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Corticotropin-releasing factor in the dorsal raphe nucleus increases medial prefrontal cortical serotonin via type 2 receptors and median raphe nucleus activity

Gina L. Forster[1], **Ronald B. Pringle**[1], **Nicholas J. Mouw**[1], **Shawn M. Vuong**[1], **Michael J. Watt**[1], **Andrew R. Burke**[1], **Christopher A. Lowry**[2,3], **Cliff H. Summers**[1,4], **Kenneth J. Renner**[1,4]

[1]Division of Basic Biomedical Sciences and Neuroscience Group, Sanford School of Medicine, University of South Dakota, 414 E. Clark Street, Vermillion, SD 57069, USA

[2]Department of Integrative Physiology, University of Colorado at Boulder, Boulder, CO, USA

[3]Henry Wellcome Laboratories for Integrative Neuroscience and Endocrinology, University of Bristol, Bristol, UK

[4]Department of Biology and Neuroscience Group, University of South Dakota, Vermillion, SD, USA

Abstract

Interactions between central corticotropin-releasing factor (CRF) and serotonergic systems are believed to be important for mediating fear and anxiety behaviors. Recently we demonstrated that infusions of CRF into the rat dorsal raphe nucleus result in a delayed increase in serotonin release within the medial prefrontal cortex that coincided with a reduction in fear behavior. The current studies were designed to study the CRF receptor mechanisms and pathways involved in this serotonergic response. Infusions of CRF (0.5 μg/0.5 μL) were made into the dorsal raphe nucleus of urethane-anesthetized rats following either inactivation of the median raphe nucleus by muscimol (25 ng/0.25 μL) or antagonism of CRF receptor type 1 or CRF receptor type 2 in the dorsal raphe nucleus with antalarmin (25–50 ng/0.5 μL) or antisauvagine-30 (2 μg/0.5 μL), respectively. Medial prefrontal cortex serotonin levels were measured using *in-vivo* microdialysis and high-performance liquid chromatography with electrochemical detection. Increased medial prefrontal cortex serotonin release elicited by CRF infusion into the dorsal raphe nucleus was abolished by inactivation of the median raphe nucleus. Furthermore, antagonism of CRF receptor type 2 but not CRF receptor type 1 in the dorsal raphe nucleus abolished CRF-induced increases in medial prefrontal cortex serotonin. Follow-up studies involved electrical stimulation of the central nucleus of the amygdala, a source of CRF afferents to the dorsal raphe nucleus. Activation of the central nucleus increased medial prefrontal cortex serotonin release. This response was blocked by CRF receptor type 2 antagonism in the dorsal raphe. Overall, these results highlight complex CRF modulation of medial prefrontal cortex serotonergic activity at the level of the raphe nuclei.

Correspondence: Dr Gina L. Forster, gforster@usd.edu.

Keywords

central nucleus of the amygdala; microdialysis; muscimol; rat; stress

Introduction

Corticotropin-releasing factor (CRF) plays an important role in mediating stress-induced alterations in serotonergic activity (Hammack et al., 2002, 2003; Price *et al.*, 2002; Mo *et al.*, 2008). Central serotonin (5-HT) is critical for regulating fear and anxiety behaviors (Carrasco & Van de Kar, 2003; Millan, 2003). The dorsal raphe nucleus (dRN) is a main source of serotonergic projections to forebrain systems mediating anxiety and fear responses (Lowry *et al.*, 2005). Infusion of CRF or CRF-related peptides into the dRN alters limbic 5-HT release (Amat *et al.*, 2004; Forster et al., 2006; Lukkes *et al.*, 2008). Furthermore, stress-induced alterations of 5-HT release within forebrain limbic regions are suppressed by central CRF receptor antagonism (Price *et al.*, 2002; Mo et al., 2008).

The medial prefrontal cortex (mPFC) plays a complex but significant role in regulating endocrine and behavioral responses to stressors (Sullivan & Gratton, 2002). Interestingly, mPFC 5-HT levels increase when conditioned or CRF-induced freezing behavior ceases (Hashimoto *et al.*, 1999; Forster *et al.*, 2006), suggesting that mPFC 5-HT activity is important for limiting fear responses. Certainly, inactivation of the mPFC potentiates fear conditioning and impairs extinction of fear behavior (Amat *et al.*, 2005; Quirk *et al.*, 2006). The pathways and mechanisms regulating mPFC 5-HT release may represent important components mediating adaptive fear responses.

Recently, CRF infusions into the dRN were shown to increase mPFC 5-HT levels, which peak at 80 min post-infusion (Forster *et al.*, 2006). The delayed 5-HT response suggests that CRF actions in the dRN may be mediated through interactions with other brain regions. Like the dRN, the median raphe nucleus (mRN) provides serotonergic innervation of the mPFC (Azmitia & Segal, 1978; Meloni *et al.*, 2008). The mRN receives serotonergic and possibly GABAergic innervation from the dRN (Adell *et al.*, 2002; Tischler & Morin, 2003). Therefore, we tested the hypothesis that intra-dRN CRF infusions increase mPFC 5-HT through interactions with the mRN.

The second aim was to determine the CRF receptor subtype(s) in the dRN responsible for delayed mPFC 5-HT increases. Both CRF receptor type 1 (CRF_1) and CRF receptor type 2 (CRF_2) are found in the dRN (Day *et al.*, 2004) and have opposing effects on 5-HT release (Lukkes *et al.*, 2008). Activation of CRF_2 receptors in the dRN enhances firing rates of 5-HT neurons (Pernar *et al.*, 2004), increases *c-Fos* expression in 5-HT neurons (Staub *et al.*, 2006) and evokes 5-HT release in the nucleus accumbens and basolateral amygdala (Amat *et al.*, 2004; Lukkes *et al.*, 2008). We tested the hypothesis that CRF activation of CRF_2 receptors within the dRN increases 5-HT release in the mPFC. Finally, the role of the central nucleus of the amygdala (CeA) in CRF-elicited increases in mPFC 5-HT was examined, as the CeA provides a major source of CRF to the dRN (Gray, 1993). Furthermore, lesions that encompass the CeA prevent increases in mPFC 5-HT levels during a conditioned

fear paradigm (Goldstein *et al.*, 1996). We tested the hypothesis that CRF derived from stimulation of the CeA activates CRF_2 receptors in the dRN to increase mPFC 5-HT levels.

Materials and methods

Animals

Adult male Sprague Dawley rats (250–350 g) were purchased from the University of South Dakota Animal Resource Center. Rats were housed in pairs, maintained at 22°C, on a reverse 12 h light/12 h dark cycle with free access to food and water. The procedures were approved by the Institutional Animal Care and Use Committee of the University of South Dakota, and were carried out in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

Experiment 1: contribution of the mRN to increased mPFC 5-HT in response to CRF infusion into the dRN

In this experiment, we tested the hypothesis that the mRN is critical for the delayed increases in mPFC 5-HT levels following CRF infusion into the dRN. The delayed increases in mPFC 5-HT have been previously linked with the cessation of fear-like behavior (Forster *et al.*, 2006). Pharmacological blockade (muscimol) of the mRN was used to determine the contribution of the mRN to basal mPFC 5-HT release and also to test the effect of mRN inactivation on mPFC 5-HT levels as induced by CRF microinfusion into the dRN of urethane-anesthetized rats.

Surgery—Twenty-four rats were anesthetized with urethane (1.5 g/kg, i.p.) and placed within a small mammal stereotaxic frame (Kopf, Tujunga, CA, USA). Anesthesia (up to 1.8 g/kg, i.p.) was maintained throughout the entire experiment, with body temperature held at 37°C by a temperature-controlled heating pad (Harvard Apparatus, Holliston, MA, USA). Laboratory-made dual stainless-steel guide cannulae (26 gauge) were stereotaxically implanted so that the lateral guide cannula was positioned 2 mm above the mRN (anterioposterior (AP), −7.8 mm from bregma; mediolateral (ML), −3.8 mm from midline at a 25° angle lateral to medial; dorsoventral (DV), −6.2 mm from dura; Paxinos & Watson, 1997) and the medial guide cannula positioned 2 mm above the dRN (AP, −7.8 mm from bregma; ML, −2.8 mm from midline at a 25° angle lateral to medial; DV, −4.4 mm from dura; Paxinos & Watson, 1997). A laboratory-made concentric microdialysis probe (4.0 mm exposed membrane length, 5000 MW cut-off, typical recovery 20%; Forster *et al.*, 2006) was directly inserted into the mPFC (AP, +3.2 mm from bregma; ML, −0.5 mm from midline; −5.4 mm from dura; Paxinos & Watson, 1997). Artificial cerebrospinal fluid (aCSF) was perfused through the probe at a rate of 0.4 μL/min via PE20 tubing connected to a 1 mL syringe within a microinfusion pump (Harvard Apparatus).

Microdialysis and drug infusion—Silica cannulae (194 μm o.d., 2 mm longer than guides; Polymicro Technologies, Phoenix, AZ, USA) were fixed to PE20 tubing, connected to a 5 μL Hamilton syringe and drug or vehicle was back-loaded into the cannula before implantation as described previously (Forster & Blaha, 2000). Drug-filled silica cannulae were lowered through the mRN and dRN guide cannulae 3 h following probe

implantation. At 1 h following cannulae insertion (Lukkes *et al.*, 2008), dialysates (8 μL) were collected from the mPFC at 20 min intervals and 5-HT levels were measured using high-performance liquid chromatography with electrochemical detection (see below for details). After collection of at least three comparable baseline samples, a 0.25-μL microinfusion of muscimol or vehicle was made into the mRN followed, in some cases, by a 0.5-μL microinfusion of CRF into the dRN 10 min later (drug concentrations and treatment groups for Experiment 1 are listed in Table 1). All drug infusions into the raphe nuclei were made at a rate of 0.5 μL/min using a microinfusion pump (Stoelting, Wood Dale, IL, USA). Dialysates were collected until mPFC 5-HT returned to baseline levels. In the case of rats where no alterations in mPFC 5-HT were observed, eight post-injection samples were collected.

