the BAL measured on the last sampling day was higher than 100 mg%.

Experiment 1: *in vitro* measurements of CRF-IR release from amygdala. *In vitro* experiments were performed according to the method of Calogero et al. (1989), with modification. The experiment was conducted at 37°C under O_2/CO_2 (95:5%), and consisted of serial passage of the minced tissue through different wells of a 48-multiwell plate (Costar, Cambridge, MA) previously precoated with incubation buffer containing 1% BSA. Each well contained 700 μl medium, and tissue minces were passed through wells at 20 min interval. At the end of each incubation interval the tissue was removed and placed on the next well for the following 20 min interval. Immediately after removing the tissue, the incubation medium was collected in tubes, frozen on dry ice, and stored at -70°C until CRF radioimmunoassay. A set of preliminary experiments indicated that stabilization of spontaneous CRF-IR release occurs between 80 and 100 min after the beginning of the experiment. The levels of CRF-IR measured in the following incubation interval (100–120 min) was considered the reference basal value for comparison with the stimulated levels. Stimulation was performed in the subsequent interval (120–140 min) incubating the sections in medium containing the K^+ -channel blocker 4-aminopyridine. A dose-response curve was obtained by incubating minced amygdala with 4-aminopyridine at concentrations of 0.1, 1.0, or 10.0 mM. The Ca^{2+} -dependency of CRF-IR release produced by 4-aminopyridine (1.0 mM) was studied by incubating the tissue with the Ca^{2+} channel blocker CoCl_2 (10 mM). Incubation with CoCl_2 begun during the interval before exposure to 4-aminopyridine. Each experimental group consisted of amygdala minces collected from five to six animals, each tube containing slices from a single animal.

Experiment 2: effects of restraint stress on CRF-IR release from the amygdala. This experiment was performed in the home cage of seven rats previously implanted with a guide cannula. Microdialysis probes were slowly inserted 12 hr prior to the start of sampling and secured in the guide cannula under brief, shallow halothane anesthesia. During this period, the probe was perfused with ACSF at a flow rate of 0.7 $\mu\text{l}/\text{min}$. One hour before sampling, the perfusion medium was changed to a freshly prepared medium containing anti-CRF serum, and the flow rate was increased to 3.0 $\mu\text{l}/\text{min}$. This flow rate was previously shown to yield optimum recovery (Merlo Pich et al., 1993). Fractions were collected every 20 min, at a volume of approximately 60 μl . After a period of basal collection (five to six samples), animals were restrained for 20 min by immobilizing their paws. At the end of the stress period rats were returned to their home cage and sampling was continued for approximately 2–3 hr. Perfusion medium was then substituted with a solution containing 10 mM 4-aminopyridine, and perfusion was continued

for 1 hr. At the end of the test the animals were anesthetized and sacrificed as described.

Experiment 3: effects of ethanol withdrawal on CRF-IR release from the amygdala. In this experiment, rats exposed to ethanol diet ($n = 9$) or control diet ($n = 8$) were implanted with guide cannulae 1 week before the experiment. On the testing day, 12 hr prior to the start of sampling, microdialysis probes were secured in the guide cannula under halothane anesthesia. The probe was perfused with ACSF at a rate of 0.7 $\mu\text{l}/\text{min}$ overnight. On the next morning, 1 hr before sampling, the perfusion medium was changed to a freshly prepared medium containing anti-CRF serum, and the flow rate was increased to 3.0 $\mu\text{l}/\text{min}$. Fractions of 60 μl were then collected every 20 min. All experiments started with collection of five basal fractions, during a period of 2 hr in which ethanol diet was available. At the end of this period the bottle containing ethanol diet was removed and replaced by control diet. Perfusion medium was then changed, and perfusion continued at the same flow rate using regular ACSF. Standard ACSF and solution containing anti-CRF serum were substituted every 2 hr, and this procedure was repeated four times during the experiment. Thus, CRF-IR measurements were performed over four periods of 2 hr interspersed with nonsampling 2 hr periods over a total of 12 hr. The first fraction collected after each change of the perfusion medium was discarded to avoid carryover effects from dialysate remaining in the tubing dead volume. The four-fraction collection periods corresponded to the 2–4 hr, 6–8 hr, and 10–12 hr intervals after withdrawal. At the end of the experiment, rats were perfused with ACSF over night at 0.7 $\mu\text{l}/\text{min}$ before sacrifice. During withdrawal each rat was observed for physical signs of withdrawal (Baldwin et al., 1991).

