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Activation of raphe efferents to the medial prefrontal cortex by CRF; correlation with anxiety-like behavior

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Abstract

Background: Parallel lines of research suggest that dysfunction affecting both corticotropin-releasing factor (CRF) and serotonin (5-HT) systems is involved in the pathophysiology of psychiatric illnesses such as anxiety and depression. The effect of CRF on behavior, and on the accompanying change in activity of 5-HT neurons in the dorsal and median raphe nuclei (DR and MR) that project to the ventral medial prefrontal cortex (mPFC), a brain area implicated in mood and anxiety disorders, was studied.

Methods: Male Sprague-Dawley rats with intra-mPFC deposits of fluorescent microspheres received injections of CRF (1 µg, icv) and were tested for CRF-enhanced startle, a behavioral assay believed to reflect stress- or anxiety-like states. C-Fos immunohistochemistry was used to measure CRF-induced activity in retrogradely-labeled neurons in the DR and MR and correlate this level of activity with the level of CRF-enhanced startle.

Results: CRF-enhanced startle was accompanied by an increase c-Fos expression in retrogradely-labeled cells in the raphe. In the DR and MR, there was a clear topography of activation, with a higher percent activation in retrogradely-labeled neurons in caudal sections. In the caudal DR, this effect was positively correlated with the level of CRF-enhanced startle. Coexpression of retrogradely-labeled cells with tryptophan hydroxylase showed that the majority (> 90%) of raphe efferents to the mPFC were from serotonergic neurons.

Conclusions: These data indicate that CRF activates a subpopulation of cortical-projecting 5-HT raphe neurons, and suggest that increased 5-HT release in the mPFC may be an important component driving some types of anxiety-like behaviors.

Keywords

raphe; c-Fos; serotonin; startle; prefrontal cortex; stress

Introduction

The neuropeptide corticotropin-releasing factor (CRF) has been widely implicated in the development and manifestation of psychiatric illnesses such as anxiety and depression [1;2],

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highly comorbid disorders that may share common elements of an underlying pathophysiology [3]. The link between CRF and anxiety/mood disorders is evident in studies demonstrating that administration of CRF or CRF antagonists, or genetic manipulations that change the expression levels of CRF receptors, can dramatically alter behavior in several animal models of anxiety and depression. In general, these studies have shown that increases in CRF-neurotransmission have anxiety- and depressive-like effects whereas decreases in CRF-neurotransmission have anxiolytic- and antidepressive-like effects [4-8]. Taken together, these preclinical findings complement reports from clinical studies where an association between hyperactive CRF systems and some types of anxiety (e.g. post-traumatic stress disorder [PTSD]) and depression (e.g. major depression) have been found [9-11].

CRFergic systems are also known to interact with other neurotransmitter systems associated with anxiety and depression, such as the serotonin (5-hydroxytryptamine, 5-HT) system [12-14], and it is possible that CRF-5-HT interactions play a role in the constellation of symptoms seen in these diseases [15;16]. In animals, administration of CRF or related peptides (e.g. urocortins) can have either excitatory or inhibitory effects on 5-HT neurons of the dorsal raphe nucleus depending on the preparation (*in vitro* vs. *in vivo*) and dose used: low doses inhibit, whereas high doses tend to excite 5-HT neurons [17-19]. In addition, CRF administration has been shown to increase, decrease, or have no effect on 5-HT release depending on the raphe projection area examined [20-23]. In the medial prefrontal cortex (mPFC), a brain area implicated in mood and anxiety disorders [24;25] and a major target of ascending 5-HT pathways [26], studies have shown an increase in 5-HT or 5-HT metabolites after administration of CRF [27;28]. Interestingly, the time course of CRF-induced 5-HT release in the mPFC (peak effects occurring 80 min after CRF infusion; [27]) is temporally similar to the behavioral effects of CRF on the acoustic startle response (i.e. potentiation) seen in many laboratories [29], including ours [30]. This observation suggests that time-dependent activation by CRF of raphe efferents to the mPFC may be an important component driving some types of anxiety-like behavior. To address this possibility, we used the novel approach of combining retrograde tracing with c-Fos immunohistochemistry (as a marker of neuronal activity [31;32]) to examine the effect of CRF on raphe efferents to the mPFC in behaving rats.