Experiment 2: role of dRN CRF_1 and CRF_2 receptors in CRF-induced increases in mPFC 5-HT

This experiment was designed to test the hypothesis that activation of CRF_2 receptors in the dRN is required for delayed increases in mPFC 5-HT levels as induced by CRF microinfusion into the dRN. Accordingly, the effects of CRF_1 and CRF_2 receptor antagonists on intra-dRN CRF-induced changes in mPFC 5-HT levels were assessed in urethane-anesthetized rats.

Surgery—Twenty-eight rats were anesthetized with urethane and placed within a small mammal stereotaxic frame as described for Experiment 1. Laboratory-made 26 gauge dual stainless-steel guide cannulae, constructed so that the tips of the two guide cannulae were immediately adjacent to one another to ensure infusion into the same region of the dRN, were implanted 2 mm above the dRN (AP, −7.9 mm from bregma; ML, −2.8 mm from midline at a 25° angle lateral to medial; DV, −4.4 mm from dura; Paxinos & Watson, 1997). A concentric microdialysis probe was inserted into the mPFC as described for Experiment 1.

Microdialysis and drug infusion—Drug cannulae were lowered into the dRN 3 h following probe implantation and dialysates were collected 1 h later, as described for Experiment 1. After collection of at least three comparable baseline samples, a 0.5 μL microinfusion of either the CRF_1 receptor antagonist antalarmin, the CRF_2 receptor antagonist antisauvagine-30 (ASV-30) or vehicle was made into the dRN, followed 10 min later by a 0.5-μL microinfusion of CRF into the dRN (see Table 2 for drug concentrations and experimental design).

Experiment 3: the effects of CeA stimulation on mPFC 5-HT: role of CRF_2 receptors in the dRN

This experiment tested the hypothesis that electrical stimulation of the CeA (using stimulation parameters mimicking firing rates induced by stress) would produce similar delayed increases in mPFC 5-HT as seen with CRF infusions into the dRN. Follow-up experiments tested the hypothesis that CeA-induced increases in mPFC 5-HT were mediated by CRF_2 receptors in the dRN. The effects of CeA electrical stimulation on mPFC 5-HT levels, and the effects of CRF_2 receptor antagonists on CeA-induced changes in mPFC 5-HT levels, were assessed in urethane-anesthetized rats. Converging evidence suggests that

activation of the CeA during stress increases circulating corticosterone through the activation of the paraventricular nucleus of the hypothalamus (Feldman *et al.*, 1995; Lowry, 2002; Herman *et al.*, 2005). The effects of CeA stimulation on circulating plasma corticosterone levels were also measured to confirm that our stimulation parameters induced a hormonal response that is consistent with a stress response (Sinton *et al.*, 2000).

Surgery—Fifty-five rats were anesthetized with urethane and placed within a small mammal stereotaxic frame as described for Experiment 1. A concentric bipolar stimulating electrode (SNE-100; RMI, Tujunga, CA, USA) was implanted into the CeA (AP, −2.6 mm from bregma; ML, −4.3 mm from midline; DV, −7.0 mm from dura; Paxinos & Watson, 1997). A laboratory-made single stainless-steel guide cannula (26 gauge) was implanted above the dRN and a concentric microdialysis probe was inserted into the mPFC, as described for Experiment 1 and Experiment 2.

Microdialysis, drug infusion and electrical stimulation—Drug cannula insertion and dialysate collection were performed as described in Experiment 1. After collection of at least three comparable baseline samples, a 0.5 μL microinfusion was made into the dRN of either the general $CRF_{1/2}$ receptor antagonist d-Phe12, Nle21, 38, αMeLeu37-CRF (D-phe-CRF), the CRF_2 receptor antagonist ASV-30 or vehicle, followed 10 min later by electrical stimulation of the CeA (see Table 3 for drug concentrations and treatment groups). Additional groups received microinfusions without stimulating the bipolar electrode implanted into the CeA (Table 3). We have previously demonstrated that the CRF_2 receptor antagonist ASV-30 does not affect basal 5-HT release when infused into the dRN alone (Lukkes *et al.*, 2008). However, to assess the effects of the $CRF_{1/2}$ receptor antagonist D-phe-CRF on basal 5-HT release, a group with D-phe-CRF infusion alone was included in this experiment.

Electrical stimulation of the CeA consisted of a continuous 5 Hz train of pulses (0.2 ms pulse duration; 800 μA) applied over a 3-min period (total of 720 pulses) with an optical isolator via a programmable pulse generator (IsoFlex/Master-8; AMPI, Israel). These parameters were designed to mimic those recorded from the CeA during restraint stress (Henke, 1983). The radius of neuronal activation from the stimulating electrode tip for similar stimulation intensities has been calculated at 700 μm (Yeomans *et al.*, 1988).

Collection of plasma—Plasma was collected from the caudal tail vein from rats that received vehicle (aCSF) into the dRN (vehicle without stimulation) and from rats that received vehicle into the dRN prior to stimulation of the CeA, for later analysis of circulating corticosterone levels. At 30 min following CeA stimulation (equivalent to 40 min after vehicle infusion), a small tail incision was made and blood collected with heparin-coated micro-hematocrit capillary tubes (Fisher Scientific, Pittsburgh, PA, USA) for 2 min. After the blood was centrifuged (1118 *g* for 10 min), plasma was drawn off and stored in 10 μL aliquots at −80°C until assayed.

High-performance liquid chromatography measurement of 5-HT

The detection of 5-HT in dialysates was accomplished using high-performance liquid chromatography with electrochemical detection (Bradberry *et al.*, 1991; Forster *et al.*, 2006). The mobile phase [containing (per liter): 300 mg EDTA, 432 mg sodium octanesulfonate, 4.8 g NaH_2PO_4, 300 μL triethylamine and 122 mL acetonitrile, pH 5.35; all obtained from Sigma, St Louis, MO, USA] was pumped through a UniJet 3 μm C_{18} microbore column (Bioanalytical Systems; West Lafayette, IN, USA) under nitrogen gas pressure (2000 psi). Dialysates were injected onto the chromatographic system using a rheodyne injector via a 5 μL loop (Bioanalytical Systems). The perfusate rate of 0.4 μL/min resulted in the collection of approximately 8 μL of dialysate/20 min to ensure that the loop was overfilled during each sample period. Following separation by the column, 5-HT was detected by a glassy carbon electrode (Bioanalytical Systems), which was maintained at +0.5 V with respect to an $Ag/AgCl_2$ reference electrode using an LC-4C potentiostat (Bioanalytical Systems). The voltage output was recorded by CSW32 v1.4 Chromatography Station for Windows (DataApex, Prague, Czech Republic). 5-HT peaks were identified by comparison to a 5-HT standard (7.9 pg/5 μL 5-HT). The 2 : 1 signal-to-noise detection limit for 5-HT using this system was 0.11 ± 0.10 pg and the mean ± SEM baseline level of mPFC 5-HT was 0.69 ± 0.11 pg/5 μL (uncorrected for recovery).

Measurement of plasma corticosterone levels

Measurement of plasma corticosterone levels was performed using a corticosterone enzyme-linked immunoassay kit (R&D Systems, Minneapolis, MN, USA) according to the manufacturer's instructions. Briefly, 10 μL of plasma and 0.5 μL of steroid displacement reagent were diluted with 990 μL of assay buffer for a 100-fold dilution. Duplicates of samples, controls and corticosterone standards (0–2000 ng/mL) were assayed. Plasma corticosterone levels were detected by absorbance of samples at 405 nm (wavelength correction set at 595 nm), using an automated plate reader and KinetiCalc Jr software (Bio-Tek Instruments, Winooski, VT, USA). Absorbance values from samples were applied to the standard curve generated ($r^2 = 0.99$) and corticosterone levels were expressed as ng/mL. Absorbance values were also used to calculate the percentage of maximum binding (23.2%) and percentage of non-specific binding (2.8%). Both of these values were within the manufacturer's range. The detection limit sensitivity of this assay was 27.0 pg/mL.

Histology

Upon completion of experiments, rats were killed by overdose with sodium pentobarbital (0.5 mL Fatal Plus, i.p.; Vortech, Dearborn, MI, USA) and the brains were removed and fixed in 10% buffered formalin (Fisher Scientific). Brains were sectioned frozen at 60 μm on a sliding microtome and then examined under a light microscope by two experimenters blind to treatment to determine placements of probes, cannulae and stimulating electrodes. Only data from rats with correct probe, cannulae and stimulating electrode placements were included in the following analyses.

Data analysis

The height of the 5-HT peaks in three baseline dialysis samples were averaged and post-drug 5-HT peak heights were calculated as a percentage change from mean baseline levels for each animal. For each experiment, 5-HT levels were analysed with respect to time (within-subject factor) and treatment group (between-subject factor) using two-way ANOVA with one repeated measure. When a significant effect of treatment was observed, a one-way ANOVA was used to compare treatment groups for each separate time-point. Significant effects of treatment at a given time-point were further analysed by Student–Newman Keul's (SNK) multiple comparison procedure. When a significant effect of time was noted, a one-way ANOVA with one repeated measure was performed across time for each given treatment. Resulting significant time-points were identified by Dunnett's *post-hoc* test for multiple comparisons with a single control, where the sample collected immediately before the first drug infusion served as the control sample. Corticosterone levels of CeA-stimulated and non-stimulated rats were compared using an unpaired *t*-test. All analyses were performed using SigmaStat v.2.03, with the alpha level set at 0.05.

