Corticotropin-Releasing Factor Increases *In Vitro* Firing Rates of Serotonergic Neurons in the Rat Dorsal Raphe Nucleus: Evidence for Activation of a Topographically Organized Mesolimbocortical Serotonergic System

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In vivo studies suggest that the stress-related neuropeptide corticotropin-releasing factor (CRF) modulates serotonergic neurotransmission. To investigate the underlying mechanisms for this interaction, the present study examined the effects of CRF *in vitro* on dorsal raphe neurons that displayed electrophysiological and pharmacological properties consistent with a serotonergic phenotype. In the presence of either 1 or 2 mM Ca^{2+} , perfusion of ovine CRF or rat/human CRF rapidly and reversibly increased firing rates of a subpopulation (19 of 70, 27%) of serotonergic neurons predominantly located in the ventral portion of the dorsal raphe nucleus. For a given responsive neuron, the excitatory effects of CRF were reproducible, and there was no tachyphylaxis. Excitatory effects were dose-dependent (over the range of 0.1–1.6 μM) and were completely absent after exposure to the competitive CRF receptor antagonists α -helical CRF_{9–41} or rat/human [D-Phe¹², Nle^{21,38}, α -Me-Leu³⁷]-CRF_{12–41}. Both the proportion of responsive neurons and the magnitude of excitatory

responses to CRF in the ventral portion of the caudal dorsal raphe nucleus were markedly potentiated in slices prepared from animals previously exposed to isolation and daily restraint stress for 5 d. Immunohistochemical staining of the recorded slices revealed close associations between CRF-immunoreactive varicose axons and tryptophan hydroxylase-immunoreactive neurons in the area of the recordings, providing anatomical evidence for potential direct actions of CRF on serotonergic neurons. The electrophysiological properties and the distribution of responsive neurons within the dorsal raphe nucleus are consistent with the hypothesis that endogenous CRF activates a topographically organized mesolimbocortical serotonergic system.

Key words: anxiety; conditioned fear; corticotropin-releasing hormone; CRF; CRH; drug addiction; drug withdrawal; serotonin; mesolimbic; mesolimbocortical; serotonergic; restraint; isolation; sensitization; stress

Corticotropin-releasing factor (CRF) is a 41 amino acid neuropeptide with diverse physiological and behavioral functions. It is the principal regulator of the hypothalamo-pituitary-adrenal axis and is an important modulator of autonomic and behavioral responses to stressful stimuli (Owens and Nemeroff, 1991; Contarino et al., 1999), including anxiety and aversive states associated with drug withdrawal (Heinrichs et al., 1995; Sarnyai et al., 1995) and stress-induced relapse to drug-seeking behavior (Shaham et al., 1997). One mechanism through which CRF may modulate a broad spectrum of physiological and behavioral responses is via actions on ascending neuromodulatory systems, such as serotonergic systems.

Several lines of evidence support the hypothesis that CRF plays a role in regulating serotonergic neurotransmission. First, moderate to high densities of CRF-immunoreactive neuronal cell bodies and fibers are associated with serotonergic neurons in brainstem raphe structures (Cummings et al., 1983; Sakanaka et al., 1986, 1987; Austin et al., 1997; Ruggiero et al., 1999). Second, CRF₁ and CRF₂ receptor binding sites, receptor mRNA expression, and CRF₁ receptor-immunoreactive neurons have been identified in raphe nuclei (De Souza et al., 1985; Chalmers et al., 1995; Vaughan et al., 1995; Bonaz and Rivest, 1998; Bittencourt and Sawchenko, 2000; Chen et al., 2000), raising the possibility that CRF or CRF-

like peptides may have direct receptor-mediated actions on serotonergic neurons. Third, stress-related stimuli, particularly behavioral paradigms associated with increased anxiety or conditioned fear (Pezzone et al., 1993; Silveira et al., 1993; Beck and Fibiger, 1995; Matsuda et al., 1996; Beckett et al., 1997; Campeau and Watson, 1997; Kollack-Walker et al., 1997; Martinez et al., 1998; Nikulina et al., 1998; Chung et al., 1999, 2000; Grahm et al., 1999), including opiate withdrawal (Chieng et al., 1995; Chahl et al., 1996) and intracerebroventricular infusion of CRF or CRF-like peptides (Vaughan et al., 1995; Bittencourt and Sawchenko, 2000), activate immediate-early gene expression within the dorsal raphe nucleus. Fourth, exogenous CRF or CRF-like peptides alter serotonin metabolism or neurotransmission in studies using *ex vivo* tryptophan hydroxylase activity assays (Singh et al., 1992) and *in vivo* microdialysis (Price et al., 1998).

Based on these findings and evidence that stress-related stimuli increase serotonergic neurotransmission in the median and dorsal raphe nuclei (Adell et al., 1997; Maswood et al., 1998) and limbic forebrain regions, especially in response to intense, uncontrollable, or unpredictable stimuli (Adell et al., 1988b; Inoue et al., 1994; Amat et al., 1998a,b), one hypothesis is that stress increases serotonergic neurotransmission via the actions of CRF on subpopulations of serotonergic neurons that contribute to the mesolimbocortical serotonergic innervation of the forebrain. Using *in vitro* electrophysiological techniques, the present study investigated the possibility that CRF modulates the activity of serotonergic neurons in an area of the dorsal raphe nucleus known to express CRF receptors and to contribute to the mesolimbocortical serotonergic innervation of the forebrain. Furthermore, the locations of responsive neurons were compared with the distribution of endogenous CRF-immunoreactive fibers and tryptophan hydroxylase-immunoreactive neurons in the same slices.

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Figure 1. CRF increases the firing rate of a subpopulation of serotonergic neurons in the dorsal raphe nucleus. *A, B*, The majority of serotonergic neurons studied in the dorsal raphe nucleus were unaffected by application of rhCRF or oCRF. *PE* (–), Removal of phenylephrine from the aCSF. *C*, In contrast, a small subpopulation of serotonergic neurons responded with a rapid, reversible increase in firing rate. In this example, the excitatory effects of oCRF were reversed by previous application of the long-acting CRF receptor antagonist D-Phe-CRF_{12–41}. Coapplication of 1.2 μ M oCRF and 2 μ M D-Phe-CRF_{12–41} resulted in a decreased duration of the excitatory effects of CRF, whereas a subsequent coapplication of 1.2 μ M oCRF and 4 μ M D-Phe-CRF_{12–41} resulted in a complete inhibition of the excitatory effects of oCRF. Responses to subsequent applications of 1.2 μ M oCRF every 15 min up to 1 hr were also inhibited. *D*, Diagrammatic illustration summarizing the recorded locations of 70 serotonergic neurons studied during the application of rhCRF or oCRF to slices from group-housed rats (*open symbols*, nonresponsive neurons; *filled symbols*, responsive neurons). A higher percentage of neurons in the ventral and interfascicular regions of the dorsal raphe nucleus (*DRV*) were stimulated by CRF compared with neurons in the dorsal region of the dorsal raphe nucleus (*DRD*). *Aq*, Aqueduct; *mlf*, medial longitudinal fasciculus.

MATERIALS AND METHODS

Animals and housing conditions. Because the excitability of serotonergic systems is dependent on previous housing experience (Fulford and Marsden, 1998), animal housing conditions were consistent throughout these studies. Adult male Wistar rats were obtained from a colony maintained at the University of Bristol. After weaning, animals were handled once per week for weighing; at that time each week, animals were sorted according to weight classes and were housed six per cage (RC1 cages, 56 $\times$ 38 $\times$ 20 cm). Cage litter was changed twice per week. Animals were maintained under standard lighting conditions (14/10 hr light/dark cycle, lights on at 5:00 A.M.). On the morning of the experiment, a single animal (225–300 gm) was removed from group housing, weighed, and transported to the experimental room. In initial studies, 26 animals were used. In subsequent studies (see below), stressed animals ($n = 12$) were compared with control animals ($n = 10$); for these studies, the last animal in each control cage was not used.