Statistical analysis. The effects of various doses of 4-aminopyridine on CRF release *in vitro* were analyzed using one-way ANOVA, while a mixed factorial ANOVA was used to study the interaction of CoCl_2 on 4-aminopyridine-stimulated release. The effect of restraint stress on CRF-IR release *in vivo* was assessed using one-way ANOVA for repeated measurements. The data collected in the experiment performed on ethanol-dependent rats were analyzed using a mixed factorial ANOVA with "diet" as between-subject factor and "time of withdrawal" as within-subject factor. In all experiments significant differences among individual means were determined by Tukey's post hoc test.

Results

Experiment 1: *in vitro* CRF-IR release from amygdala

Figure 1 shows the effects of 4-aminopyridine on CRF release from slices of amygdala tissues *in vitro*. Inclusion of 4-aminopyridine in the incubation medium significantly increased CRF-IR release in a dose-response fashion ($p < 0.01$). Significant effects were observed at 1.0 mM ($p < 0.05$) and 10.0 mM ($p < 0.01$) 4-aminopyridine concentrations, while the lowest dose (0.1 mM) was without significant effect. In the presence of CoCl_2 , the stimulating effects of 1.0 mM 4-aminopyridine were completely antagonized ($p < 0.05$). These results confirm the Ca^{2+} dependency of CRF-IR release from slices of rat amygdala *in vitro*.

Experiment 2: effects of restraint stress on CRF-IR release from the amygdala

Postmortem anatomical evaluation of the placement of the "active" zone of the microdialysis probes in rats exposed to restraint stress revealed that the target region was an area defined by the lateral component of the Ce and the medial border of the BL (Fig. 2). A graphical representation of the locations of the microdialysis membrane region in the brain of all rats of this experiment is shown in Figure 3B. Two rats were not included in the data analysis because of anatomical misplacement of the probe.

Basal and stress-induced CRF-IR levels in dialysate from the amygdala of awake rats over time are shown in Figure 3A. Basal CRF-IR dialysate concentrations in undisturbed rats were 1.19 ± 0.15 fmol/50 μl . These values represent the mean and the standard error of all the fraction values measured before stress

Figure 1. *A*, Effects of incubation with 4-aminopyridine at different concentrations (mM) on CRF-IR release from slices of the rat amygdala tissue *in vitro*. Amygdala slices were incubated in regular medium, and then exposed to 4-aminopyridine during the following 20 min interval. Since basal CRF-IR levels did not differ from those measured in the stimulation interval when 4-aminopyridine was absent (0 mM), only the results obtained during the stimulation intervals are shown. Each bar represents the mean $\pm$ SEM of four to six rats (*, $p < 0.05$; **, $p < 0.01$; vs basal CRF-IR levels, Tukey's test). *B*, Effects of cobalt chloride (CoCl₂) on CRF-IR release induced by 4-aminopyridine from slices of amygdala tissue *in vitro*. Basal CRF-IR levels are represented on the left, when 4-aminopyridine was absent, while stimulated CRF-IR levels are shown on the right, in the presence of 1 mM 4-aminopyridine. Incubation with 10 mM CoCl₂ (open bars) abolished the effects of 4-aminopyridine. Each bar represents the mean $\pm$ SEM of five rats (*, $p < 0.05$, stimulated vs basal CRF-IR levels; #, $p < 0.05$; regular medium vs CoCl₂ during stimulation).

in all rats. Restraint stress (20 min) produced a significant increase of CRF-IR dialysate levels to 4.54 ± 1.33 fmol/50 μ l ($p < 0.01$). After stress, dialysate CRF-IR levels returned to levels close to the basal values in about 1 hr. During the following intervals, perfusion with 4-aminopyridine markedly increased CRF-IR concentrations in the dialysate collected over a 1 hr sampling period (4.83 ± 0.92 fmol/50 μ l, $p < 0.01$). All rats in

which CRF-IR release was increased by restraint stress also showed significant increases of CRF-IR levels during 4-aminopyridine perfusion. Observation of the rats during the experiment indicated that perfusion with 4-aminopyridine produced behavioral signs of increased arousal, inducing episodes of locomotor activity alternated with periods of immobility, and occasional limbic seizures.

Figure 2. Anatomical estimation of the "active" zone of microdialysis probes implanted in rats and aimed at the amygdala. A series of consecutive brain sections bearing the trace of the microdialysis probe were stained with cresyl violet. Sections were selected on the basis of the most ventral location of the probe tip. The sections with the deepest trace was selected (*A*), and a segment of 2 mm then drawn vertically, tracing the estimated location of the active zone (broken line). The same procedure was performed on more rostral sections, maintaining the stereotactic and anatomical alignment (*B* and *C*). From this analysis the estimated region of location of the microdialysis membrane was an area defined by the lateral component of the central nucleus of the amygdala (*Ce*) and the medial border of the basolateral nucleus of the amygdala (*BL*).