Materials and Methods

Animals

The animals were male Sprague-Dawley rats (Charles River, Raleigh, NC) weighing ~350 g and were housed in group cages of four rats each until surgery. Animals were maintained on a 12-hr light/dark cycle (lights on at 07.00h) with food and water continuously available.

Apparatus—The equipment used to measure the effect of CRF on the acoustic startle reflex is identical to that described elsewhere [30].

Surgery

FluoSphere deposits in the mPFC—Rats were anesthetized with Nembutal (50 mg/kg, intraperitoneally [ip]) and placed in a Kopf stereotaxic instrument (Model 900) with blunt ear bars. The skin was retracted and a hole was drilled in the skull above the mPFC. A stainless steel infusion cannula (30 gauge; **150 µm internal diameter**) attached to a Hamilton microsyringe (10 µl) by polyethylene tubing was lowered into the brain, and the infralimbic (IL) division of the mPFC was targeted using the following coordinates: +2.8 mm rostral to bregma, −0.6 mm lateral to the midline, −4.8 mm ventral to dura. A Harvard Apparatus (Model 22) infusion pump was used to deliver 0.2 µl of the FluoSpheres (FS, 0.04 µm diameter polystyrene beads; Molecular Probes, Eugene, OR), diluted 1:1 in 100 mM sodium citrate (pH 7.0) directly into the mPFC at a rate of .1 µl/min for 2 min. Following the injection, the infusion

cannula was left in place for 10 min to avoid leakage upon removal, and the skull hole was filled with sterile Gelfoam (Pharmacia & Upjohn, Kalamazoo, MI).

Intracerebroventricular (icv) cannulation—Following the FS deposit in the mPFC, a stainless steel guide cannula (23 gauge; Plastics One, Roanoke, VA) with an internal dummy stylet extending 1.5 mm beyond the guide cannula tip was lowered into the brain aimed at the lateral ventricle using the following coordinates: −0.8 mm caudal to bregma, + 1.3 mm lateral to the midline, −3.5 mm ventral to dura. Three stainless steel screws (size 0-80; Small Parts, Miami Lakes, FL) were also placed in the skull to anchor the guide cannula, and Loctite adhesive (Newington, CT) and dental acrylic (Stoelting, Wood Dale, IL) were used to cement the cannula in place. Animals were placed under a heating lamp, and after recovery the rats were singly-housed in plastic cages (45 × 24 × 20 cm) with wood-shaving bedding.

Procedure

Handling—One week after surgery, rats were given pre-test handling sessions on three consecutive days to familiarize them to the apparatus and the startle stimuli. These sessions also served to habituate c-Fos activation that occurs due to the stress of handling. To do this, rats were placed in the cages and given a 5-min acclimation period followed by the presentation of 100 startle stimuli at each of three different intensities (95, 100 and 105 dB) in a semirandom order with a 30-s interstimulus interval (ISI). We used the startle data acquired on these pre-test handling days (average across the three days) to match the animals so that the two treatment groups had nearly equivalent levels of baseline startle (Pre-CRF match; see Fig 1).

Testing—Twenty-four hours after the last pre-test handling day, rats were returned to the startle testing room and received icv infusions of either vehicle (artificial cerebrospinal fluid [aCSF]; Harvard Bioscience, Holliston, MA) or CRF (1 µg; American Peptide Company, Sunnyvale, CA) according to previously described methods [30]. Following icv infusion, rats were immediately placed in the startle cages and given a 5-min acclimation period followed by presentation of two habituating startle stimuli (100 dB, 30-s ISI). Rats were then presented with 300 startle stimuli at each of three different intensities (95, 100 and 105 dB) in a semirandom order with a 30-s ISI for a total test session of 150 min. Immediately following the end of the test session, animals were overdosed with pentobarbital (130 mg/kg, ip) and perfused intracardially with 0.9% saline (200 ml) followed by 4% paraformaldehyde (500 ml). The brains were removed and stored (4°C) for 3-4 days in a 30% sucrose/0.1M PBS (pH 7.4) solution.