Brain slice preparation. Tissue slices were prepared at ~7:00 A.M. After rapid decapitation, the brain was removed, and coronal slices (400 μ m) were cut using a vibratome. With few exceptions, only sections containing the midline decussating fibers of the superior cerebellar peduncle were selected for recordings. In studies involving isolation rearing and restraint, recordings were focused on the caudal margin of this region (at and caudal to bregma –8.00 mm; Paxinos and Watson, 1998). The midbrain slice was placed immediately in artificial CSF (aCSF) consisting of (in mM): 124 NaCl, 3.25 KCl, 2.4 MgSO₄, 1 or 2 CaCl₂, 1.25 KH₂PO₄, 10 D-glucose, and 26 NaHCO₃, equilibrated with 95% O₂–5% CO₂, at room temperature. Cortical tissues were removed, and then slices were transferred to a sloping perfusion chamber maintained at 37°C and perfused with oxygenated aCSF for at least 1 hr before changing the medium to aCSF containing 3 μ M phenylephrine hydrochloride (an α_1 adrenergic agonist). Unless otherwise stated, all recordings were made in the presence of 3 μ M phenylephrine, which increases spontaneous discharge rates of dorsal raphe 5-hydroxytryptamine (5-HT) neurons to levels observed *in vivo* (Vandermaelen and Aghajanian, 1983).

Extracellular recording. Extracellular recordings were made using glass microelectrodes filled with 0.5 M NaCl and coupled to an alternating current differential preamplifier (1000 $\times$). Units were carefully screened for properties consistent with a serotonergic phenotype (Vandermaelen and Aghajanian, 1983). Once located, the activity of an individual neuron was recorded for ~5 min to obtain baseline data, before perfusing with 50 μ M 5-HT for 2 min. Single units with biphasic (positive–negative) or

triphasic (positive–negative–positive) action potentials, which were reversibly inhibited by 5-HT and demonstrated the highly regular and relatively slow (0.5–2.8 spikes/sec in 2 mM Ca²⁺) firing patterns characteristic of 5-HT raphe neurons *in vivo* (Jacobs and Fornal, 1991), were accepted as serotonergic. In the presence of 1 mM Ca²⁺ (which, compared with 2 mM Ca²⁺, elevated the baseline firing rate of serotonergic neurons), many cells were tested for a decrease in spontaneous activity after removal of phenylephrine from the medium as further evidence of a serotonergic phenotype. Single units were discriminated using an amplitude window, and data were recorded using Spike 2 software (version 2.02; Cambridge Electronics Design, Cambridge, UK).

Drugs. 5-HT, rat/human CRF (rhCRF), phenylephrine hydrochloride, and bovine serum albumin (BSA) were purchased from Sigma (Poole, UK). Ovine CRF (oCRF) and rat/human [D-Phe¹², Nle^{21,38}, α -Me-Leu³⁷]-CRF_{12–41} (D-Phe-CRF_{12–41}) were purchased from Bachem (Saffron Walden, UK). α -Helical CRF_{9–41} was a gift from J. Rivier (Salk Institute for Biological Studies, La Jolla, CA). Unless otherwise indicated, all drugs were applied for 2 min. The aim of the current study was to determine whether CRF has a receptor-mediated effect on serotonergic neuron firing rate. Ovine CRF was used in the majority of cases because oCRF (compared with rhCRF) has a very low affinity for CRF-binding protein (CRF-BP) (Behan et al., 1996), thus avoiding indirect, nonreceptor-mediated actions of the CRF ligand. Such interactions between rhCRF and CRF-BP could confound results because CRF-BP mRNA expression levels are high within the dorsal raphe nucleus (Potter et al., 1992), and CRF-BP may play a role in regulating CRF responses via mechanisms not involving CRF₁ or CRF₂ receptors (Chan et al., 1999). The records have been corrected for the lag time of the perfusion system (~120 sec). In a few studies, BSA was added to the aCSF (3.125 μ g/ml) to prevent possible interactions between neuropeptides and the polytetrafluoroethylene tubing used to convey the drug solution to the chamber, but this was found not to be necessary to observe neuropeptide effects.

Analysis of firing rates and responses. Neurons with stable firing rates allowing unambiguous interpretation of responses were considered suitable for analysis. For each neuron, baseline firing rate was calculated over the 300 sec period before the first 5-HT application and over the 120 sec period before other drug applications. The response of a neuron to 5-HT was calculated as the mean percentage increase or decrease in firing rate for the first 2 min that the tissue was exposed to the drug compared with baseline. The response of a neuron to other drug applications was evaluated as either (1) as above, or (2) the maximal percentage increase or

Table 1. Effects of oCRF and rhCRF on the *in vitro* firing rates of identified serotonergic neurons in brainstem slices prepared from group-housed control animals

CRF	100 nM	200 nM	1 μ M	Total responses
1 mM Ca^{2+}				
rhCRF	4 (–)	8 (–), 4 (↑)	5 (–), 1 (↓)	17 (–), 4 (↑), 1 (↓)
oCRF	7 (–)		2 (–)	9 (–)
2 mM Ca^{2+}				
rhCRF			2 (–), 2 (↑)	2 (–), 2 (↑)
oCRF	15 (–), 11 (↑), 1 (↓)		6 (–), 2 (↑)	21 (–), 13 (↑), 1 (↓)

Number of serotonergic neurons that were unaffected (–), stimulated (↑), or inhibited (↓) by application of CRF peptides. Only the first CRF application for each neuron is indicated (for 70 neurons).

decrease in firing rate compared with baseline. For a neuron to be considered to be responsive to a treatment, the firing rate had to be reversibly increased or decreased at least for the period of time that the tissue was exposed to the drug.

Isolation housing and daily restraint for 5 d. Previous studies have implicated mesolimbocortical serotonergic systems in the development of behavioral sensitization to a novel stressor after previous exposure to stress (cross-sensitization) (for review, see Maier, 1993; Graeff et al., 1996) (see also Chung et al., 2000). Stress-induced behavioral sensitization is evident 24 or 48 hr after exposure to stress and is thought to involve a hypersensitivity of dorsal raphe serotonergic neurons attributable to a desensitization of somatodendritic 5-HT_{1A} autoreceptors (for review, see Maier, 1993) (see also Laaris et al., 1997, 1999). Based on these observations, we proposed to investigate the effect that a repeated stress had on the sensitivity of serotonergic neuronal firing rates to CRF *in vitro*. In doing this, we selected restraint stress; daily restraint stress, particularly of longer duration (30 min or longer), consistently alters indices of mesolimbocortical serotonergic function and subsequent behavioral responses to heterotypic stressors. (1) Both acute and repeated daily restraint stress activate mesolimbocortical serotonergic systems (Joseph and Kennett, 1983; Mitchell and Thomas, 1988; Clement et al., 1993, 1998; Chamas et al., 1999), consistent with restraint-induced increases in *c-fos* expression within the dorsal raphe nucleus (Senba et al., 1993; Watanabe et al., 1994; Cullinan et al., 1995; Krukoff and Khalili, 1997). (2) Repeated restraint stress results in a facilitation of mesolimbocortical serotonergic activity after subsequent exposure to a novel stressor 20 hr later (Adell et al., 1988a). (3) Restraint

stress induces behavioral sensitization (indicated by increased measures of anxiety or fear upon subsequent exposure to a novel stressor). For example, 1 hr restraint followed by 24 hr isolation housing, but not 15 min restraint alone, is “anxiogenic” in the elevated plus maze (McBlane and Handley, 1994). In addition, 2 hr restraint followed by 24 hr isolation housing results in reduced locomotion in an open field (Kennett et al., 1987), a behavioral effect subsequently proposed as a model of stress-induced anxiety (Carli et al., 1989). (4) Restraint stress (30 min) followed by 24 hr isolation produces a functional desensitization of somatodendritic 5-HT_{1A} autoreceptors as shown by the reduced potency of ipsapirone, a 5-HT_{1A} receptor agonist, to inhibit the *in vitro* firing rates of serotonergic neurons in the dorsal raphe nucleus (Laaris et al., 1999). In contrast, 30 or 90 min restraint stress alone is ineffective (Laaris et al., 1997, 1999). Based on consideration of these and other previous studies, we investigated the effects of CRF on *in vitro* serotonergic neuronal firing rates in the dorsal raphe nucleus in control animals and in animals subjected to 5 d daily 1 hr restraint sessions, with isolation housing during interim periods.