Drugs

Urethane, 5-HT creatinine sulfate, rat-human CRF, muscimol, antalarmin hydrochloride and ASV-30 trifluoroacetate salt were purchased from Sigma. D-phe-CRF was purchased from Bachem (Torrance, CA, USA). Muscimol, CRF and D-phe-CRF were dissolved in aCSF (pH 7.4) to working concentrations. Urethane was dissolved in distilled water to a concentration of 0.345 g/mL, whereas 5-HT was dissolved in sodium acetate buffer, pH 5.0, to a concentration of 15.8 pg/μL with the final working concentration diluted to 1.58 pg/μL in aCSF.

Antalarmin was dissolved in a 5% ethanol/5% Cremopher EL (Sigma)/aCSF vehicle, whereas ASV-30 was dissolved in a 2% ethanol/aCSF vehicle. The doses of CRF, CRF receptor antagonists (and their vehicles) and muscimol used in the current experiments were based on previous reports where infusions of these drugs into the raphe nuclei were performed (Nishikawa & Scatton, 1986; Hammack *et al.*, 2002; Forster *et al.*, 2006; Lukkes *et al.*, 2008). Two doses of antalarmin were used to confirm that the lack of effect observed with this CRF_1 receptor antagonist was not due to insufficient antagonist concentration.

Results

Experiment 1: contribution of the mRN to increased mPFC 5-HT in response to CRF infusion into the dRN

Microdialysis probe membranes were located within the mPFC from 3.7 to 2.7 mm anterior from bregma (Paxinos & Watson, 1997), encompassing the cingulate, prelimbic and infralimbic (IL) cortices (Fig. 1A). The tips of dRN infusion cannulae were located between 7.64 and 8.00 mm posterior from bregma (Paxinos & Watson, 1997) but the majority of infusions were made at the 8.00 mm posterior from bregma level (Fig. 1B). This more posterior level of the dRN corresponds to the location of neurons that project to the mPFC and are activated by intracerebroventricular administration of CRF (Van Bockstaele *et al.*, 1993; Meloni *et al.*, 2008). Although close to the cerebral aqueduct, infusions made from

cannulae placed on an angle minimize the possibility that CRF could reach the aqueduct via dorsal diffusion along the cannula track (Forster *et al.*, 2006; Lukkes *et al.*, 2008). Furthermore, we have shown previously that infusions of CRF in 0.5 μL volumes made into the central gray areas adjacent to the dRN do not affect 5-HT release (Forster *et al.*, 2006; Lukkes *et al.*, 2008). This suggests that regions adjacent to the dRN are not responsible for the effects of CRF on mPFC 5-HT levels and/or that diffusion of CRF administered in a 0.5 μL volume is restricted.

The tips of the mRN infusion cannulae ranged from 7.64 to 8.30 mm posterior from bregma (Paxinos & Watson, 1997), with the large majority of infusions made at 8.30 mm posterior from bregma (Fig. 1B). These infusion sites were located at least 1 mm ventral to the most ventral portion of the dRN and the infusion cannula was inserted at an angle to avoid the dRN, minimizing the possibility that the infusion volume of 0.25 μL within the mRN could diffuse dorsally and affect activity in the dRN. The distribution of microdialysis probes and drug cannulae was similar for the different pharmacological treatment groups.

Pharmacological blockade of the mRN by muscimol (25 ng/0.25 μL) resulted in a transient decrease n mPFC 5-HT release (Fig. 2A). There was a significant interaction between drug treatment and time ($F_{11,98} = 7.343$; $P < 0.030$) on mPFC 5-HT levels. Muscimol infused into the mRN caused a decrease in mPFC 5-HT levels, significant at after 20 min compared with vehicle infusions into the mRN (SNK, $P < 0.001$; Fig. 2A).

Consistent with our earlier findings (Forster et al., 2006), microinfusion of CRF (0.5 μg/0.5 μL) into the dRN resulted in the typical delayed increase in mPFC 5-HT levels (Fig 2B). This effect was blocked by muscimol (25 ng/0.25 μL) inactivation of the mRN 10 min prior to CRF microinfusion (Fig. 2B). Significant main effects of drug treatment ($F_{1,10} = 41.301$; $P < 0.001$), time ($F_{11,85} = 4.573$; $P < 0.001$) and a significant interaction between treatment and time ($F_{11,85} = 11.756$; $P < 0.001$) were apparent. Microinfusion of CRF into the dRN following vehicle microinfusion into the mRN resulted in a significant increase in mPFC 5-HT levels over time ($F_{11,39} = 11.025$; $P < 0.001$). Microinfusion of CRF into the dRN resulted in increased mPFC 5-HT levels at 40–100 min post-infusion when compared with pre-infusion levels (Dunnett's, $P < 0.05$) and with rats pre-infused with muscimol into the mRN (SNK, $P < 0.001$; Fig. 2B). Rats pre-treated with muscimol in the mRN prior to intra-dRN CRF infusion showed a significant decrease in mPFC 5-HT levels over time ($F_{11,46} = 6.350$; $P < 0.001$). This effect was significant at 20 min post-infusion when compared with pre-infusion levels (Dunnett's, $P < 0.05$; Fig. 2B). Muscimol pre-treated rats receiving intra-dRN CRF infusion also showed significantly decreased mPFC 5-HT levels compared with vehicle pre-treated rats receiving intra-dRN CRF infusions in the sample collected during infusion (time 0) and at 20 min post-infusion (SNK, $P < 0.01$, Fig. 2B).

Experiment 2: role of dRN CRF_1 and CRF_2 receptors in CRF-induced increases in mPFC 5-HT

Similar to Experiment 1, microdialysis probe membranes were located within the mPFC, 3.7 to 2.7 mm anterior from bregma (Paxinos & Watson, 1997), and sampled from the cingulate, prelimbic and IL cortices (Fig. 3A). Also comparable to the first experiment, the tips of dual-infusion dRN cannulae were located between 7.64 and 8.00 mm posterior from bregma

(Paxinos & Watson, 1997), with the majority of infusions made at the 8.00 mm posterior from bregma level (Fig. 3B). The distribution of microdialysis probes and drug cannulae were similar in the different pharmacological treatment groups.

Pre-treatment of the dRN with the CRF_2 receptor antagonist ASV-30 (2 μg/0.5 μL) completely abolished the delayed increases in mPFC 5-HT release as induced by CRF infusion (0.5 μg/0.5 μL) into the dRN (Fig. 4A). There were significant main effects of drug treatment ($F_{1,10} = 9.706$; $P = 0.011$) and time ($F_{11,98} = 6.157$; $P < 0.001$), and a significant interaction between treatment and time ($F_{11,98} = 6.855$; $P < 0.001$). Microinfusions of CRF into the dRN following vehicle infusion resulted in a significant increase in mPFC 5-HT levels over time ($F_{11,48} = 9.214$; $P < 0.001$), which were significant at 40–60 min post-infusion when compared with pre-infusion levels (Dunnett's, $P < 0.05$; Fig. 4A). In contrast, mPFC 5-HT levels were not significantly altered over time in rats pre-treated with ASV-30 prior to CRF infusion into the dRN ($F_{11,50} = 0.630$; $P = 0.794$). When vehicle and ASV-30 groups were compared at each time-point, vehicle pre-treated rats exhibited significantly higher CRF-induced mPFC 5-HT levels at 40–100 min post-CRF infusion (SNK, $P < 0.01$; Fig. 4A).

In contrast to CRF_2 receptor antagonism, pre-treatment of the dRN with the CRF_1 receptor antagonist antalarmin (25 and 50 ng/0.5 μL) had no effect on CRF-induced mPFC 5-HT release (Fig. 4B). There was no significant main effect of drug treatment ($F_{2,13} = 0.704$; $P = 0.512$) or a significant interaction between treatment and time ($F_{22,129} = 0.748$; $P = 0.782$). However, a significant effect of time on 5-HT levels was observed ($F_{11,129} = 22.405$; $P < 0.001$). Separate one-way ANOVA with one repeated measure showed that all three treatment groups exhibited significant CRF-induced increases in mPFC 5-HT levels across time (vehicle and CRF: $F_{11,48} = 9.586$, $P < 0.001$; 25 ng antalarmin and CRF: $F_{11,52} = 12.015$, $P < 0.001$; 50 ng antalarmin and CRF: $F_{11,29} = 4.383$, $P < 0.001$).

Experiment 3: effects of CeA stimulation on mPFC 5-HT: role of CRF_2 receptors in the dRN

Microdialysis probe membranes were located within the mPFC from 3.7 to 2.7 mm anterior from bregma (Paxinos & Watson, 1997) and encompassed the cingulate, prelimbic and IL cortices (Fig. 5A), comparable to Experiments 1 and 2. Stimulating electrode tips were distributed throughout the CeA from 2.30 to 3.14 mm posterior to bregma (Paxinos & Watson, 1997), with the majority of stimulating electrode tips localized to 2.30–2.80 mm posterior to bregma (Fig. 5B). The tips of dRN infusion cannulae were located between 7.64 and 8.00 mm posterior from bregma (Paxinos & Watson, 1997), with infusions made in comparable regions to Experiments 1 and 2 (Fig. 5C). The placement of microdialysis probes, stimulating electrodes and drug cannulae was similar in all treatment groups.

To determine whether the stimulation parameters used for the following studies resulted in activation of the hypothalamic-pituitary-adrenal axis, plasma corticosterone levels were measured in rats that received vehicle infusion into the dRN alone or vehicle infusion into the dRN prior to CeA stimulation. Plasma concentrations of corticosterone were significantly elevated 30 min following CeA stimulation (mean ± SEM, 298.33 ± 25.90) when compared with vehicle treatment alone (mean ± SEM, 101.39 ± 19.37; $P < 0.001$).