Figure 3. *A*, Effects of restraint stress (20 min) and 4-aminopyridine (4-AP, 10 mM) perfusion on CRF-IR levels as measured by intracranial microdialysis in the rat amygdala. Fractions were collected every 20 min. CRF-IR levels measured during stress and 4-AP were significantly different from basal levels ($p < 0.05$). *B*, Distribution of the anatomical localizations of the "active" zone of microdialysis probes according to anatomical trace analysis carried out as described in Figure 2. Only microdialysis probe traces of rats included in the restraint stress experiment are reported ($n = 5$).

Experiment 3: effects of ethanol withdrawal on CRF-IR release from the amygdala

Exposure to the ethanol diet produced moderately high BAL levels in exposed rats (126 ± 13 mg%). No differences in body weight between the ethanol and control diet groups as assessed the day before the experiment was noted. Observation of experimental rats during microdialysis experiments showed that, during the 6–12 hr period of withdrawal, ethanol-exposed rats were awake most of the time, displayed low levels of spontaneous locomotor activity, exhibited occasional body tremor and stiffness of the tail. Conversely, control rats were frequently sleeping, and no body tremor or stiffness of the tail were ob-

served. Seven rats (three control and four ethanol dependent rats) were excluded from further analysis because of surgical failure ($n = 2$), probe disconnection, or membrane rupture ($n = 2$), radioimmunoassay errors ($n = 1$), or inadequate BAL ($n = 2$), resulting a sample of five ethanol-dependent rats and five control rats. The initial fractions collected during the 2–4 hr period of three rats included in the experiment were not available for assay, and therefore this collection interval was excluded from the analysis. Postmortem anatomical evaluation of microdialysis probe placements indicated that the tips were located between the Ce and the BL (Fig. 4), as observed in rats exposed to restraint stress.

Figure 4. Anatomical localizations of the "active" zone of microdialysis probes according to anatomical trace analysis carried out as described in Figure 2. Only the microdialysis probe traces of rats included in the ethanol experiment are reported ($n = 10$).

Figure 5. Effects of ethanol withdrawal on CRF-IR levels in the rat amygdala as determined by microdialysis. Dialysate was collected over four 2 hr periods regularly alternated with nonsampling 2 hr periods. The four sampling periods correspond to the basal collection (before removal of ethanol), and 2–4 hr, 6–8 hr, and 10–12 hr after withdrawal. Fractions were collected every 20 min. Data are represented as mean $\pm$ SEM ($n = 5/\text{group}$). ANOVA confirmed significant differences between the two groups over time ($p < 0.05$).

The results of the microdialysis experiments are shown in Figure 5. The average basal CRF-IR levels measured in dialysate from the amygdala were 1.30 ± 0.19 fmol/50 μl and 2.11 ± 0.43 fmol/50 μl in control diet and ethanol diet-exposed rats, respectively. Although this difference was not statistically significant, ANOVA indicated that the overall difference between the two treatment groups during withdrawal was significant [$F(1,8) = 7.65$, $p < 0.05$]. Accordingly, there was a significant time-dependent increase in CRF-IR levels measured in dialysate ($p < 0.01$). This effect over time was mainly due to the increase of CRF-IR levels measured in ethanol-exposed rats during withdrawal, as indicated by significant “treatment $\times$ time” interaction ($p < 0.01$). Post hoc analysis of simple effects showed that the two groups were significantly different during the 10–12 hr period of withdrawal ($p < 0.01$), while only marginally significant increases in CRF-IR levels were obtained during the 6–8 hr period of withdrawal ($0.10 > p > 0.05$).

Discussion

The results presented in this article confirm and extend previous observations that depolarizing agents can induce CRF-IR release from slices of rat amygdala *in vitro* in a Ca^{2+} -dependent manner. The stimulant action of 4-aminopyridine on CRF-IR release was also present in awake rats studied with intracranial microdialysis. More interestingly, a transient increase of CRF-IR release was observed when rats implanted with a microdialysis probe in the amygdala were exposed to restraint stress for 20 min. In contrast, a progressive and prolonged increase of CRF-IR release was measured in ethanol-dependent rats during ethanol withdrawal, with a time course corresponding to the emergence and progression of behavioral signs of withdrawal. These findings indicate that CRF-IR can be released from the rat amygdala in

response to stress produced by exogenous or endogenous stimuli that result in increased arousal and emotionality.