Control animals ($n = 10$) were taken directly from group housing as described above. Animals subjected to isolation housing and daily restraint (60 min) for 5 d (stressed animals, $n = 12$) were removed singly from group housing on the appropriate day, transferred to the experimental room, and placed in a Plexiglas tube. After 60 min, animals were isolated in cages (RB3 cages, 45 × 28 × 20 cm) and placed in a holding room with standard environmental conditions. Brain slices were prepared on the sixth day, 24 hr after the fifth and final restraint session. Although slices from control and stressed rats were challenged with varying concentrations of oCRF

Figure 2. Comparison of the effects of CRF on the firing rates of serotonergic neurons in control and stressed rats, based on electrophysiological recordings in the ventral portion of the caudal dorsal raphe nucleus (DRV). *A*, Application of 400 nM (top) or 1.2 μ M (bottom) oCRF had no effect on the firing rate of the majority of serotonergic neurons recorded from control animals. *B*, Application of 400 nM (top) or 1.2 μ M (bottom) oCRF reversibly increased the firing rate of a proportion of serotonergic neurons recorded from stressed animals. *C*, CRF dose-dependently increased the firing rate of a serotonergic neuron in the ventral portion of the caudal dorsal raphe nucleus. *D*, Dose-response curves (0.4–2 μ M oCRF) for three serotonergic neurons in the ventral portion of the caudal dorsal raphe nucleus in slices from stressed rats. *E*, Mean change in firing rate of the first cell tested in each animal at 400 and 1200 nM oCRF. Serotonergic neurons responded to 400 nM or 1.2 μ M oCRF with greater increases in firing rate in stressed rats (stippled bars) compared with control rats (solid bars). * $p \leq 0.01$, ** $p \leq 0.001$.

Figure 3. Immunohistochemical double-labeling of associations between CRF-immunoreactive fibers (SG, blue reaction product) and tryptophan hydroxylase-immunoreactive neurons (DAB, brown reaction product) within tissues used previously for electrophysiological recordings. *A*, *C*, and *D* are from the same 30 μ m section at approximately bregma -8.00 mm. *A*, Schematic illustration of the location of the recording electrode during recording of the unit illustrated in Figure 1*C*. *B*, Camera lucida drawing of tryptophan hydroxylase-immunoreactive neurons from an alternate section to that illustrated in *A*. Superimposed on this drawing are indications of the locations of dense bands of CRF-immunoreactive fibers (dotted ovals; illustrated for 5 cases), which ascend through the DpMe and then the VLPAG at progressively more caudal anatomical levels. These fibers, the pattern of tryptophan hydroxylase immunoreactive staining, and other anatomical features permitted precise identification of the rostrocaudal levels of recordings in these animals. *C*, Higher magnification of the interfascicular region of the dorsal raphe nucleus illustrated in *A*. CRF-immunoreactive varicose fibers were visible throughout the interfascicular region of the dorsal raphe nucleus. Illustrated are close associations between CRF-immunoreactive varicose fibers and some tryptophan hydroxylase-immunoreactive neurons (arrowheads), raising the possibility that synaptic specializations may exist between CRF fibers and a subpopulation of serotonergic neurons. *D*, Higher magnification of the dorsomedial portion of the dorsal raphe nucleus illustrated in *A*. CRF-immunoreactive varicose fibers were visible throughout the dorsal part of the dorsal raphe nucleus, although electrophysiological recordings failed to identify a significant population of CRF-responsive serotonergic neurons in the dorsal part of the dorsal raphe nucleus. *E*, Tryptophan hydroxylase-immunoreactive neuronal cell bodies and fibers and CRF-immunoreactive varicose fibers in the dorsal raphe nucleus at bregma -7.80 mm. Note the dense CRF-immunoreactive fibers throughout the VLPAG and the bundle of CRF-immunoreactive fibers in the DpMe (dotted oval). Also, tryptophan hydroxylase-immunoreactive neurons were more dense within the DRV than at bregma -8.00 mm (*A*). Rectangle indicates region illustrated in *F*. *F*, Higher magnification of the VLPAG and the ventrolateral part of the dorsal raphe nucleus. CRF-immunoreactive varicose fibers were visible throughout this

(0.4–4 μM), two doses (0.4 and 1.2 μM) were chosen for routine application and subsequent statistical analyses. The concentrations of oCRF used to elicit neuronal responses were similar to those that have been shown to depolarize and increase the spontaneous firing rates of both CA1 and CA3 hippocampal pyramidal neurons ($1.2\text{--}5 \times 10^{-7}$ M) (Aldenhoff et al., 1983; Siggins et al., 1985) and those that have been shown to decrease the afterhyperpolarization in cerebellar Purkinje neurons ($0.5\text{--}1 \times 10^{-6}$ M CRF) (Fox and Gruol, 1993).

From the 22 animals studied, 15 slices were saved to allow identification of the neuroanatomical location of the recordings and to allow double-labeling of slice preparations for CRF- and tryptophan hydroxylase-immunoreactive neuronal cell bodies and fibers.

Double-labeling immunohistochemistry. After recording, tissue sections were placed in 4% paraformaldehyde solution in 0.1 M phosphate buffer for 24 hr and then transferred to 0.1 M phosphate buffer containing 30% sucrose and 0.1% sodium azide.

Double-labeling of tissues (for CRF and tryptophan hydroxylase immunoreactivity) was performed as follows. Fixed sections used previously for electrophysiological recordings were frozen directly on a preleveled platform of OCT compound (Tissue-Tek; Sakura Finetechnical Co. Ltd.), and 30 μm sections were cut using a cryostat. Alternate sections from each slice were collected in 0.05 M PBS in gelatin-coated 24-well polystyrene tissue culture plates. One set of sections was used for immunohistochemical double-labeling for CRF and tryptophan hydroxylase. Labeling for CRF was performed by preincubating with freshly prepared 1% H_2O_2 in PBS for 20 min, followed by incubation with anti-CRF rabbit polyclonal antibody (IHC-8561; Peninsula Laboratories, Belmont, CA) diluted 1:16,000 in PBS containing 0.3% Triton X-100–0.04% BSA (PBST-BSA) and 0.01% NaN_3 in PBS for 14 hr. Sections were washed using PBST-BSA and then incubated with biotinylated donkey anti-rabbit antibody (771-065-152; Jackson ImmunoResearch, West Grove, PA) for 90 min. Sections were washed again using PBST-BSA and then incubated with ABC reagent (PK-6101; Vector Laboratories, Burlingame, CA) for 90 min. After washing with PBST-BSA and then PBS, sections were incubated with substrate (SG peroxidase substrate, SK-4700; Vector Laboratories) for 10 min. Before immunohistochemical labeling of the same sections for tryptophan hydroxylase, sections were treated with 0.75% H_2O_2 in 0.1 M sodium phosphate buffer for 30 min. Sections were then washed with PBS and incubated with anti-tryptophan hydroxylase sheep polyclonal antibody (9260–2505; Biogenesis, Sandown, NH) diluted 1:12,800 in PBST with 0.01% NaN_3 for 14 hr. Sections were washed using PBST and incubated with biotinylated rabbit anti-sheep antibody (PK-6106; Vector Laboratories) for 2 hr. Sections were washed again using PBST and incubated with ABC reagent (45 μl of Reagent A, 45 μl of Reagent B in 2.5 ml PBST, diluted 36-fold in PBST just before use) for 2 hr. After washing with PBST and 0.1 M sodium phosphate buffer, sections were incubated with substrate (0.02% 3,3'-diaminobenzidine tetrahydrochloride, D-5637; Sigma) and 0.003% H_2O_2 in 0.1 M sodium phosphate buffer for 7 min. Sections were transferred to gelatin-coated glass slides and mounted with coverslips using DPX mounting medium (BDH Laboratory Supplies, Poole, UK).

Statistics. Comparisons of independent observations were made using Student's *t* tests or Fisher's exact probability test, when appropriate (SYSTAT for Windows; Statistics, version 5; SYSTAT Inc., Evanston, IL).