Statistical analysis—Startle amplitude data were expressed as the mean averaged across the three startle-eliciting intensities across time. The effect of CRF on startle was evaluated using analyses of variance (ANOVA) with treatment group (Vehicle and CRF) as a between-subjects factor and blocks of time (3, 50-min blocks) as a within-subjects factor. Subsequent multiple comparisons for significant differences between the Vehicle and CRF groups at each block of time were made using Dunn's test.

Anatomical Studies

We used the nomenclature, delineation of structures, and stereotaxic reference system of Paxinos and Watson (1997) [33]. In addition, we used a neuroanatomic map of the DR adapted from Abrams et al., (2004) [34] to define rostral (−7.37 to −7.64), middle (−7.73 to 8.27) and caudal levels (−8.36 to −8.54) of the DR; numbers are in millimeters caudal to bregma. Although the map of Abrams et al., (2004) comprises many more sections through the DR (from −6.92 to −9.26 mm), we limited our analysis to those sections with the greatest densities of 5-HT neurons.

Retrograde labeling and c-Fos / tryptophan hydroxylase (TrpOH) immunohistochemistry—Please see Supplementary information for immunohistochemical methods.

Results

Behavior

Figure 1 illustrates the effect of icv CRF (1 μ g) on startle. A two-way ANOVA with treatment as a between-subjects factor and blocks of time (1-3) as a within-subjects factor revealed a significant main effect of treatment ($F_{(1,13)} = 14.8$; $p < 0.005$), block of time ($F_{(2,13)} = 3.9$; $p < 0.05$), and a significant treatment by block interaction ($F_{(2,26)} = 10.1$; $p < 0.005$). Multiple comparisons showed no significant differences between groups during the first 50-min block of time. Further comparisons showed that startle was significantly elevated in the CRF group compared to the vehicle group across the next two blocks of time ($p < 0.005$), with the peak effect occurring at approximately 60 min and lasting for the duration of the test session (full time course illustrated in Fig 1B). The behavior observed in this study is similar to that observed in previous studies from our laboratory [30], indicating that FS deposits in the mPFC had no untoward effects on the ability of CRF to potentiate the startle response.

Anatomy

CRF-induced c-Fos expression in the raphe—Vehicle-treated rats showed almost no c-Fos expression in the DR (Fig 2A) or MR (data not shown), likely due to handling the rats on multiple pre-test days. These data suggest that the icv infusion itself, or the presentation of the startle-eliciting stimuli, was not sufficient to induce c-Fos activation in the raphe in these well-handled rats. As such, we did not quantify the expression of c-Fos in FS-filled raphe efferents from this group. In contrast, CRF-treated rats showed heavy c-Fos expression throughout the DR (Fig 2B), and to a lesser extent, the MR. Because CRF-enhanced startle has a delayed onset to peak effect (~ 60 -80 min; see Fig 1B), and the c-Fos protein requires 60-90 minutes for maximal expression [35], sacrificing animals at 150 min after CRF infusion would reveal neuronal activation occurring during this peak behavioral effect.

FluoSphere deposits in the mPFC—FS deposits in the mPFC were primarily restricted to the infralimbic (IL) division; a representative image from one of these cases is shown in Fig 3A. The centers of the deposits were most often found in layer 5 of the IL with some mediolateral spread into layers 2-6 of cortex. The use of a fine gauge stainless steel cannula, to prevent clogging of the FS beads, most likely contributed to the relatively long dorsoventral extent of the deposit center. However, this had the favorable outcome of depositing the FluoSpheres in a narrow but long track throughout most of the dorsoventral extent of the IL. There was minimal spread of the tracer into the ventrally located dorsal peduncular cortex and negligible deposit artifact along the cannula track through the prelimbic (PL) and cingulate (Cg) subdivisions.