The origin of CRF-IR measured in the present experiments is likely to be in the nerve terminals of the Ce. This interpretation is based on immunocytochemical evidence that shows a dense plexus of positive neurons, fibers, and terminals in the Ce (Sakanaka et al., 1988; Cassel and Gray, 1989; Gray, 1990). The assessment of microdialysis probe locations within the amygdala indicated that CRF-IR levels in perfusate were detectable when the probe was placed in close proximity to the Ce. In two cases of anatomical misplacement, in which the active part of the probe was located in the cortex, the levels of CRF-IR were almost undetectable (data not shown). The anatomical specificity of CRF-IR measurement with microdialysis in the rat brain was previously studied in anesthetized rats. CRF-IR levels were undetectable in perfusate from probes placed in the upper third ventricle and dorsal striatum, while CRF-IR levels of about 1 fmol/50 μl were measured in the mediobasal hypothalamus and Ce (Merlo Pich et al., 1993). CRF-IR release from the mediobasal hypothalamus was also measured in anesthetized rats (Gabr et al., 1994), and in awake rats using the push-pull cannula (Ixart et al., 1987). In both present experiments involving awake rats, basal dialysate CRF-IR levels were within the range measured in the perfusate from the amygdala of anesthetized rats.

The synaptic origin of CRF-IR is further supported by the observation of enhanced CRF-IR release by depolarizing stimuli, and its dependency upon Ca^{2+} availability. Ca^{2+} -dependent K^{+} -induced release of CRF has been demonstrated in amygdala primary culture from embryonic explants (Cratty and Brikle, 1994), in slices of amygdala tissue from adult rat brain (Smith et al., 1986), and in perfusate from a microdialysis probe located in the Ce of anesthetized rats (Merlo Pich et al., 1993; Richter et

al., submitted). In the present work, these observations were extended by showing that also the K⁺-channel blocker 4-aminopyridine stimulates CRF-IR release in the amygdala. CRF-IR increases produced by 4-aminopyridine were completely blocked by the Ca²⁺ antagonist CoCl₂ *in vitro*. Preliminary observations indicate that the same blocking effect of CoCl₂ was present in anesthetized rats implanted with microdialysis probes in the amygdala (R. Richter, unpublished data). It is worth to note that the 4-aminopyridine-dependent release of CRF-IR may not be a direct effect. In fact, 4-aminopyridine is known to release norepinephrine and acetylcholine *in vitro* (Drukarch et al., 1989; Heemskerk et al., 1990). Since norepinephrine or acetylcholine can produce CRF-IR release from the CNS neurons (Tsagarakis et al., 1988; Hu et al., 1992), it cannot be excluded that these or other neurotransmitters mediate the effects of 4-aminopyridine on CRF release within the amygdala.

Previous findings of high concentrations of CRF binding sites within the amygdaloid nuclei, particularly in the basolateral nucleus (BL) (De Souza, 1987; Perrin et al., 1993), strongly suggest a role for locally released CRF-IR on amygdaloid neuron activity. In brain slice preparations CRF produces postsynaptic depolarization in most of amygdala cells (Eberly et al., 1983; Rainnie et al., 1992), but presynaptic inhibitory effects on CRF-IR neurons have also been proposed (Wiersma et al., 1993). CRF binding sites are located on the perikarya, dendrites, and terminals of peptide-containing medium spiny neurons of the Ce, including CRF-IR neurons. Incidentally, receptors on CRF-IR neurons may represent autoreceptors that regulate CRF-IR release, locally (Wiersma et al., 1993) or in their projection fields in hypothalamic and brainstem nuclei. Thus, a rise in extracellular CRF-IR levels in the Ce may contribute to activate peptide-containing medium spiny neurons of the amygdala, activation recently demonstrated using Fos immunostaining technique in rats exposed to restraint stress (Honkaniemi, 1992). However, by acting on autoreceptors, CRF may also participate in the shutdown of CRF-IR release from CRF-IR neurons. To further complicate the picture of the role of CRF in the amygdala, BL neurons are also likely to be affected by CRF-IR. Since microdialysis experiments have shown that CRF-IR levels increased in a sampling region between Ce and BL during stress, it is reasonable to suggest that locally released CRF-IR can diffuse and modulate also BL neurons, possibly in a nonsynaptic way (Fuxe and Agnati, 1991). Since BL neurons massively project to Ce, but Ce neurons do not project to BL (McDonald, 1992), it is possible that CRF-IR diffusing from Ce can retrogradely affect BL neurons, providing a local nonsynaptic feedback mechanism. All these observations point to a complex modulatory role of CRF within the amygdala.