RESULTS

Characterization of serotonergic neuron firing rates in the dorsal raphe nucleus

In initial studies, the effects of CRF were assessed using recordings made from 70 neurons with electrophysiological and pharmacological properties consistent with a serotonergic phenotype. Recordings were made throughout the midline and paramidline dorsal raphe nucleus. The effects of CRF were examined under two primary conditions, i.e., aCSF containing either 1 or 2 mM Ca^{2+} . Because previous *in vitro* electrophysiological studies of serotonergic neurons have principally used aCSF containing 2 mM Ca^{2+} (Vandermaelen and Aghajanian, 1983), we briefly describe fundamental differences in serotonergic neurons under these two conditions. As expected (Vandermaelen and Aghajanian, 1983), neurons recorded in the presence of 1 mM Ca^{2+} had higher mean firing rates (3.1 ± 0.2 spikes/sec, range of 2.4–4.7 spikes/sec, $n = 41$ neurons) than neurons recorded in the presence of 2 mM Ca^{2+} (1.6 ± 0.1 spikes/sec, range of 0.5–2.8 spikes/sec, $n = 71$ neurons; $p < 0.001$). Data only include units that were inhibited by a 2 min application of 50 μM 5-HT, although the magnitude of responses of

individual neurons varied considerably during the brief exposure to 5-HT. In studies using aCSF containing 1 mM Ca^{2+} , neurons that were inhibited by 5-HT also displayed a reversible decline in activity after removal of phenylephrine from the aCSF ($n = 18$) (Fig. 1A). The 5-HT_{1A} receptor agonist 8-hydroxy-2-dipropylamino-tetralin caused a dose-dependent decrease in firing rate (minimum effective dose of 20 nM), with 50 and 100 nM concentrations resulting in robust and prolonged inhibition (lasting ~30 and 60 min, respectively) of serotonergic neuronal firing rate.

In the presence of either 1 or 2 mM Ca^{2+} , the majority of serotonergic neurons [26 of 31 (84%) and 23 of 39 (59%), respectively] showed no change in firing rate after application of rhCRF (Fig. 1A) or oCRF (Fig. 1B) at doses ranging from 100 nM to 1 μM (Table 1). A recent study found that neurotensin excites dorsal raphe 5-HT neurons but only in the absence of phenylephrine (Jolas and Aghajanian 1997a). To determine whether a similar occlusion phenomenon was occurring in the present study, 18 neurons recorded in 1 mM Ca^{2+} aCSF were tested with rhCRF (100 nM to 1 μM) in the absence of α -1 adrenoreceptor stimulation. Three of these neurons were stimulated by rhCRF (data not shown), and the remaining neurons tested were unresponsive.

Two neurons responded to rhCRF or oCRF (one neuron for each peptide) with a decrease in firing rate, but the latency of these effects and the duration of the inhibition were different, suggesting separate mechanisms for these effects. In contrast, 19 of 70 neurons responded to application of rhCRF or oCRF with a rapid increase in firing rate that returned to baseline soon after drug offset (Fig. 1C,D). Previous application of either the CRF receptor antagonists α -helical CRF_{9–41} or rat/human D-Phe-CRF_{12–41} resulted in long-lasting inhibition of the excitatory effects of oCRF on the firing rates of serotonergic neurons ($n = 4$) (Fig. 1C). A striking feature of the excitatory responses to CRF was that responsive neurons were selectively clustered in the ventral portion of the dorsal raphe nucleus [1 of 26 (4%) responded in the dorsal division versus 18 of 44 (41%) in the ventral division; Fisher's exact probability test, $t = 0.001$] (Fig. 1D). This difference in the proportion of CRF-responsive neurons was observed despite the presence of tryptophan hydroxylase-immunoreactive perikarya and neurons with electrophysiological characteristics of serotonergic neurons in both the dorsal and ventral divisions of the dorsal raphe nucleus (see below).

Effects of oCRF on serotonergic neuronal firing rates in control and stressed animals

Baseline firing rates of serotonergic neurons from control and stressed animals were comparable (control, 1.6 ± 0.1 Hz, range of 0.7–2.5 Hz, $n = 24$ neurons; stressed, 1.4 ± 0.1 Hz, range of 0.6–2.6 Hz, $n = 48$ neurons). Likewise, the percent inhibition of firing rate by 5-HT was similar (mean value $\pm$ SEM during 2 min exposure to 5-HT; control, $62.5 \pm 5.7\%$; stressed, $60.0 \pm 4.1\%$), although use of a supramaximal dose of 5-HT (50 μM) may have prevented detection of group differences in 5-HT_{1A} sensitivity (cf. Laaris et al., 1997, 1999). Selection of tissue slices for recording was made by identifying the first section from the caudal direction to contain the midline decussating fibers of the superior cerebellar peduncle; recordings were made from the caudal surface of that slice. This resulted in consistent sampling from approximately bregma -8.00 to -8.40 mm.

Two doses of oCRF (400 nM and 1.2 μM) were selected for testing the effects of stress on the sensitivity of serotonergic neurons to the stimulatory effects of CRF (Fig. 2). Based on analysis of the first cell tested (using 1.2 μM oCRF) in each animal, a higher proportion of neurons recorded from slices from stressed animals displayed excitatory responses to oCRF compared with controls (0

region and were particularly dense in the VLPAG; pericellular baskets of CRF-immunoreactive varicosities resulted in a dense patch-like pattern of immunolabeling. *Aq*, Aqueduct; *DLPAG*, dorsolateral periaqueductal gray; *DpMe*, deep mesencephalic nucleus; *DRD*, dorsal raphe nucleus, dorsal part; *DRV*, dorsal raphe nucleus, ventral part; *DRVl*, dorsal raphe nucleus, ventrolateral part; *dtg*, dorsal tegmental bundle; *LPAG*, lateral periaqueductal gray; *me5*, mesencephalic trigeminal tract; *mll*, medial longitudinal fasciculus; *VLPAG*, ventrolateral periaqueductal gray. Scale bars: A, B, E, 400 μm ; C, D, F, 50 μm .

of 7 control animals vs 6 of 10 stressed animals; Fischer's exact probability test, $t = 0.035$). In addition, mean increases in firing rates after oCRF exposure at both doses tested were significantly greater in stressed animals than in controls (Fig. 2E). In some animals, additional doses of oCRF were tested, revealing that the stimulatory effects of oCRF on serotonergic cell firing rate were dose-responsive, with maximal responses at concentrations of 1.6–2 μM (Fig. 2C,D).

Because initial studies revealed that the topographical location of serotonergic neurons may be correlated with responsivity to CRF *in vitro*, sections from several animals were processed for immunohistochemical double-labeling for tryptophan hydroxylase and CRF to determine the spatial relationships between CRF-immunoreactive fibers and serotonergic neurons. Although most sections were used for electrophysiological recordings for up to 12–16 hr, the distribution of tryptophan hydroxylase-immunoreactive neuronal cell bodies and CRF-immunoreactive neuronal cell bodies and fibers were identical to previous descriptions of serotonergic and CRF systems (Fig. 3) (cf. Sakanaka et al., 1986, 1987; Jacobs and Azmitia, 1992). As described previously, a very high density of CRF-immunoreactive fibers was located in the ventral part of the deep mesencephalic nucleus (DpMe) (Fig. 3), shifting mediodorsally in the caudal direction. Also, as described previously (Sakanaka et al., 1986, 1987), the ventral portions of the central gray, especially the ventrolateral periaqueductal gray (VLPAG), had a high density of CRF-immunoreactive fibers at the level of the dorsal raphe nucleus. The VLPAG contains GABAergic and glutamatergic neurons known to provide input to serotonergic neurons in the dorsal raphe nucleus (Jolas and Aghajanian, 1997b), raising the possibility that endogenous CRF may have indirect local effects on dorsal raphe nucleus serotonergic neurons. However, in the ventral and interfascicular portions of the caudal dorsal raphe nucleus, the region in which a high density of CRF-responsive serotonergic neurons were found, immunohistochemical labeling revealed close anatomical associations between CRF-immunoreactive varicose fibers and tryptophan hydroxylase-immunoreactive perikarya, raising the possibility that endogenous CRF may have direct effects on dorsal raphe nucleus serotonergic neurons (Fig. 3).

DISCUSSION

This study provides the first description of the effects of the stress-related neuropeptide CRF on serotonergic neuronal activity *in vitro*. The effects of CRF were principally excitatory and limited to a subpopulation of serotonergic neurons. The responsive neurons were differentially distributed with a greater proportion in the ventral and interfascicular regions of the caudal dorsal raphe nucleus compared with the dorsomedial region. Furthermore, serotonergic responses to CRF were enhanced after exposure of rats to isolation housing and repeated restraint stress for 5 d. Together with convergent evidence from multiple disciplines, these observations suggest that CRF actions on serotonergic neurons may play an important role in behavioral responses associated with anxiety and conditioned fear, extending previous hypothetical models for the complex neurobiological mechanisms underlying these behavioral states (Gray, 1982; Davis, 1998). In marked contrast to previous hypotheses for serotonergic function (Jacobs and Fornal, 1995; Rueter et al., 1997; Jacobs and Fornal, 1999), the present study suggests that topographically organized subpopulations of serotonergic neurons may be dedicated to particular functions associated with stress responses, including behavioral sensitization and behavioral adaptation to previous stress.