Electrical stimulation of the CeA resulted in a transient decrease and then a delayed increase in mPFC 5-HT levels, with the latter effect abolished by pre-treatment of the dRN with the $CRF_{1/2}$ general receptor antagonist D-phe-CRF (50 ng/0.5 μL) (Fig. 6A). Significant main effects of drug treatment ($F_{3,28} = 5.653$; $P = 0.004$) and time ($F_{12,293} = 3.481$; $P < 0.001$), and a significant interaction between treatment and time ($F_{36,293} = 4.292$; $P < 0.001$) were observed. Infusion of vehicle alone into the dRN had no effect on mPFC 5-HT levels over time ($F_{12,78} = 1.479$; $P = 0.150$). However, analysis with a one-way A revealed a significant effect of infusion of 50 ng D-phe-CRF alone into the dRN ($F_{12,74} = 2.953$; $P = 0.002$). Dunnett's *post-hoc* tests did not identify any time-point following D-phe-CRF infusion that was significantly different from pre-infusion levels ($P > 0.05$), suggesting a slight but gradual decline in 5-HT levels over time in this group (Fig. 6A). Importantly, mPFC 5-HT levels collected from vehicle- and D-phe-CRF-infused groups (in the absence of CeA stimulation) did not differ from one another at any time-point (SNK, $P > 0.05$; Fig. 6A). A significant effect on 5-HT levels over time was observed for rats that were pre-infused with vehicle into the dRN and received CeA stimulation ($F_{12,68} = 6.615$; $P < 0.001$; Fig. 6A). *Post-hoc* tests revealed a significant decrease in mPFC 5-HT levels at 20 min post-stimulation and a significant increase in mPFC 5-HT levels at 80–100 min post-stimulation (Dunnett's, $P < 0.05$; Fig. 6A). There was also a significant effect on 5-HT levels over time for rats that were pre-treated with D-phe-CRF prior to CeA stimulation ($F_{12,73} = 2.044$; $P = 0.032$). However, a decrease in mPFC 5-HT levels at 20 min post-stimulation (Dunnett's, $P < 0.05$) was the only significant change observed in this group (Fig. 6A). When vehicle and D-phe-CRF pre-treated groups that received CeA stimulation were compared, only rats that received vehicle infusion with stimulation exhibited greater 5-HT release at 80–120 min post-stimulation (SNK, $P < 0.001$; Fig. 6A).

In contrast to the CeA-evoked increase in mPFC 5-HT release, D-phe-CRF had no effect on the transient decrease in mPFC 5-HT observed at 20 min post-stimulation when compared with vehicle pre-treated stimulated rats (SNK, $P = 0.084$; Fig. 6A). Both vehicle and D-phe-CRF pre-treated groups showed decreased mPFC 5-HT levels at 20 min following stimulation when compared with both vehicle and D-phe-CRF groups that did not receive stimulation (SNK, $P < 0.01$; Fig. 6A).

Delayed increases in mPFC 5-HT levels induced by electrical stimulation of the CeA were also completely suppressed by pre-treatment of the dRN with the CRF_2 receptor antagonist ASV-30 (2 μg/0.5 μL; Fig. 6B). There were significant main effects of drug treatment ($F_{2,20} = 6.585$; $P = 0.006$) and time ($F_{12,219} = 3.817$; $P < 0.001$), and also a significant interaction between treatment and time ($F_{24,219} = 3.834$; $P < 0.001$). Infusion of vehicle alone into the dRN had a significant effect on mPFC 5-HT levels ($F_{12,77} = 2.121$; $P = 0.025$). Similar to D-phe-CRF infusions alone (see Fig. 6A), Dunnett's *post-hoc* tests did not identify any time-point post-vehicle infusion that was significantly different from pre-infusion levels (P > 0.05), suggesting a slight decline in 5-HT levels over time in this group (Fig. 6B). A significant effect on 5-HT levels over time was observed for rats that were pre-infused with vehicle into the dRN and received CeA stimulation ($F_{12,77} = 5.186$; $P < 0.001$; Fig. 6B). Comparable to the D-phe-CRF experiments described above (Fig. 6A), *post-hoc* tests revealed a significant decrease in mPFC 5-HT levels at 20 min post-stimulation and a significant increase in mPFC 5-HT levels at 80–100 min post-stimulation compared with

prestimulation levels (Dunnett's, $P < 0.05$; Fig. 6B). There was also a significant effect on 5-HT levels over time for rats that were pre-treated with ASV-30 prior to CeA stimulation ($F_{12,73} = 2.169$; $P = 0.024$), which was accounted for by a significant decrease in mPFC 5-HT levels at 20 min post-stimulation (Dunnett's, $P < 0$ .05; Fig. 6B). Also comparable to the effects of D-phe-CRF (Fig. 6A), pre-treatment of the dRN with ASV-30 had no effect on the transient decrease in mPFC observed at 20 min post-stimulation when compared with vehicle pre-treated CeA-stimulated rats (SNK, $P = 0.565$; Fig. 6B). However, of the stimulation groups, only vehicle pre-treated rats exhibited higher mPFC 5-HT levels at 80–140 min post-stimulation when compared with non-stimulated control groups (SNK, $P < 0.01$; Fig. 6B), suggesting that pre-treatment of the dRN with ASV-30 blocked the delayed excitatory effects of CeA stimulation on mPFC 5-HT release.

Discussion

Microinfusion of CRF (0.5 lg) into the dRN of urethane-anesthetized rats produced a delayed, prolonged increase in mPFC 5-HT release. The delayed time-course of this effect (40–100 min post-infusion) was similar to that observed with the same dose of CRF injected into the dRN of freely-moving rats (Forster *et al.*, 2006). Interestingly, the delayed effect of 0.5 μg CRF infusion into the dRN appears to be specific to the mPFC as the same concentration of CRF infused into the dRN results in immediate increases in nucleus accumbens and CeA 5-HT release (Forster *et al.*, 2006; Lukkes *et al.*, 2008). The major difference between the current study and the previous mPFC report is that the maximal mPFC 5-HT increase in anesthetized rats was consistently observed at 40 min post-CRF infusion (Figs 2 and 4), whereas in non-anesthetized rats this maximal value was noted at 80 min post-CRF injection (Forster *et al.*, 2006). This difference may be an effect of the anesthetic on CRF-elicited mPFC 5-HT release or, conversely, the effects of behavioral performance on mPFC 5-HT levels. Regardless, the similar delayed time-course of CRF-elicited effects in both anesthetized and freely-moving rats suggested that the use of anesthetized preparations would provide useful data when examining the mechanisms by which CRF increases mPFC 5-HT levels.

The current series of experiments in urethane-anesthetized rats demonstrated that: (i) activity of the mRN was required for delayed increases in 5-HT levels within the mPFC as induced by CRF infusion into the dRN; (ii) CRF-induced increases in mPFC 5-HT levels were dependent on CRF_2 receptors in the dRN; (iii) electrical stimulation of the CeA also produced delayed and prolonged increases in mPFC 5-HT levels and (iv) CeA-elicited increases in mPFC 5-HT levels were dependent upon CRF_2 receptors in the dRN.

Pharmacological inactivation of the mRN by muscimol resulted in a significant and transient decrease in mPFC 5-HT levels. This finding is consistent with the observation that the mRN provides serotonergic innervation to the mPFC (Azmitia & Segal, 1978; Meloni *et al.*, 2008). Furthermore, this suggests that activity of the mRN tonically regulates mPFC 5-HT release. The effects of CRF infused into the dRN were completely abolished when the mRN was inactivated by muscimol. These findings imply that mRN activity is required during the same time period as the actions of CRF in the dRN. Importantly, after intra-mRN muscimol microinfusion alone, mPFC 5-HT levels recovered to baseline levels prior to the normal

onset of intra-dRN CRF-elicited increases in mPFC 5-HT. This implies that the lack of CRF effect following mRN inactivation cannot be explained by reduced tonic levels of mPFC 5-HT. The finding that activation of the mRN is required for increased mPFC 5-HT release is consistent with the hypothesis that the mRN and mPFC are a part of a limbic circuitry critical for adaptive coping in response to both acute and chronic stress (Graeff *et al.*, 1996; Lowry, 2002; Netto *et al.*, 2002; Amat *et al.*, 2005; Forster *et al.*, 2006).

The CRF-mediated increases in mPFC 5-HT levels were abolished by pre-treatment of the dRN with the CRF_2 receptor antagonist ASV-30. In contrast, microinfusions into the dRN of the CRF_1 receptor antagonist antalarmin at two different doses had no effect on CRF-elicited mPFC 5-HT release. Pre-treatment of the dRN with the lowest dose of antalarmin (25 ng) has been shown to completely block CRF-induced decreases in accumbal 5-HT levels (Lukkes *et al.*, 2008), indicating that the doses of antalarmin used in the current study were sufficient to block CRF_1-mediated effects. Overall, these results suggest that CRF_2 receptors in the dRN mediate the excitatory effects of CRF on mPFC 5-HT release and that CRF_1 receptors play no role in this response. The current findings add to a growing body of literature suggesting that activation of CRF_2 receptors in the dRN stimulates 5-HT release in different brain regions (Amat *et al.*, 2004; Pernar *et al.*, 2004; Staub *et al.*, 2006; Lukkes *et al.*, 2008).