The present results complement a series of behavioral experiments aimed to understand the involvement of CRF in emotionality. When the CRF antagonist α CRF(9–41) was microinjected within the amygdala of rats exposed to social stress, a dose-dependent decrease of the stress-induced anxiogenic-like effect was observed (Heinrichs et al., 1992). Attenuation of stress-induced immobility was also produced by intra-amygdala administration of α CRF(9–41) in the pM range (Swiergiel et al., 1993). Thus, it may be concluded that CRF in the amygdala mediates the emotional response to stress exposure (but see also Liang et al., 1992a,b).

The results of the present experiments performed in ethanol-dependent rats during ethanol withdrawal partially support this hypothesis. Chronic ethanol exposure has been associated with

emotional hyperactivity in rodents and humans (Cooper et al., 1979; Glue and Nutt, 1990). Stress-like effects produced by withdrawal from chronic ethanol have been previously studied in rats using the social interaction test (File et al., 1989) and the elevated plus-maze (Baldwin et al., 1991). In these experiments, significant withdrawal signs are observed approximately 8–10 hr after ethanol withdrawal. Recent work has shown that intra-amygdala administration of α CRF(9–41) blocks the anxiogenic-like effects of ethanol withdrawal in the elevated plus-maze 8 hr after withdrawal (Rassnick et al., 1993). Interestingly, this period corresponds to the rise in CRF-IR dialysate levels from amygdala observed in the present experiment. The most intense effects of withdrawal on CRF-IR release were observed at 10–12 hr. The peak of CRF-IR levels were two to three times higher than the peak effect in rats exposed to restraint stress, suggesting an increased activity of CRF-IR neurons in the amygdala. In rats chronically exposed to ethanol, increased sensitivity to the locomotor stimulating effects of CRF is also reported (Ehlers and Chaplin, 1987).

The mechanisms involved in the enhanced CRF-IR release are not known. Since acute administration of ethanol exerts anxiolytic-like effects, prolonged exposure to ethanol may produce adaptive changes in central neurons to counteract this inhibitory effect (Pohorecky, 1981; Koob and Bloom, 1988). According to this hypothesis, the absence of ethanol during withdrawal would produce a rebound activation of previously inhibited neurons, leading to their hyperactivity (Glue and Nutt, 1990). The increased CRF release measured in the amygdala of dependent rats during withdrawal may represent such a mechanism, in that inhibition of CRF neurons of the Ce by chronic ethanol may result in a downregulation of inhibitory receptors, such as CRF autoreceptors or GABA/benzodiazepine receptors. GABA/benzodiazepine receptors activity is known to be reduced by chronic ethanol exposure (Morrow et al., 1988). Since both Ce and BL neurons contain a very high density of GABA/benzodiazepine binding sites (Niehoff and Kuhar, 1988), it can be surmised that CRF-IR neurons are normally under the inhibitory effects of GABAergic transmission. Therefore, the reduced tonic influence of GABAergic transmission in chronic ethanol exposed rats may result in disinhibition of CRF-IR neurons.

A third possible substrate mediating the CRF-IR release in the amygdala during ethanol withdrawal is represented by monoamines, in particular, norepinephrine. In both situations of stress and ethanol withdrawal the triggering signal for CRF-IR release may be due to norepinephrine released from the terminals innervating the Ce (Fallon et al., 1988; Gavin, 1990). A recent microdialysis experiment (Tanaka et al., 1991) showed that the temporal profile of norepinephrine release from the amygdala of rats exposed to 20 min restraint stress closely matches that of CRF-IR described in the present work. *In vitro* norepinephrine evokes CRF-IR release from hypothalamic neurons (Tsagarakis et al., 1988; Hu et al., 1992), while microdialysis experiments show that CRF produces norepinephrine release from nerve terminals in prefrontal cortex and hypothalamus (Lavicky and Dunn, 1993), suggesting the existence of a reciprocal stimulating interaction between CRF and norepinephrine. Although the relevance of central norepinephrine in anxiety is still under debate, some of the behavioral effects produced by stress and ethanol withdrawal involve adrenoceptor mediation (Berridge and Dunn, 1989; Glue et al., 1989; Soderpalm and Engel, 1990).

In conclusion, a recently validated method to measure CRF-IR release with microdialysis in freely moving rats was used to

investigate the effects of restraint stress and ethanol withdrawal on CRF-IR release from the amygdala. The time course of the enhancing effects on CRF-IR release induced by restraint stress and ethanol withdrawal parallels the appearance of anxiogenic behavioral effects. Together, these results suggest that CRF-IR neurons of the amygdala participate in mediating the emotional effects of restraint stress and ethanol withdrawal.

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