The majority of serotonergic neurons in the dorsal and median raphe nuclei (designated type I serotonergic neurons) display a progressively decreasing firing rate during the inactive period of the sleep–wake cycle, becoming virtually silent during paradoxical sleep. Previous electrophysiological studies in behaving animals suggest that physical and psychological stressors have no effect on the firing rates of these serotonergic neurons within the dorsal raphe nucleus, even when associated with sympathetic nervous system activation (for review, see Jacobs and Azmitia, 1992). These

findings and others have led to the proposal that serotonergic systems play no specific role in mediating stress-induced physiological or behavioral change, but instead serotonergic neuronal activity changes as a correlate of behavioral activity (Jacobs and Fornal, 1995). An alternative hypothesis is that stress alters the firing rates of small subpopulations of serotonergic neurons that were not systematically included in the previous studies. In support of this hypothesis, subpopulations of serotonergic neurons with unique electrophysiological properties and behavioral correlates have been identified (Rasmussen et al., 1984). Serotonergic neurons of a small, topographically organized subpopulation (designated type II serotonergic neurons) display no significant change in firing rate during the inactive period of the sleep–wake cycle and remain fully active during paradoxical sleep. Furthermore, in contrast to type I serotonergic neurons, which display a rapid onset, short duration excitation after phasic auditory or visual stimuli, type II neurons display rapid onset, long duration inhibition after phasic auditory or visual stimuli. Type II serotonergic neurons are most evident in a highly confined region between the medial longitudinal fasciculi at the caudal interface of the dorsal raphe nucleus and the median raphe nucleus. It is unclear whether the neurons identified in the present study belong to the type II subpopulation of serotonergic neurons. Regardless, the distribution of CRF-responsive neurons identified in the present study corresponds well with the location of mesolimbocortical serotonergic neurons (based on retrograde tracing studies, see below), suggesting that CRF may activate mesolimbocortical serotonergic systems.

Retrograde tracing studies reveal that different subregions of the dorsal raphe nucleus receive unique, topographically organized afferent input (Peyron et al., 1998), suggesting that subregions of the dorsal raphe nucleus may be differentially regulated by stress or stress-related neuropeptides (e.g., CRF). In addition, subregions of the dorsal raphe nucleus have topographically organized efferent projections; retrograde tracing studies reveal that the ventral and interfascicular regions of the caudal dorsal raphe nucleus have projections to limbic and limbocortical sites. These sites include the cingulate and prefrontal cortices (Porriño and Goldman-Rakic, 1982; Kazakov et al., 1993; Van Bockstaele et al., 1993), entorhinal cortex (Köhler and Steinbusch, 1982), dorsal hippocampus (Azmitia, 1981; Köhler and Steinbusch, 1982), and nucleus accumbens (Van Bockstaele et al., 1993). In addition, there are extensive, topographically organized and reciprocal projections from the dorsal raphe nucleus to the central nucleus of the amygdala (Mehler 1980; Russchen, 1982; Rizvi et al., 1991; Wallace et al., 1992) and the bed nucleus of the stria terminalis (Weller and Smith, 1982; Holstege et al., 1985), limbic forebrain regions associated with conditioned fear and anxiety (Davis, 1998). Serotonergic neurons that innervate the central nucleus of the amygdala are restricted to the middle and caudal portions of the dorsal raphe nucleus (at and caudal to bregma -8.00 mm; Petrov et al., 1994), consistent with the distribution of serotonergic neurons projecting to other mesolimbocortical sites.

Stress-related behavioral paradigms, particularly those associated with increased anxiety or conditioned fear, may activate topographically organized mesolimbocortical serotonergic systems. For example, behavioral paradigms associated with increased anxiety or conditioned fear increase serotonin metabolism or release in the medial prefrontal cortex (Dunn, 1988; Inoue et al., 1993, 1994; Kawahara et al., 1993; Yoshioka et al., 1995; Goldstein et al., 1996; Adell et al., 1997), cingulate cortex (Palkovits et al., 1976), entorhinal cortex (Blanchard et al., 1991; Ge et al., 1997), nucleus accumbens (Inoue et al., 1993, 1994; Ge et al., 1997), amygdala (Blanchard et al., 1991; Kawahara et al., 1993; Ge et al., 1997; Amat et al., 1998b), and dorsal hippocampus (Joseph and Kennett, 1983; Ge et al., 1997). This topographically selective activation of serotonergic neurotransmission suggests that the serotonergic neurons activated by these stress-related stimuli may reside in the median raphe nucleus (Vertes and Martin, 1988; Vertes et al., 1999) and ventral and interfascicular regions of the caudal dorsal raphe nucleus (Pierce et al., 1976). The data from the current study support

the hypothesis that CRF acts on a topographically organized subpopulation of serotonergic neurons to activate mesolimbocortical serotonergic pathways during intense, prolonged, uncontrollable, or unpredictable stress.

The present data are consistent with previous studies of the effects of intracerebroventricular CRF infusions on *in vivo* firing rates of midline raphe neurons in a behaving amphibian, *Taricha granulosa* (Lowry et al., 1996). However, previous studies of rats suggest that intracerebroventricular CRF (Price et al., 1998) or direct microinfusion of CRF into the region of the dorsal raphe nucleus (Kirby et al., 2000) have principally inhibitory effects on the *in vivo* firing rates of serotonergic neurons. This may be attributable to sampling of more rostral dorsal raphe serotonergic neurons (−7.5 mm Bregma; Kirby et al., 2000) that are known to project preferentially to neocortical areas (Vertes, 1991), as well as to the substantia nigra and caudate putamen (Steinbusch et al., 1981; Imai et al., 1986). This interpretation is consistent with the ability of intracerebroventricular infusions of CRF to decrease extracellular serotonin concentrations in the striatum (Price et al., 1998) and lateral septum (Price and Lucki, 1998). Serotonergic innervation of the lateral septum (like that of the striatum) arises from neurons positioned more rostrally and laterally in the dorsal raphe nucleus compared with those neurons giving rise to mesolimbocortical projections (Köhler et al., 1982). These observations support the hypothesis that mesolimbocortical and mesostriatal serotonergic systems are differentially regulated by CRF; this in turn may contribute to the dissociation of mesolimbocortical and mesostriatal serotonergic activity during stress (Clement et al., 1998). Alternatively, differences between the current study and previous studies (Price et al., 1998; Kirby et al., 2000), particularly the absence of inhibitory effects of CRF *in vitro*, may reflect methodological differences between *in vitro* and *in vivo* recordings.

Previous exposure to stressful stimuli results in an upregulation of tryptophan hydroxylase mRNA levels (coding for the rate-limiting enzyme in serotonin synthesis) in the dorsal and median raphe nuclei (Chamas et al., 1999) and enhances the responsiveness of mesolimbocortical serotonergic neurotransmission to a subsequent stress (De Souza and Van Loon, 1986; Adell et al., 1988a). Intense psychophysical stress is believed to sensitize the animal so that subsequent behavioral responses to stress (including behavioral anxiety and fear) are exaggerated 24 or 48 hr later (Maier, 1993; Graeff et al., 1996). This behavioral sensitization is believed to be a result of prolonged, enhanced sensitivity of serotonergic neurons located in the caudal portion of the dorsal raphe nucleus, possibly involving a functional desensitization of somatodendritic 5-HT_{1A} receptors (Laaris et al., 1997, 1999; Grahn et al., 1999). The present study raises the possibility that CRF actions on mesolimbocortical serotonergic systems may contribute to the development and expression of stress-induced behavioral sensitization. Likewise, interactions between CRF (see introductory remarks) and serotonergic systems may play an important role in drug addiction (Walsh and Cunningham, 1997; Rocha et al., 1998), drug withdrawal (Parsons et al., 1995; Weiss et al., 1996), and stress-induced relapse to drug-seeking behavior (Erb et al., 1998).