Activation of CRF_2 receptors in the dRN may not stimulate mPFC 5-HT release through a direct pathway from the dRN to the mPFC. Instead, results from the current study demonstrate that mRN activity is necessary for dRN CRF-induced delayed increases in mPFC 5-HT levels. The dRN provides serotonergic and possibly GABAergic innervation to the mRN (Adell *et al.*, 2002; Tischler & Morin, 2003). Converging evidence suggests that 5-HT autoreceptors do not exert tonic control of mRN 5-HT neurons (Adell *et al.*, 2002) but tonic GABAergic control of mRN 5-HT neurons has been demonstrated (Forchetti & Meek, 1981). Importantly, activation of CRF_2 receptors in the dRN can inhibit non-serotonergic neurons that are thought to be GABAergic neurons (Pernar *et al.*, 2004). It is possible that CRF_2 receptor activation inhibits GABAergic neurons in the dRN that also project to the mRN, releasing the mRN from tonic dRN inhibition and allowing increased 5-HT activity within the mPFC (Fig. 7). However, the possibility that increased mPFC 5-HT levels induced by CRF are a result of direct 5-HT projections from the dRN cannot be excluded. Recently, Meloni et al. (2008) have demonstrated that intracerebroventricular infusion of CRF induces c-Fos activity in neurons within both the dRN and the mRN that directly project to the IL mPFC region. Furthermore, the large majority of dRN and mRN neurons that projected to the IL were serotonergic (Meloni *et al.*, 2008). Combined with the current findings, this raises the possibility that mRN activity is required for CRF activation of both mRN and dRN serotonergic projections to the mPFC, which may be modulated by reciprocal circuitry between the dRN and mRN (Fig. 7). Clearly, further investigation is required to elucidate the mechanisms by which the dRN and mRN interact to ultimately affect mPFC 5-HT release.

As mPFC 5-HT increases are associated with a reduction in fear behavior and CRF_2 receptors mediate increased mPFC 5-HT, it is possible that activation of CRF_2 receptors is related to limiting fear, anxiety or stress responses. In support of this idea, CRF_2

receptor knockout mice show greater anxiety-like and depression-like behavior and increased endocrine responses to stress (Kishimoto *et al.*, 2000; Bale, 2005). Interestingly, intracerebroventricular injections of the CRF_2 receptor agonist urocortin II have delayed anxiolytic effects in rats (Valdez *et al.*, 2002), which our results suggest may be related to delayed activation of mPFC 5-HT pathways as mediated by CRF_2 receptors. In contrast, other studies suggest that CRF_2 receptors in the dRN or CRF activation of mPFC-projection neurons (Meloni *et al.*, 2008) is associated with heightened stress responses. For example, Hammack *et al.* (2003) found that conditioned freezing behavior, measured 24 h following exposure of rats to uncontrollable stress in a learned helplessness paradigm, is reduced following infusion of the CRF_2 receptor antagonist ASV-30 into the dRN prior to stress exposure. Therefore, it is probable that subpopulations of CRF_2 receptors in the dRN play different and perhaps opposing roles in mediating behavioral responses to stressors.

Like CRF infusion into the dRN, electrical stimulation of the CeA resulted in a delayed and prolonged increase in mPFC 5-HT levels. One major difference in mPFC 5-HT responses between CRF infusions in the dRN compared with CeA stimulation was the magnitude of the response (approximately 100–150% increase for CRF infusions vs. approximately 50% increase for CeA stimulation). This difference is probably an effect of the quite different methodologies employed to elicit mPFC 5-HT release, i.e. direct pharmacological activation of CRF receptors in the dRN compared with indirect activation of the dRN by stimulation of CRF afferent inputs. A second noticeable difference was when the onset of the response occurred (40 min post-CRF infusion vs. 80 min post-CeA infusion). This again may be due to differences in the intensity by which each methodology activates CRF receptors in the dRN. The CeA stimulation parameters utilized were based on those recorded from the CeA of rats during restraint stress (Henke, 1983). Furthermore, these stimulation parameters increased plasma corticosterone concentrations by approximately 300%. Similar increases in plasma corticosterone have been observed immediately following and 60 min after 30 min of restraint stress (Sinton *et al.*, 2000), suggesting that our stimulation of the CeA may approximate the level of activity in this region during stress. The stimulating current (800 μA) used in the present study has been shown to spread approximately 700 μm within the brain (Yeomans., 1988). As CRF cell bodies are located throughout the amygdala complex (Gray, 1993), it is possible that the spread of current from the stimulating electrode tip resulted in stimulation of other CRF projection neurons outside the boundaries of the CeA. It is not clear whether the dRN receives CRF projections from other amgydaloid structures and future anatomical studies are warranted to elucidate the full extent of CRF projections from other regions to the dRN.

Central nucleus-stimulated increases in mPFC 5-HT levels were abolished by intra-dRN infusions of the $CRF_{1/2}$ receptor antagonist D-phe-CRF and the CRF_2 receptor antagonist ASV-30. These results suggest that CeA-stimulated increases in mPFC 5-HT release are mediated by CRF_2 receptors in the dRN. This is consistent with our demonstration that CRF-induced increases in mPFC 5-HT release are dependent upon CRF_2 receptors in the dRN. These findings also suggest that stressor-evoked increases in mPFC 5-HT, which are dependent on the activity of the CeA (Goldstein *et al.*, 1996), could be mediated by CRF_2 receptor activation in the dRN. Furthermore, as we have demonstrated that CRF-induced

increases in mPFC 5-HT require mRN activity, stress-related activation of the CeA may increase mPFC 5-HT release through CRF actions on dRN-mRN circuitry (Fig. 7).

Interestingly, an initial decrease in mPFC 5-HT release was observed following CeA stimulation. A similar response was not detected following CRF infusion into the dRN. Furthermore, the initial decrease in mPFC 5-HT following electrical stimulation of the CeA was not affected by CRF receptor antagonism in the dRN, suggesting that this response is not mediated by CRF receptors in the dRN. There are several possibilities that may account for the early decrease in mPFC 5-HT. First, electrical stimulation of the CeA may simply result in antidromic activation of 5-HT terminals in the CeA, causing increased 5-HT release in the dRN or mRN, activation of inhibitory somatodentric 5-HT autoreceptors (Adell *et al.*, 2002) and a corresponding decrease in dRN or mRN 5-HT activity. Alternatively, it is possible that stimulating the CeA results in a transient decrease in mPFC 5-HT release via activation of non-CRF pathways that target either 5-HT cell bodies in the raphe nuclei or 5-HT terminals in the mPFC. Finally, it is possible that CeA stimulation results in immediate activation of inhibitory local circuitry within the CeA to initially inhibit CeA output. Further experimentation is required to determine whether this initial decrease in mPFC 5-HT release is an important component of stress-activated CeA mediation of mPFC 5-HT activity or simply an artifact of CeA electrical stimulation.

Conclusions

The current results suggest that delayed increases in mPFC 5-HT release, previously associated with cessation of freezing behavior in rats, are mediated by CRF activation of CRF_2 receptors in the dRN, require mRN activity and can be elicited by stimulation of the CeA. This implies the involvement of complex neurocircuitry that includes, at least in part, CeA-derived CRF release in the dRN, CRF_2 receptor activation in the dRN and CRF_2 receptor mediation of mRN disinhibition to result in increased mPFC 5-HT release (Fig. 7). Although future work is required to elucidate the mechanisms by which these brain regions interact, the current study combined with previous findings provides a neural framework for how the central nervous system could produce integrated activity related to adaptive stress responses.

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Abbreviations

5-HT	serotonin (5-hydroxytryptamine)
aCSF	artificial cerebrospinal fluid
AP	anterioposterior

ASV-30	antisauvagine-30
CeA	central nucleus of the amygdala
CRF	corticotropin-releasing factor
CRF_1	corticotropin-releasing factor receptor type 1
CRF_2	corticotropin-releasing factor receptor type 2
D-phe-CRF	d-phe12, Nle21, 38, αMeleu37-CRF
dRN	dorsal raphe nucleus
DV	dorsoventral
IL	infralimbic
ML	mediolateral
mPFC	medial prefrontal cortex
mRN	median raphe nucleus
SNK	Student-Newman-Keuls