Retrograde labeling of raphe efferents to the mPFC and c-Fos activation by CRF—FS deposits restricted to the IL division of the mPFC retrogradely labeled cells throughout the rostrocaudal extent of the dorsal raphe (DR) nucleus. FS-filled neurons were found almost exclusively, and in roughly equal numbers, in the dorsal and ventral subdivisions of the DR (DRD and DRV respectively); very few cells were seen in the DR lateral wings. FS-filled neurons were also found throughout the median raphe (MR), but in fewer numbers and with much less intense labeling than that seen in the DR. The distribution of FS-filled neurons and those double-labeled for c-Fos, at different levels of the DR and MR from a representative case, is illustrated in Figure 3B. We do not believe that we have labeled a specialized population of DR neurons which project to the ventricle walls and have been shown to be able to take up

retrograde tracers that infiltrate the lumen of the ventricular system [see 36; 37]. If so, we would have expected to see pronounced bilateral labeling of DRD neurons [as shown in 36; 37], rather than the almost exclusively ipsilateral pattern of labeling of DRD neurons seen in the current study.

Table 1 shows the quantification of FS-filled neurons and those double-labeled for c-Fos, at different levels of the raphe, as well as the corresponding size/layer of the deposit center in the IL for each of the CRF-treated rats used in this study. Across animals, approximately 38% of all retrogradely labeled DR neurons were also positive for c-Fos immunolabel, and there was a clear topography of expression; the percent number of FS-filled neurons that were also c-Fos positive increased from the rostral (23%) to middle (38%) to caudal (53%) levels of the DR. Double-labeled neurons were also seen in the MR, but the overall percent of FS-filled cells that were also positive for c-Fos (23%) was less than that seen in the DR. Like the DR, caudal sections of the MR tended to have a higher percentage of double-labeled cells than rostral sections. A representative image of FS-filled cells, c-Fos positive cells, and double-labeling of these cell populations at mid-level through the DR is shown in Fig 4A-D. As shown in Table 2, over 90% of FS-filled neurons in the DR and MR were immunopositive for TrpOH (representative images from the DR are shown in Fig 4E-F), suggesting that the majority of raphe efferents to the IL are from 5-HT-containing neurons.

One of the main findings of this study was a significant correlation between the level of CRF-enhanced startle (measured as % change from Pre-CRF match test) and the degree of activation (measured as % number of FS-filled cells that also expressed c-Fos) of neurons in the caudal part of the DR. As shown in Figure 5, there was a trend for a positive relationship between the level of CRF-enhanced startle and the level of overall activation in the DR ($r^2 = .37$, $p = .10$). An examination of this relationship at different levels of the DR showed that this correlation was only significant for activation in the caudal DR ($r^2 = .5$; $F(1,6) = 6.07$; $p < .05$). There was no obvious relationship between the level of CRF-enhanced startle and activation of MR efferents to the mPFC.

The data from Table 1 show a positive relationship between the size of the deposit in the IL and the overall number of FS-filled cells in the DR, but not the MR, indicating that a bigger deposit in the IL produced a higher number of retrogradely labeled cells in the DR (see Supplementary Data Fig 1A). Despite this relationship, there was no significant correlation between the size of the deposit (and the accompanying increase in the number of retrogradely-labeled cells) and the percentage of FS-filled cells in the DR that are activated by CRF (Supplementary Data Fig 1B). Thus, we believe that the significant correlation between activation of caudal DR efferents to the mPFC and CRF-enhanced startle is not confounded by an inflated/deflated retrograde cell count due to the size of the deposit. To address this further, we examined the relationship between activation of caudal DR neurons and CRF-enhanced startle in a subset of CRF-treated rats ($n=5$) with roughly equivalent deposit sizes and levels of total retrograde tracing. These animals had between 134 and 160 total FS-filled cells, and between 26 and 40 FS-filled cells in the caudal DR, as indicated in Table 1 (rat id#s: C2-5, C2-7, C2-2, C2-9 and C2-4). In this subset of animals, there was still a strong positive relationship between the percent activation of caudal DR neurons and CRF-enhanced startle ($r^2 = .631$; $F(1,3) = 5.1$; $p < .11$; see Supplementary Fig. 2) although the correlation was not significant likely owing to the reduced power of the analysis (i.e. fewer observations).