The present studies demonstrate that the stress-related neuropeptide CRF increases neuronal firing rates of a subpopulation of serotonergic neurons in the dorsal raphe nucleus. Together with other studies, these data support the hypothesis that CRF plays a role in the activation of mesolimbocortical serotonergic systems in response to stress-related stimuli, with potential long-term behavioral consequences, including behavioral sensitization after previous exposure to stress. Consequently, the interactions between CRF and serotonergic neurons described may play important roles in stress-related psychopathology associated with intense or chronic psychosocial stressors, including generalized anxiety, anxiety associated with drug withdrawal, and stress-induced drug relapse.

REFERENCES

- Adell A, Garcia-Marquez C, Armario A, Gelpi E (1988a) Chronic stress increases serotonin and noradrenaline in rat brain and sensitizes their responses to a further acute stress. *J Neurochem* 50:1678–1681.
- Adell A, Trullas R, Gelpi E (1988b) Time course of changes in serotonin and noradrenaline in rat brain after predictable or unpredictable shock. *Brain Res* 459:54–59.
- Adell A, Casanovas JM, Artigas F (1997) Comparative study in the rat of the actions of different types of stress on the release of 5-HT in raphe nuclei and forebrain areas. *Neuropharmacology* 36:735–741.
- Aldenhoff JB, Gruol DL, Rivier J, Vale W, Siggins GR (1983) Corticotropin releasing factor decreases postburst hyperpolarizations and excites hippocampal neurons. *Science* 221:875–877.
- Amat J, Matus-Amat P, Watkins LR, Maier SF (1998a) Escapable and inescapable stress differentially and selectively alter extracellular levels of 5-HT in the ventral hippocampus and dorsal periaqueductal gray of the rat. *Brain Res* 797:12–22.
- Amat J, Matus-Amat P, Watkins LR, Maier SF (1998b) Escapable and inescapable stress differentially alter extracellular levels of 5-HT in the basolateral amygdala of the rat. *Brain Res* 812:113–120.
- Austin MC, Rhodes JL, Lewis DA (1997) Differential distribution of corticotropin-releasing hormone immunoreactive axons in monoaminergic nuclei of the human brainstem. *Neuropsychopharmacology* 17:326–341.
- Azmitia EC (1981) Bilateral serotonergic projections to the dorsal hippocampus of the rat: simultaneous localization of ³H-5HT and HRP after retrograde transport. *J Comp Neurol* 203:737–743.
- Beck CHM, Fibiger HC (1995) Conditioned fear-induced changes in behavior and in the expression of the immediate early gene *c-fos*: with and without diazepam pretreatment. *J Neurosci* 15:709–720.
- Beckett SRG, Duxon MS, Aspley S, Marsden CA (1997) Central c-Fos expression following 20kHz/ultrasound induced defence behaviour in the rat. *Brain Res Bull* 42:421–426.
- Behan DP, Grigoriadis DE, Lovenberg T, Chalmers D, Heinrichs S, Liaw C, De Souza EB (1996) Neurobiology of corticotropin releasing factor (CRF) receptors and CRF-binding protein: implications for the treatment of CNS disorders. *Mol Psychiatry* 1:265–277.
- Bittencourt JC, Sawchenko PE (2000) Do centrally administered neuropeptides access cognate receptors?: an analysis in the central corticotropin-releasing factor system. *J Neurosci* 20:1142–1156.
- Blanchard DC, Cholvanich P, Blanchard RJ, Clow DW, Hammer Jr RP, Rowlett JK, Bardo MT (1991) Serotonin, but not dopamine, metabolites are increased in selected brain regions of subordinate male rats in a colony environment. *Brain Res* 568:61–66.
- Bonaz B, Rivest S (1998) Effect of a chronic stress on CRF neuronal activity and expression of its type 1 receptor in the rat brain. *Am J Physiol* 275:R1438–R1449.
- Campeau S, Watson SJ (1997) Neuroendocrine and behavioral responses and brain pattern of *c-fos* induction associated with audiogenic stress. *J Neuroendocrinol* 9:577–588.
- Carli M, Prontera C, Samanin R (1989) Effect of 5-HT_{1A} agonists on stress-induced deficit in open field locomotor activity of rats: evidence that this model identifies anxiolytic-like activity. *Neuropharmacology* 28:471–476.
- Chahl LA, Leah J, Herdegen T, Trueman L, Lynch-Frame AM (1996) Distribution of c-Fos in guinea-pig brain following morphine withdrawal. *Brain Res* 717:127–134.
- Chalmers DT, Lovenberg TW, De Souza EB (1995) Localization of novel corticotropin-releasing factor receptor (CRF₂) mRNA expression to specific subcortical nuclei in rat brain: comparison with CRF₁ receptor mRNA expression. *J Neurosci* 15:6340–6350.
- Chamas F, Serova L, Sabban EL (1999) Tryptophan hydroxylase mRNA levels are elevated by repeated immobilization stress in rat raphe nuclei but not in pineal gland. *Neurosci Lett* 267:157–160.
- Chan RKW, Vale WW, Sawchenko PE (1999) Paradoxical activation effects of a CRF-binding protein “ligand inhibitor” in rat brain. *Soc Neurosci Abstr* 25:155.
- Chen Y, Brunson KL, Müller MB, Cariaga W, Baram TZ (2000) Immunocytochemical distribution of corticotropin-releasing hormone receptor type-1 (CRF₁)-like immunoreactivity in the mouse brain: light microscopy analysis using an antibody directed against the C-terminus. *J Comp Neurol* 420:305–323.
- Chieng B, Keay KA, Christie MJ (1995) Increased fos-like immunoreactivity in the periaqueductal gray of anaesthetized rats during opiate withdrawal. *Neurosci Lett* 183:79–82.
- Chung KKK, Martinez M, Herbert J (1999) Central serotonin depletion modulates the behavioural, endocrine and physiological responses to repeated social stress and subsequent c-Fos expression in the brains of male rats. *Neuroscience* 92:613–625.
- Chung KKK, Martinez M, Herbert J (2000) *c-fos* expression, behavioural, endocrine and autonomic responses to acute social stress in male rats after chronic restraint: modulation by serotonin. *Neuroscience* 95:453–463.
- Clement HW, Schäfer F, Ruwe C, Gemsa D, Wesemann W (1993) Stress-induced changes of extracellular 5-hydroxyindoleacetic acid concentrations followed in the nucleus raphe dorsalis and the frontal cortex of the rat. *Brain Res* 614:117–124.
- Clement HW, Kirsch M, Hasse C, Opper C, Gemsa D, Wesemann W (1998) Effect of repeated immobilization on serotonin metabolism in