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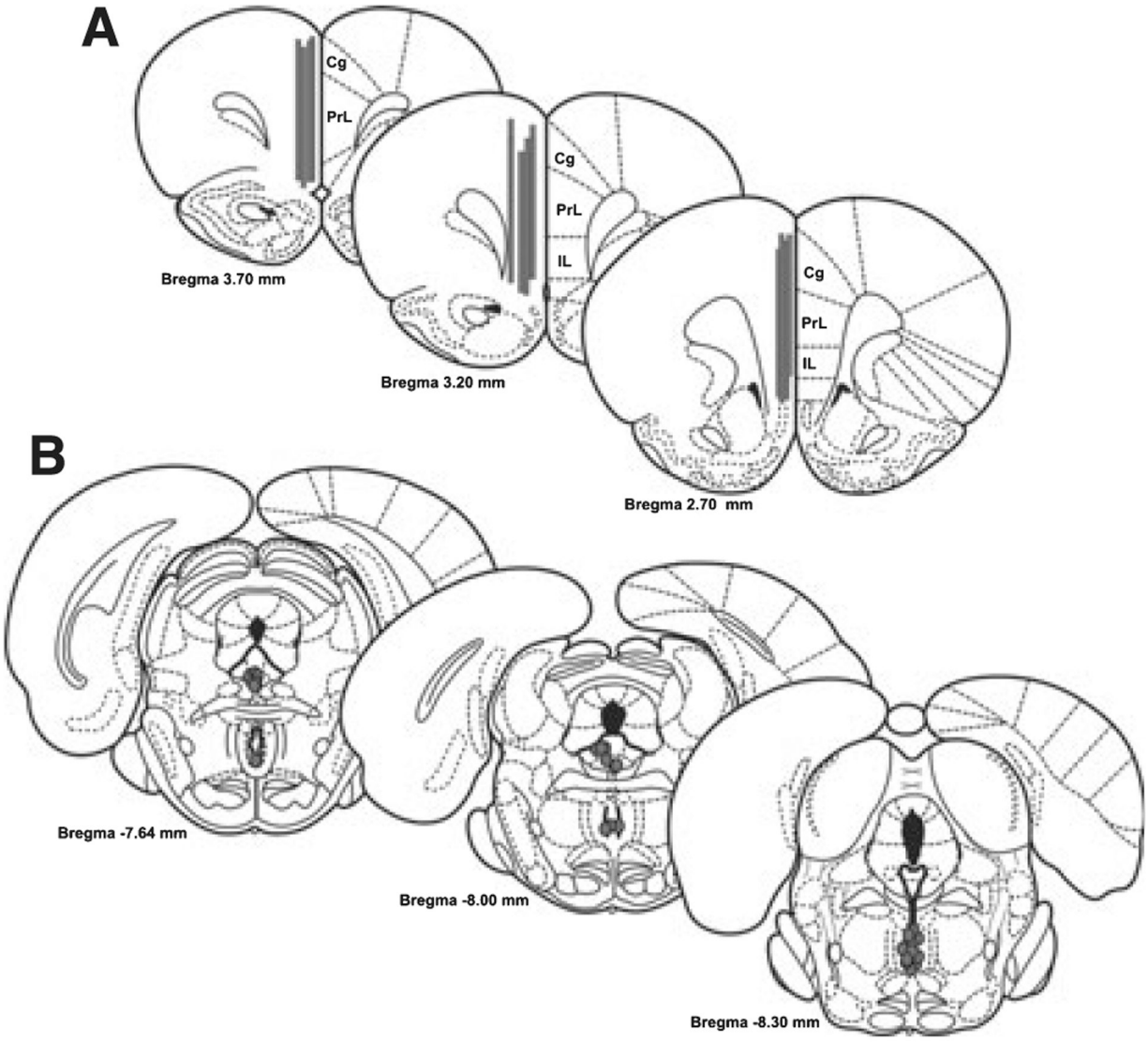

Fig. 1.

Representative coronal diagrams of microdialysis probe and drug infusion cannula placements for muscimol experiments. (A) Microdialysis probe membrane (gray bars) placement in the cingulate (Cg), prelimbic (PrL) and IL cortices of the mPFC. (B) Location of drug infusion cannula tips (gray circles) in the dRN and the more ventrally located mRN (both outlined by thick black lines). Figure adapted from Paxinos & Watson (1997).

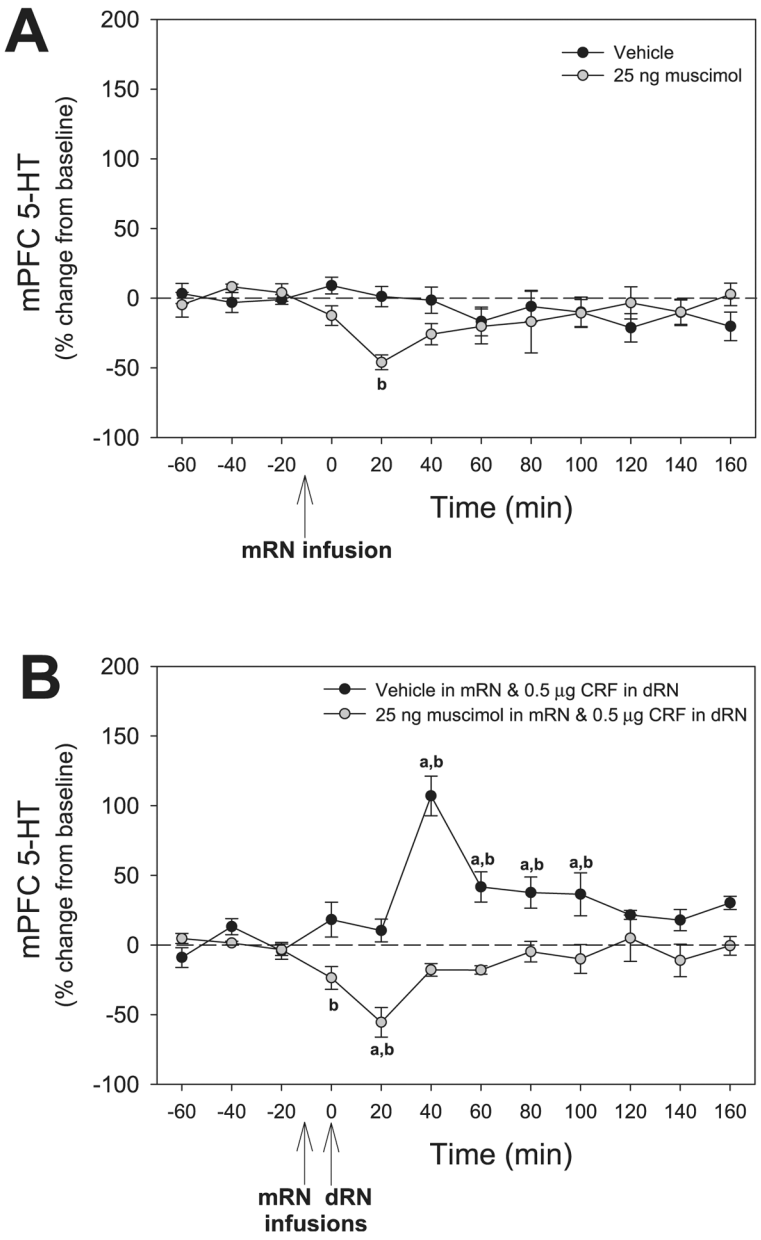

Fig. 2.

Effects of muscimol in the mRN on basal and CRF-induced mPFC 5-HT release. (A) Muscimol (25 ng/0.25 µL) infused into the mRN caused an immediate and transient decrease in mPFC 5-HT release. (B) Muscimol (25 ng /0.25 µL) infused into the mRN blocked intra-dRN CRF-induced increases in mPFC 5-HT. Data represent mean ± SEM. [a]Significantly different from pre-infusion levels. [b]Significant differences between treatment groups ($P < 0.05$).

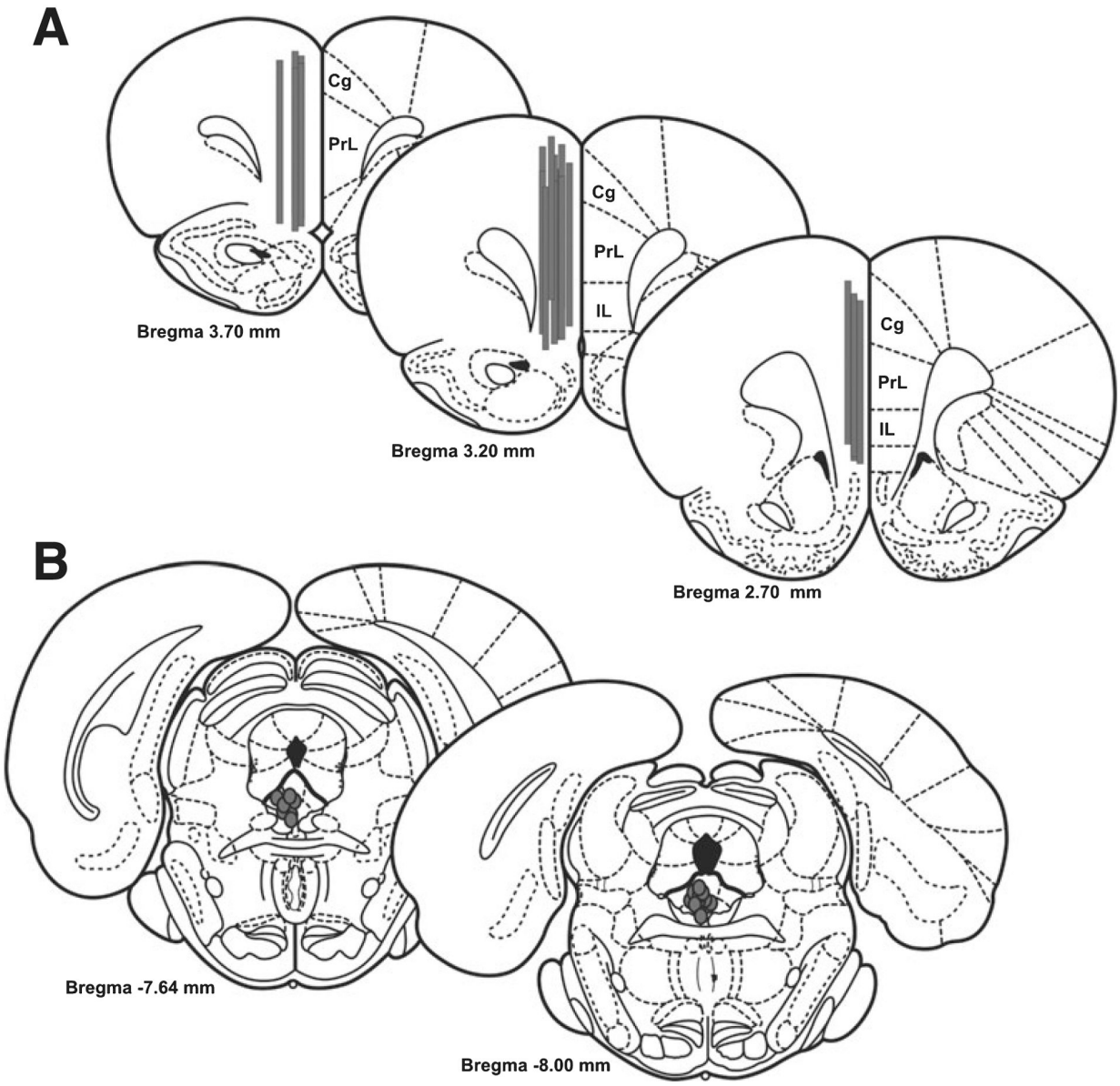

Fig. 3.