Discussion

Using c-Fos immunohistochemistry as a marker of neuronal activity, the results of the present study show that icv CRF activates a population of raphe neurons that project to the mPFC. Based on our double labeling studies of FS-filled neurons with TrpOH, the enzyme involved

in the production of 5-HT, our data suggest that the majority of CRF-activated raphe efferents to the IL division of the mPFC are serotonergic. FS-filled neurons were found at all levels of the DR, and in roughly equal numbers in two of the major subdivisions of the DR (i.e. the DRD and DRV). However, CRF-induced activation of DR neurons showed a clear rostral-caudal topography, with a progressive increase in the percent activation of raphe-mPFC projecting neurons from rostral to middle to caudal levels of the DR. There was also a significant correlation between the level of CRF-enhanced startle and the degree of activation of DR efferents to the mPFC (caudal division only). These data suggest that the level of anxiety-like behavior induced by CRF may be controlled by specific 5-HT circuits originating in the caudal parts of the DR, and projecting to the ventral mPFC. Interestingly, this CRF effect on topographically organized subpopulations of 5-HT neurons closely resembles the pattern of c-Fos activation seen in the DR after administration of other anxiogenic drugs such as m-chlorophenyl piperazine (mCPP), yohimbine, and FG-7142 [34]. Furthermore, our data overlap with the finding that neurons in these mid-to-caudal levels of the DR also have projections to other brain areas known to regulate anxiety-like behaviors, such as the basolateral amygdala, and support the hypothesis that subpopulations of 5-HT neurons are involved in functional anxiety circuits [38].

The simplest explanation to account for the effect of exogenously applied CRF on raphe neurons is through a direct action on CRF receptors located on DR and MR neurons. While immunohistochemical studies have shown that CRFergic innervation of the DR is high [39], and has been shown to make direct synaptic contact with both 5-HT and GABA-containing neurons in the DR [40], it is somewhat surprising that levels of CRF-1 receptors (the primary receptor through which CRF acts), and mRNA for this receptor, are quite low in the DR [41; 42]. Instead, mRNA for the CRF-2 receptor is found in relatively high levels in the DR, with moderate levels of both CRF-1 and CRF-2 receptor mRNA found in the MR [43]. Although CRF can act at CRF-2 receptors, these observations suggest that CRF-related peptides, such as the urocortins (Ucn, including Ucn1, Ucn2 and Ucn3), which bind with high affinity to the CRF-2 receptor [44;45], may play a prominent role in modulating the activity of raphe neurons. This idea is supported by the finding that intra-DR administration of a sufficiently high dose of Ucn2 can increase the activity of DR 5-HT neurons [18], increase c-Fos expression in these neurons, and increase 5-HT efflux in DR projection areas [46]. Likewise, icv administration of Ucn2 has been shown to increase c-Fos expression in DR 5-HT neurons with a topography remarkably similar to that seen in the present study using CRF [47]. Thus, while direct activation of DR neurons by CRF would be the most straightforward mechanism to account for our data, indirect activation through other CRF-modulated neurotransmitter systems, such as the urocortin system, is also a possibility [48].

The activation of serotonergic neurons in the DR by CRF, as indicated by c-Fos expression, would presumably lead to an increase in 5-HT release in mPFC. Although the majority of raphe efferents to the mPFC were not c-Fos positive, indicating a lack of activation (or inhibition) by CRF in this population of raphe neurons, we cannot determine what the net effect on 5-HT release in the mPFC would be, given the ratios of activated vs. non-activated neurons seen in the present study. However, microdialysis studies have shown that both direct application of CRF into the DR, as well as icv CRF, can significantly increase 5-HT and 5-HT metabolite levels in the mPFC [27;28]. Interestingly, the results of Forster et al. (2006) show that CRF-induced 5-HT release in the mPFC has a gradual onset, with a peak effect occurring approximately 80 min after CRF administration. Although the mechanisms underlying this delayed effect of CRF on 5-HT release in the mPFC are unknown, it is relevant to note that the time-course of this physiological response closely mirrors the behavioral effect of CRF on startle. As shown consistently in a number of reports, the effects of CRF on startle are not immediate, rather, the startle-enhancing effects of CRF (1 µg) begin approximately 30 min after infusion, grow steadily to a peak effect at 60-80 min, and remain elevated for 2-3 hrs at