- different rat brain areas and on serum corticosterone. *J Neural Transm* 105:1155–1170.
- Contarino A, Dellu F, Koob GF, Smith GW, Lee K-F, Vale W, Gold LH (1999) Reduced anxiety-like and cognitive performance in mice lacking the corticotropin-releasing factor receptor 1. *Brain Res* 835:1–9.
- Cullinan WE, Herman JP, Battaglia DF, Akil H, Watson SJ (1995) Pattern and time course of immediate early gene expression in rat brain following acute stress. *Neuroscience* 64:477–505.
- Cummings S, Elde R, Ells J, Lindall A (1983) Corticotropin-releasing factor immunoreactivity is widely distributed within the central nervous system of the rat: an immunohistochemical study. *J Neurosci* 3:1355–1368.
- Davis M (1998) Are different parts of the extended amygdala involved in fear versus anxiety? *Biol Psychiatry* 44:1239–1247.
- De Souza EB, Van Loon GR (1986) Brain serotonin and catecholamine responses to repeated stress in rats. *Brain Res* 367:77–86.
- De Souza EB, Insel TR, Perrin MH, Rivier J, Vale WW, Kuhar MJ (1985) Corticotropin-releasing factor receptors are widely distributed within the rat central nervous system: an autoradiographic study. *J Neurosci* 5:3189–3203.
- Dunn AJ (1988) Changes in plasma and brain tryptophan and brain serotonin and 5-hydroxyindoleacetic acid after footshock stress. *Life Sci* 42:1847–1853.
- Erb S, Shaham Y, Stewart J (1998) The role of corticotropin-releasing factor and corticosterone in stress- and cocaine-induced relapse to cocaine seeking in rats. *J Neurosci* 18:5529–5536.
- Fox EA, Gruol DL (1993) Corticotropin-releasing factor suppresses the afterhyperpolarization in cerebellar Purkinje neurons. *Neurosci Lett* 149:103–107.
- Fulford AJ, Marsden CA (1998) Conditioned release of 5-hydroxytryptamine *in vivo* into the nucleus accumbens following isolation-rearing in the rat. *Neuroscience* 83:481–487.
- Ge J, Barnes NM, Costall B, Naylor RJ (1997) Effect of aversive stimulation on 5-hydroxytryptamine and dopamine metabolism in the rat brain. *Pharmacol Biochem Behav* 58:775–783.
- Goldstein LE, Rasmusson AM, Bunney BS, Roth RH (1996) Role of the amygdala in the coordination of behavioral, neuroendocrine, and prefrontal cortical monoamine responses to psychological stress in the rat. *J Neurosci* 16:4787–4798.
- Graeff FG, Guimarães FS, De Andrade TGCS, Deakin JFW (1996) Role of 5-HT in stress, anxiety, and depression. *Pharmacol Biochem Behav* 54:129–141.
- Grahn RE, Will MJ, Hammack SE, Maswood S, McQueen MB, Watkins LR, Maier SF (1999) Activation of serotonin-immunoreactive cells in the dorsal raphe nucleus in rats exposed to an uncontrollable stressor. *Brain Res* 826:35–43.
- Gray JA (1982) *The neuropsychology of anxiety: an enquiry into the functions of the septo-hippocampal system*. Oxford: Clarendon.
- Heinrichs SC, Menzaghi F, Schulteis G, Koob GF, Stinus L (1995) Suppression of corticotropin-releasing factor in the amygdala attenuates aversive consequences of morphine withdrawal. *Behav Pharmacol* 6:74–80.
- Holstege G, Meiners L, Tan K (1985) Projections of the bed nucleus of the stria terminalis to the mesencephalon, pons, and medulla oblongata in the cat. *Exp Brain Res* 58:379–391.
- Imai H, Steindler DA, Kitai ST (1986) The organization of divergent axonal projections from the midbrain raphe nuclei in the rat. *J Comp Neurol* 243:363–380.
- Inoue T, Koyama T, Yamashita I (1993) Effect of conditioned fear stress on serotonin metabolism in the rat brain. *Pharmacol Biochem Behav* 44:371–374.
- Inoue T, Tsuchiya K, Koyama T (1994) Regional changes in dopamine and serotonin activation with various intensity of physical and psychological stress in the rat brain. *Pharmacol Biochem Behav* 49:911–920.
- Jacobs BL, Azmitia EC (1992) Structure and function of the brain serotonin system. *Physiol Rev* 72:165–229.
- Jacobs BL, Fornal CA (1991) Activity of brain serotonergic neurons in the behaving animal. *Pharmacol Rev* 43:563–578.
- Jacobs BL, Fornal CA (1995) Activation of 5-HT neuronal activity during motor behavior. *Semin Neurosci* 7:401–408.
- Jacobs BL, Fornal CA (1999) Activity of serotonergic neurons in behaving animals. *Neuropsychopharmacology [Suppl 2]* 21:9S–15S.
- Jolas T, Aghajanian GK (1997a) Neurotensin and the serotonergic system. *Prog Neurobiol* 52:455–468.
- Jolas T, Aghajanian GK (1997b) Opioids suppress spontaneous and NMDA-induced inhibitory postsynaptic currents in the dorsal raphe nucleus of the rat *in vitro*. *Brain Res* 755:229–245.
- Joseph MH, Kennett GA (1983) Stress-induced release of 5-HT in the hippocampus and its dependence on increased tryptophan availability: an *in vivo* electrochemical study. *Brain Res* 270:251–257.
- Kawahara H, Yoshida M, Yokoo H, Nishi M, Tanaka M (1993) Psychological stress increases serotonin release in the rat amygdala and prefrontal cortex assessed by *in vivo* microdialysis. *Neurosci Lett* 162:81–84.
- Kazakov VN, Kravtsov PYa, Krakhotkina ED, Maisky VA (1993) Sources of cortical, hypothalamic and spinal serotonergic projections: topographical organization within the nucleus raphe dorsalis. *Neuroscience* 56:157–164.
- Kennett GA, Dourish CT, Curzon G (1987) Antidepressant-like action of 5-HT_{1A} agonists and conventional antidepressants in an animal model of depression. *Eur J Pharmacol* 134:265–274.
- Kirby LG, Rice KC, Valentino RJ (2000) Effects of corticotropin-releasing factor on neuronal activity in the serotonergic dorsal raphe nucleus. *Neuropsychopharmacology* 22:148–162.
- Köhler C, Steinbusch H (1982) Identification of serotonin and non-serotonin-containing neurons of the mid-brain raphe projecting to the entorhinal area and the hippocampal formation. A combined immunohistochemical and fluorescent retrograde tracing study in the rat brain. *Neuroscience* 7:951–975.
- Köhler C, Chan-Palay V, Steinbusch H (1982) The distribution and origin of serotonin-containing fibers in the septal area: a combined immunohistochemical and fluorescent retrograde tracing study in the rat. *J Comp Neurol* 209:91–111.
- Kollack-Walker S, Watson SJ, Akil H (1997) Social stress in hamsters: defeat activates specific neurocircuits within the brain. *J Neurosci* 17:8842–8855.
- Krukoff TL, Khalili P (1997) Stress-induced activation of nitric oxide-producing neurons in the rat brain. *J Comp Neurol* 377:509–519.
- Laaris N, Le Poul E, Hamon M, Lanfumey L (1997) Stress-induced alterations of somatodendritic 5-HT_{1A} autoreceptor sensitivity in the rat dorsal raphe nucleus—*in vitro* electrophysiological evidence. *Fundam Clin Pharmacol* 11:206–214.
- Laaris N, Le Poul E, Laporte AM, Hamon M, Lanfumey L (1999) Differential effects of stress on presynaptic and postsynaptic 5-hydroxytryptamine-1A receptors in the rat brain: an *in vitro* electrophysiological study. *Neuroscience* 91:947–958.
- Lowry CA, Rose JD, Moore FL (1996) Corticotropin-releasing factor enhances locomotion and medullary neuronal firing in an amphibian. *Horm Behav* 30:50–59.
- Lowry CA, Rodda JE, Lightman SL, Ingram CD (1999) Corticotropin-releasing factor (CRF) increases *in vitro* firing rates of serotonergic neurons in the rat dorsal raphe nucleus: evidence for selective activation of a topographically organized mesolimbocortical serotonergic system. *Soc Neurosci Abstr* 25:75.
- Maier S (1993) Learned helplessness: relationships with fear and anxiety. In: *Stress: from synapse to syndrome* (Stanford SC, Salmon P, eds), pp 207–243. London: Academic.
- Martinez M, Phillips PJ, Herbert J (1998) Adaptation in patterns of *c-fos* expression in the brain associated with exposure to either single or repeated social stress in male rats. *Eur J Neurosci* 10:20–33.
- Maswood S, Barter JE, Watkins LR, Maier SF (1998) Exposure to inescapable but not escapable shock increases extracellular levels of 5-HT in the dorsal raphe nucleus of the rat. *Brain Res* 783:115–120.
- Matsuda S, Peng H, Yoshimura H, Wen T-C, Fukuda T, Sakanaka M (1996) Persistent *c-fos* expression in the brains of mice with chronic social stress. *Neurosci Res* 26:157–170.
- McBlane JW, Handley SL (1994) Effects of two stressors on behaviour in the elevated X-maze: preliminary investigation of their interaction with 8-OH-DPAT. *Psychopharmacology* 116:173–182.
- Mehler WR (1980) Subcortical afferent connections of the amygdala in the monkey. *J Comp Neurol* 190:733–762.
- Mitchell SN, Thomas PJ (1988) Effect of restraint stress and anxiolytics on 5-HT turnover in rat brain. *Pharmacology* 37:105–113.
- Nikulina EM, Marchand JE, Kream RM, Miczek KA (1998) Behavioral sensitization to cocaine after a brief social stress is accompanied by changes in Fos expression in the murine brainstem. *Brain Res* 810:200–210.
- Owens MJ, Nemeroff CB (1991) Physiology and pharmacology of corticotropin-releasing factor. *Pharmacol Rev* 43:425–473.
- Palkovits M, Brownstein M, Kizer JS, Saavedra JM, Kopin IJ (1976) Effect of stress on serotonin concentration and tryptophan hydroxylase activity of brain nuclei. *Neuroendocrinology* 22:298–304.
- Parsons LH, Koob GF, Weiss F (1995) Serotonin dysfunction in the nucleus accumbens of rats during withdrawal after unlimited access to intravenous cocaine. *J Pharmacol Exp Ther* 274:1182–1191.
- Paxinos G, Watson C (1998) *The rat brain in stereotaxic coordinates*. San Diego: Academic.
- Petrov T, Krukoff TL, Jhamandas JH (1994) Chemically defined collateral projections from the pons to the central nucleus of the amygdala and hypothalamic paraventricular nucleus in the rat. *Cell Tissue Res* 277:289–295.
- Peyron C, Petit J-M, Rampon C, Jouvet M, Luppi P-H (1998) Forebrain afferents to the rat dorsal raphe nucleus demonstrated by retrograde and anterograde tracing methods. *Neuroscience* 82:443–468.
- Pezzone MA, Lee W-S, Hoffman GE, Pezzone KM, Rabin BS (1993) Activation of brainstem catecholaminergic neurons by conditioned and unconditioned aversive stimuli as revealed by c-Fos immunoreactivity. *Brain Res* 608:310–318.
- Pierce ET, Foote WE, Hobson JA (1976) The efferent connection of the nucleus raphe dorsalis. *Brain Res* 107:137–144.
- Porrino LJ, Goldman-Rakic PS (1982) Brainstem innervation of prefrontal and anterior cingulate cortex in the rhesus monkey revealed by retrograde transport of HRP. *J Comp Neurol* 205:63–76.
- Potter E, Behan DP, Linton EA, Lowry PJ, Sawchenko PE, Vale WW (1992) The central distribution of a corticotropin-releasing factor (CRF)-binding protein predicts multiple sites and modes of interaction with CRF. *Proc Natl Acad Sci USA* 89:4192–4196.