Representative coronal diagrams of microdialysis probe and drug infusion cannula placements for CRF receptor antagonist experiments. (A) Microdialysis probe membrane (gray bars) placement in the cingulate (Cg), prelimbic (PrL) and IL cortices of the mPFC. (B) Location of drug dual-infusion cannula tips (gray circles) in the dRN (outlined by thick black lines). Figure adapted from Paxinos & Watson (1997).

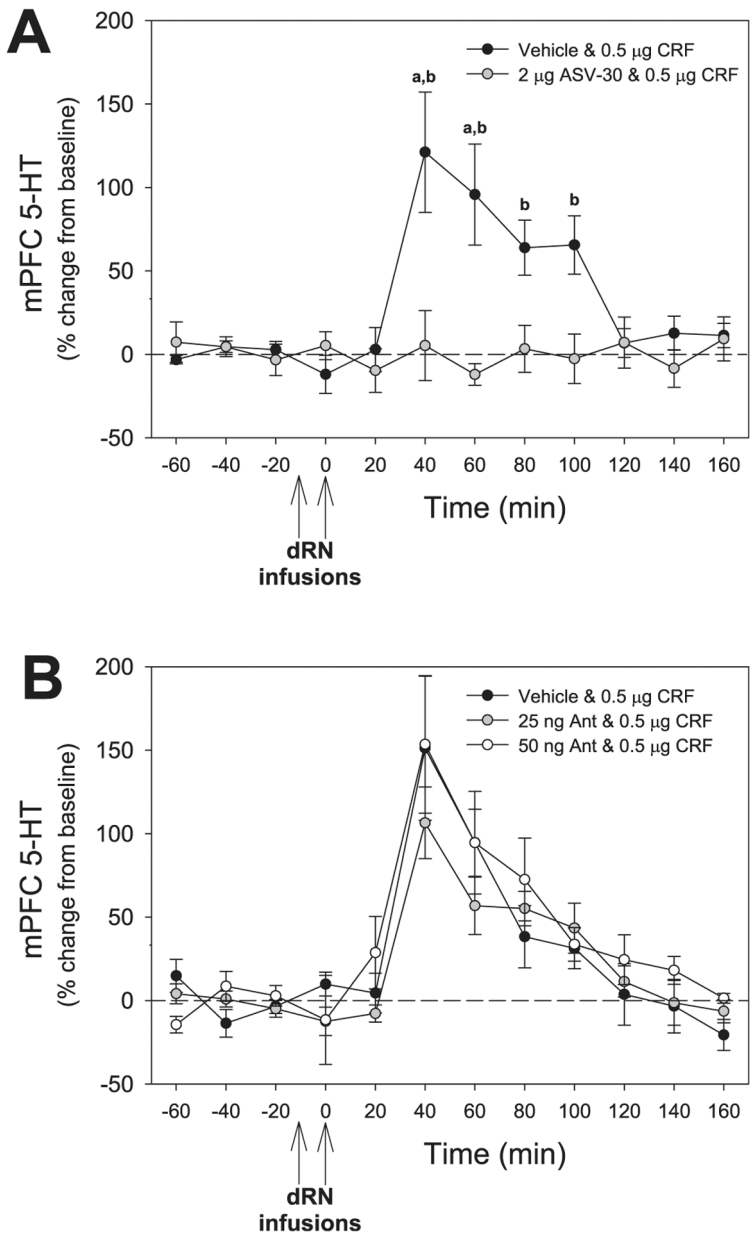

Fig. 4.

Effects of CRF antagonism in the dRN on CRF-elicited 5-HT release in the mPFC. (A) Pre-treatment of the dRN with the CRF_2 antagonist ASV-30 (2 lg /0.5 µL) blocked the stimulatory effect of intra-dRN CRF (0.5 lg /0.5 µL) on mPFC 5-HT levels. [a]Significantly different from pre-infusion level. [b]Significant differences between treatment groups ($P < 0.05$). (B) Pre-treatment of the dRN with the CRF_1 antagonist antalarmin (Ant) (25 or 50 ng/0.5 µL) did not alter CRF-induced (0.5 µg /0.5 µL) increases in mPFC 5-HT. All treatments resulted in a significant increase in mPFC 5-HT release over time ($P < 0.001$). Data represent mean ± SEM.

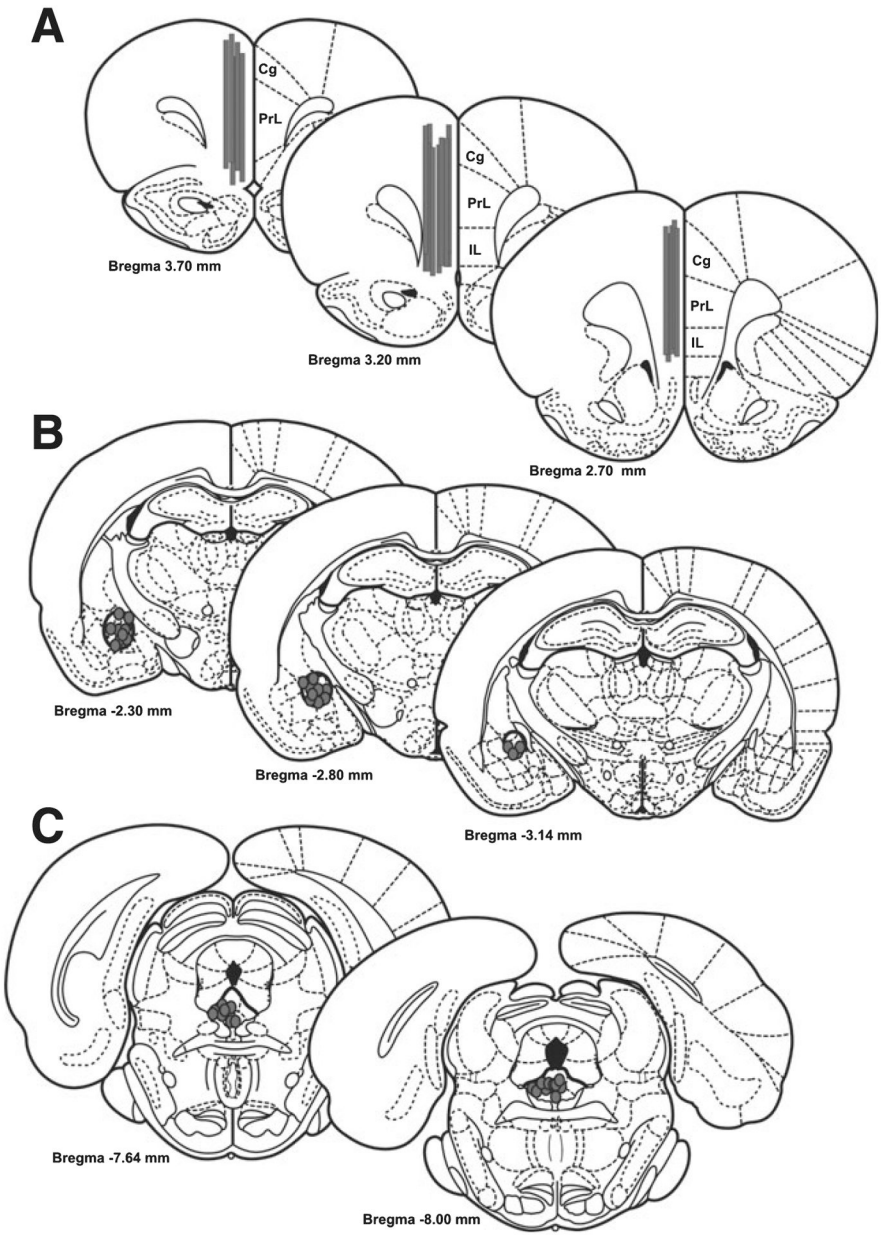

Fig. 5.

Representative coronal diagrams of microdialysis probe, stimulating electrode and drug infusion cannula placements for CeA stimulation experiments.(A) Microdialysis probe membrane (gray bars) placement in the cingulate (Cg), prelimbic (PrL) and IL cortices of the mPFC. (B) Placement of the stimulating electrode tips (gray circles) in the CeA (outlined by thick black lines). (C) Location of dual-infusion cannula tips (gray circles) in the dRN (outlined by thick black lines). Figure adapted from Paxinos & Watson (1997).