which time they begin to return to baseline [30;49]. Because CRF in these behavioral studies was usually given icv, it is unknown if these delayed behavioral effects were due to the amount of time required for CRF to infiltrate the brain parenchyma from the ventricular system, or was a function of time-dependent recruitment by CRF of other neurotransmitter systems that were directly responsible for the potentiated response. The results of the present study, in association with the results of Forster et al., (2006), raise the intriguing possibility that CRF-induced activation of specific 5-HT inputs to the mPFC is a rate-limiting step in the expression of anxiety-like behaviors such as CRF-enhanced startle.

What then might be the effect of an increase in 5-HT on mPFC neurons, and how might that be translated into the generation of anxiety-like behaviors? The results from several studies have shown that electrical stimulation of either the DR or MR inhibits a majority of pyramidal neurons in the mPFC [50-52], an effect likely mediated by 5-HT, as the inhibition was reduced by destruction of ascending 5-HT pathways and by systemic administration of 5-HT_{1A} receptor antagonists. Other electrophysiological studies have also demonstrated inhibition of mPFC pyramidal neurons after direct application of 5-HT *in vivo* [53-55], although excitatory effects of 5-HT on pyramidal neurons have also been seen *in vitro* [56]. Possible mechanisms for the inhibitory effects of 5-HT on mPFC pyramidal neurons include direct effects, mediated by 5-HT_{1A} receptors, or indirect effects, mediated through activation of GABAergic interneurons via 5-HT_{2A} and/or 5HT-3 receptors [57-59]. Interestingly, in other studies from our laboratory, we have noticed a conspicuous paucity of c-Fos positive cells in the IL and PL subdivisions of the mPFC 150 min after icv CRF administration (Meloni and Carlezon, unpublished results). These observations suggest that the majority of cells in the ventral mPFC, of which pyramidal neurons are the major cell type, are either **not involved in CRF-mediated effects** (i.e. their cellular activity is not changed), or are actively inhibited as a result of icv CRF administration and putative 5-HT release from raphe afferents. Unfortunately, the use of c-Fos alone cannot distinguish between these two possibilities and future studies are planned to examine the effect of direct intra-mPFC injections of 5-HT agonists/antagonists on CRF-enhanced startle. Although our data show a significant correlation between caudal DR activation and CRF-enhanced startle, it is entirely possible that enhanced 5-HT release in the mPFC may be playing an adaptive role involved in the recovery from stress and anxiety [60;61]. Hence, these future studies will allow us to better determine if 5-HT release in the mPFC is a mechanism by which CRF elicits its anxiogenic-like behavioral effects, or is involved in the recovery from this heightened anxiety-like state.

5-HT-mediated alterations in the descending excitatory drive from pyramidal neurons in the mPFC to limbic areas such as the extended amygdala (e.g. the central nucleus of the amygdala [CeA] and bed nucleus of the stria terminalis [BNST]) would have profound effects on emotionality. For example, there is strong evidence to suggest that the input from the ventral mPFC to the CeA is an important axis for controlling the extinction of conditioned fear [62]. Furthermore, dysfunction within this particular anxiety-control circuit may underlie the development and/or expression of PTSD [63], a disorder in which CRF has been implicated [64;65]. Although not yet fully explored, dysregulation of the strong descending input from the IL to the BNST [66], an area known to play a role in the expression of unconditioned anxiety-like behaviors [67], including CRF-enhanced startle [29], could represent another axis of pathology for anxiety disorders. Further studies are clearly needed to help elucidate the functional interactions between CRF and 5-HT occurring with raphe-cortical-limbic circuits. Such studies have the potential to identify new targets and new therapies in the treatment of mood and anxiety disorders.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Effect of CRF on startle