- Price ML, Lucki I (1998) Regulation of serotonin (5-HT) release in the lateral septum by forced swimming involves corticotropin-releasing factor (CRF). *Soc Neurosci Abstr* 24:2048.
- Price ML, Curtis AL, Kirby LG, Valentino RJ, Lucki I (1998) Effects of corticotropin-releasing factor on brain serotonergic activity. *Neuropsychopharmacology* 18:492–502.
- Rasmussen K, Heym J, Jacobs BL (1984) Activity of serotonin-containing neurons in nucleus centralis superior of freely moving cats. *Exp Neurol* 83:302–317.
- Rizvi TA, Ennis M, Behbehani MM, Shipley MT (1991) Connections between the central nucleus of the amygdala and the midbrain periaqueductal gray: topography and reciprocity. *J Comp Neurol* 303:121–131.
- Rocha BA, Searce-Levie K, Lucas JJ, Hiroi N, Castanon N, Crabbe JC, Nestler EJ, Hen R (1998) Increased vulnerability to cocaine in mice lacking the serotonin-1B receptor. *Nature* 393:175–178.
- Rueter LE, Fornal CA, Jacobs BL (1997) A critical review of 5-HT brain microdialysis and behavior. *Rev Neurosci* 8:117–137.
- Ruggiero DA, Underwood MD, Rice PM, Mann JJ, Arango V (1999) Corticotropin-releasing hormone and serotonin interact in the human brainstem: behavioral implications. *Neuroscience* 91:1343–1354.
- Russchen FT (1982) Amygdalopetal projections in the cat. II. Subcortical afferent connections. A study with retrograde tracing techniques. *J Comp Neurol* 207:157–176.
- Sakanaka M, Shibasaki T, Lederis K (1986) Distribution and efferent projections of corticotropin-releasing factor-like immunoreactivity in the rat amygdaloid complex. *Brain Res* 382:213–238.
- Sakanaka M, Shibasaki T, Lederis K (1987) Corticotropin releasing factor-like immunoreactivity in the rat brain as revealed by a modified cobalt-glucose oxidase-diaminobenzidine method. *J Comp Neurol* 260:256–298.
- Sarnyai Z, Biró É, Gardi J, Vecsernyés M, Julesz J, Telegdy G (1995) Brain corticotropin-releasing factor mediates “anxiety-like” behavior induced by cocaine withdrawal in rats. *Brain Res* 675:89–97.
- Senba E, Matsunaga K, Tohyama M, Noguchi K (1993) Stress-induced *c-fos* expression in the rat brain: activation mechanism of sympathetic pathway. *Brain Res Bull* 31:329–344.
- Shaham Y, Funk D, Erb S, Brown TJ, Walker CD, Stewart J (1997) Corticotropin-releasing factor, but not corticosterone, is involved in stress-induced relapse to heroin-seeking in rats. *J Neurosci* 17:2605–2614.
- Siggins GR, Gruol D, Aldenhoff J, Pittman Q (1985) Electrophysiological actions of corticotropin-releasing factor in the central nervous system. *Fed Proc* 44:237–242.
- Silveira MC, Sandner G, Graeff FG (1993) Induction of Fos immunoreactivity in the brain by exposure to the elevated plus-maze. *Behav Brain Res* 56:115–118.
- Singh VB, Hao-Phan T, Corley KC, Boadle-Biber MC (1992) Increase in cortical and midbrain tryptophan hydroxylase activity by intracerebroventricular administration of corticotropin releasing factor: block by adrenalectomy, by RU 38486 and by bilateral lesions to the central nucleus of the amygdala. *Neurochem Int* 20:81–92.
- Steinbusch HWM, Nieuwenhuys R, Verhofstad AAJ, Van Der Kooy D (1981) The nucleus raphe dorsalis of the rat and its projection upon the caudatoputamen. A combined cytoarchitectonic, immunohistochemical and retrograde transport study. *J Physiol (Paris)* 77:157–174.
- Van Bockstaele EJ, Biswas A, Pickel VM (1993) Topography of serotonin neurons in the dorsal raphe nucleus that send axon collaterals to the rat prefrontal cortex and nucleus accumbens. *Brain Res* 624:188–198.
- Vandermaelen CP, Aghajanian GK (1983) Electrophysiological and pharmacological characterization of serotonergic dorsal raphe neurons recorded extracellularly and intracellularly in rat brain slices. *Brain Res* 289:109–119.
- Vaughan J, Donaldson C, Bittencourt J, Perrin MH, Lewis K, Sutton S, Chan R, Turnbull AV, Lovejoy D, Rivier C, Rivier J, Sawchenko PE, Vale W (1995) Urocortin, a mammalian neuropeptide related to fish urotensin I and to corticotropin-releasing factor. *Nature* 378:287–292.
- Vertes RP (1991) A PHA-L analysis of ascending projections of the dorsal raphe nucleus in the rat. *J Comp Neurol* 313:643–668.
- Vertes RP, Martin GF (1988) Autoradiographic analysis of ascending projections from the pontine and mesencephalic reticular formation and the median raphe nucleus in the rat. *J Comp Neurol* 275:511–541.
- Vertes RP, Fortin WJ, Crane AM (1999) Projections of the median raphe nucleus in the rat. *J Comp Neurol* 407:555–582.
- Wallace DM, Magnuson DJ, Gray TS (1992) Organization of amygdaloid projections to brainstem dopaminergic, noradrenergic, and adrenergic cell groups in the rat. *Brain Res Bull* 28:447–454.
- Walsh SL, Cunningham KA (1997) Serotonergic mechanisms involved in the discriminative stimulus, reinforcing and subjective effects of cocaine. *Psychopharmacology* 130:41–58.
- Watanabe Y, Stone E, McEwen BS (1994) Induction and habituation of *c-fos* and *zif/268* by acute and repeated stressors. *NeuroReport* 5:1321–1324.
- Weiss F, Parsons LH, Schulteis G, Hyytiä P, Lorang MT, Bloom FE, Koob GF (1996) Ethanol self-administration restores withdrawal-associated deficiencies in accumbal dopamine and 5-hydroxytryptamine release in dependent rats. *J Neurosci* 16:3474–3485.
- Weller KL, Smith DA (1982) Afferent connections to the bed nucleus of the stria terminalis. *Brain Res* 232:255–270.
- Yoshioka M, Masumoto M, Togashi H, Saito H (1995) Effects of conditioned fear stress on 5-HT release in the rat prefrontal cortex. *Pharmacol Biochem Behav* 51:515–519.