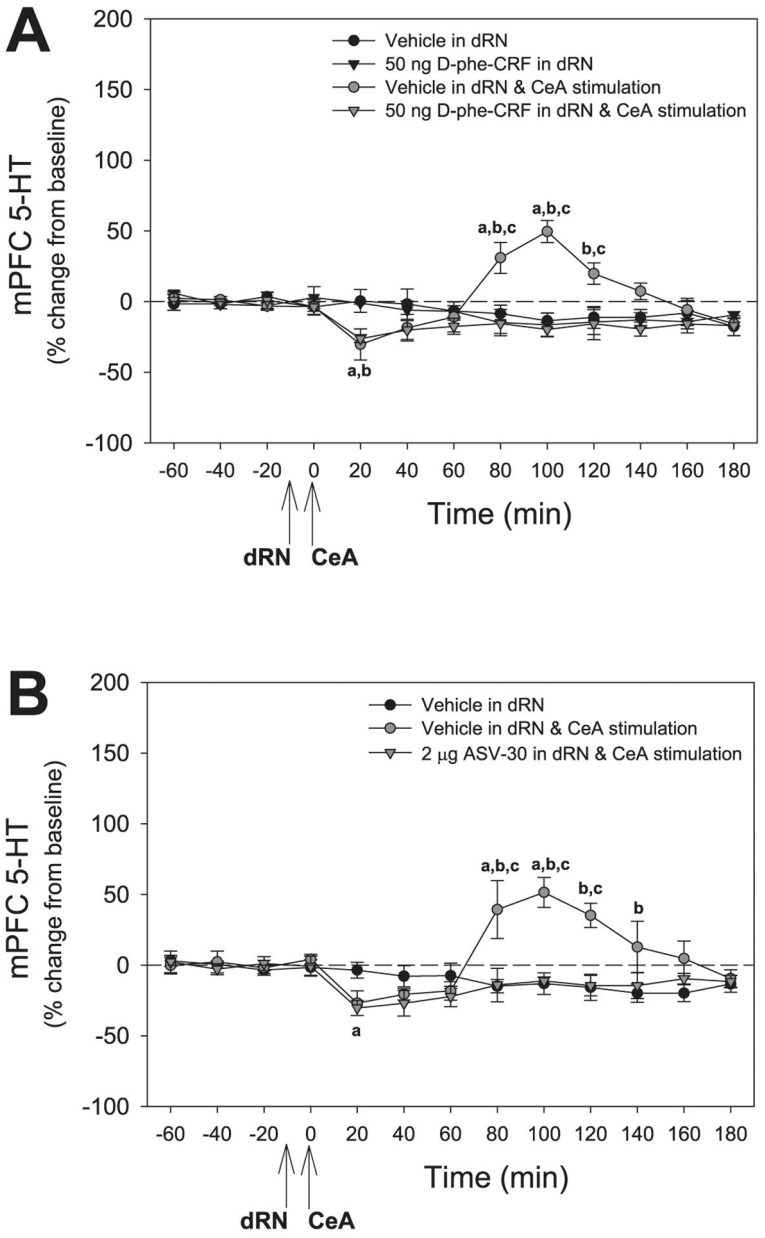

Fig. 6.

Effects of electrical stimulation of the CeA on mPFC 5-HT release (mediation by CRF receptors in the dRN). (A) Pre-treatment of the dRN with the general CRF_1 and CRF_2 antagonist D-phe-CRF (50 ng/0.5 μL) blocked the excitatory effect on mPFC 5-HT levels elicited by stimulation of the CeA. (B) Pre-treatment of the dRN with CRF_2 antagonist ASV-30 (2 μg/0.5 μL) also blocked the excitatory effect on mPFC 5-HT levels elicited by stimulation of the CeA. Data represent mean ± SEM. [a]Significantly different from pre-infusion levels. [b]Significant differences between stimulated and non-stimulated treatment groups. [c]Significant differences between vehicle + stimulation and CRF receptor antagonist + stimulation groups ($P < 0.05$).

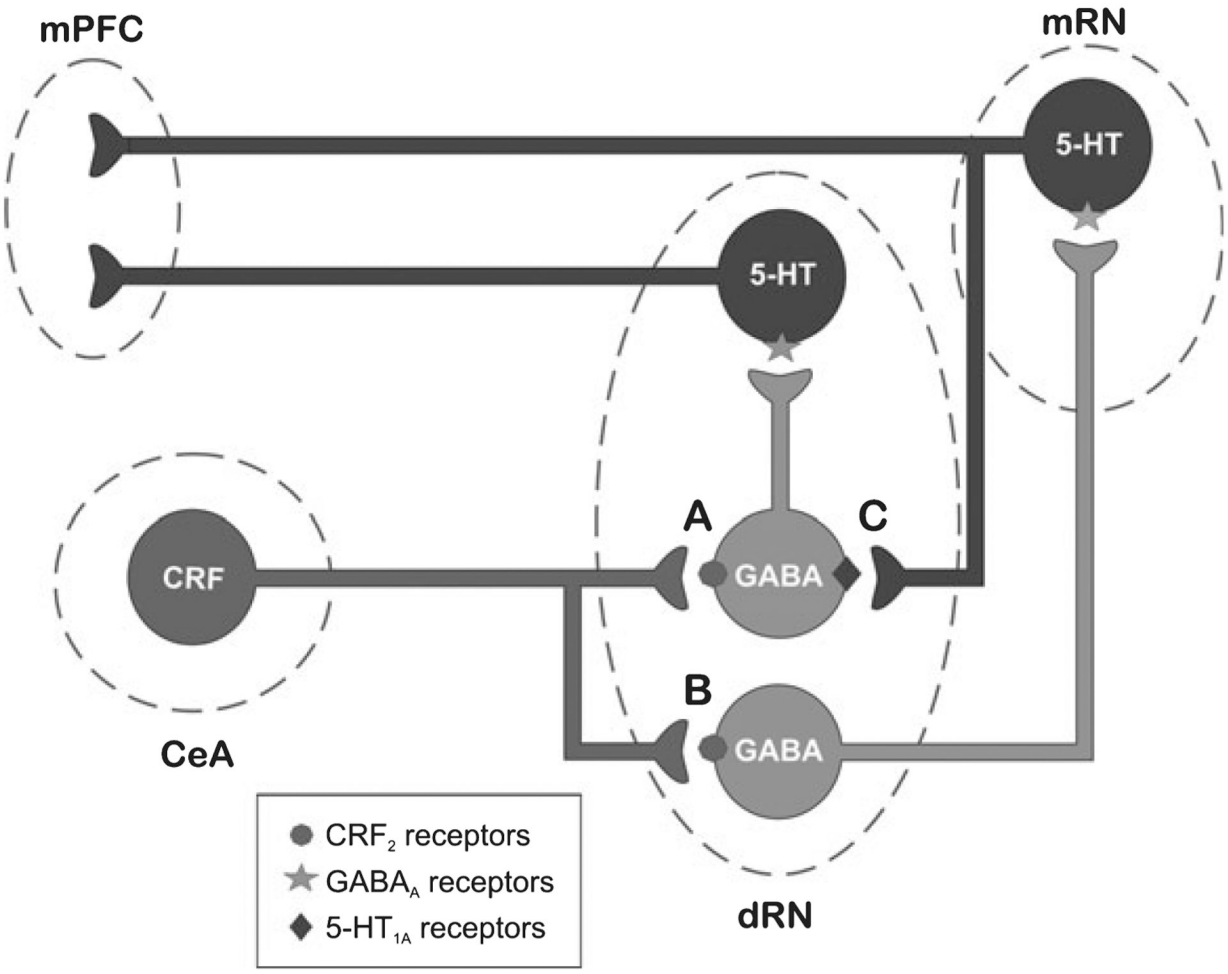

Fig. 7.

Schematic diagram illustrating the proposed neurocircuitry underlying CRF-mediated 5-HT release in the mPFC. Electrophysiological evidence (Pernar et al., 2004) suggests that activation of CRF_2 receptors in the dRN inhibits GABAergic neurons (A), which disinhibits 5-HT neurons in the dRN. This mechanism probably underlies the immediate CRF-induced 5-HT release in many regions of the limbic system, such as the nucleus accumbens (Lukkes *et al.*, 2008) and the CeA (Forster *et al.*, 2006), regions that are predominantly innervated by the dRN (Van Bockstaele *et al.*, 1993; Halberstadt & Balaban, 2006). As the mPFC is innervated by both the dRN and mRN (Meloni *et al.*, 2008) and 5-HT release in the mPFC induced by CRF infusion into the dRN requires mRN activity, the current study suggests an alternative mechanism for delayed mPFC 5-HT release as induced by CRF. It is possible that CRF_2 activation inhibits GABAergic neurons in the dRN that project to the 5-HT neurons of the mRN (B), disinhibiting 5-HT neurons in the mRN to result in increased mPFC 5-HT release. As CRF activates 5-HT neurons in the dRN and mRN that project to the mPFC (Meloni *et al.*, 2008), a complementary hypothesis is that disinhibition of mRN 5-HT neurons also results in activation of 5-HT afferents to the dRN (Mosko et al., 1977). (C) Therefore, activation of 5-HT_{1A} receptors localized on GABAergic neurons (Day *et al.*,

2004) could release 5-HT neurons in the dRN from local GABAergic inhibition, further contributing to increased mPFC 5-HT release.

Table 1.

Design of muscimol experiments

Median raphe infusion	Dorsal raphe infusion	*N*
Vehicle (0.25 μL)	–	6
Muscimol (25 ng/0.25 μL)	–	6
Vehicle (0.25 μL)	CRF (0.5 μg/0.5 μL)	6
Muscimol (25 ng/0.25 μL)	CRF (0.5 μg/0.5 μL)	6

Table 2.

Design of CRF antagonist experiments

Dorsal raphe pre-infusion	Dorsal raphe infusion	*N*
Vehicle (0.5 μL)	CRF (0.5 μg/0.5 μL)	6
ASV-30 (2 μg/0.5 μL)	CRF (0.5 μg/0.5 μL)	6
Vehicle (0.5 μL)	CRF (0.5 μg/0.5 μL)	6
Antalarmin (25 ng/0.5 μL)	CRF (0.5 μg/0.5 μL)	6
Antalarmin (50 ng/0.5 μL)	CRF (0.5 μg/0.5 μL)	4

Table 3.

Design of CeA stimulation experiments

Dorsal raphe pre-infusion	CeA stimulation	*N*
Vehicle (0.5 μL)	No	8
Vehicle (0.5 μL)	Yes	8
D-phe-CRF (50 ng/0.5 μL)	No	8
D-phe-CRF (50 ng/0.5 μL)	Yes	8
Vehicle (0.5 μL)	No	8
Vehicle (0.5 μL)	Yes	8
ASV-30 (2 μg/0.5 μL)	Yes	7