(A) Animals were matched into two groups (Vehicle, $n = 7$; CRF, $n = 8$) having equivalent levels of baseline startle (Pre-CRF match). Twenty-four hours later, animals received icv infusions of either vehicle (artificial cerebrospinal fluid, aCSF) or CRF ($1 \mu\text{g}$), were tested for startle, and then sacrificed 150 min later in order to examine the level of CRF-induced c-Fos expression in raphe efferents to the mPFC. Data are collapsed across blocks of 50 min for statistical comparisons; (B) Full timecourse of the effect of CRF on startle. ** $p < 0.005$, *** $p < 0.0005$ compared to Vehicle group.

Figure 2.

Representative coronal brain sections through the dorsal raphe (DR) demonstrating the lack of c-Fos expression in vehicle-treated rats (A) compared with CRF-treated (B) rats. For this comparison, c-Fos immunohistochemistry was performed according to previously described methods [68]. DRD, dorsal raphe, dorsal; DRV, dorsal raphe, ventral; Aq, cerebral aqueduct. Scale bar, 100 μ m

Figure 3.

(A) Coronal brain section showing discrete deposit of retrograde tracer (red FluoSpheres; FS) in the infralimbic division (IL) of the mPFC. The fluorescent image was overlaid onto an image from the same section after cresyl violet staining to determine the extent of the tracer deposit, and transcribed onto an atlas plate from the atlas of [33]. (B) Representative illustration showing the distribution of FS-filled cells (open circles), and FS-filled cells that were c-Fos positive after CRF infusion (filled circles), at different rostro-caudal levels through the dorsal raphe (DR) and median raphe (MR) in one of the animals used in this study. Numbers are in millimeters relative to bregma. DR subdivisions: dorsal (DRD), ventral (DRV), lateral (DRLV). PMR, paramedian raphe nucleus; Aq, cerebral aqueduct; Cg, cingulate cortex; DP, dorsal penduncular cortex; PrL, prelimbic cortex. Scale bar, 500 μ m.

Figure 4.

(A) Coronal section through the dorsal raphe (dorsal and ventral division; DRD and DRV respectively) showing FS-filled cells (marked with arrowheads) labeled after deposit of retrograde tracer (red FluoSpheres; FS) in the mPFC (see Fig 3). (B) c-Fos-positive cells (indicated by green fluorescence in nucleus of cells) showing activation by CRF in same section as that shown in A. (C) Merged image of A and B showing c-Fos activation in many FS-filled cells; double-labeled cells are marked by asterisks in arrowheads. (D) High magnification of section outlined in C showing FS-filled cells double-labeled for c-Fos. Arrowheads with asterisks mark DR efferents to the mPFC activated by CRF. (E & F) To determine if FS-filled cells projecting from the raphe to the mPFC were 5-HT-containing neurons, alternate sections were processed for tryptophan hydroxylase (TrpOH) immunohistochemistry. The majority of FS-filled cells (marked with arrowheads in E) were also positive for TrpOH; asterisks in F denotes FS+/TrpOH+ neurons. Scale bars: A-C, E-F, 50 μm; D, 25 μm

Figure 5.

Correlations between CRF-enhanced startle (collapsed across blocks of time and expressed as percent difference from Pre-CRF match) and the level of c-Fos activation in raphe efferents to the mPFC. There was a trend for a positive relationship between the level of CRF-enhanced startle and the amount of c-Fos activation at different levels through the DR (filled diamonds, solid trend-line), however, this correlation was only significant for activation in the caudal DR ($r^2 = 0.5$; $p < 0.05$). A relationship between the level of CRF-enhanced startle and c-Fos activation in MR efferents to the mPFC was less obvious (open squares, dashed trend-line).

Table 1

Quantification of FluoSphere-filled cells (FS+) and those double labeled for c-Fos in the raphe after FS deposit in the mPFC

Rat id# ^a	C2-5	C2-1	C2-6	C2-7	C2-2	C2-9	C2-4	C2-3	Average ±SE
% CRF-enhanced startle ^b	304%	187%	107%	106%	76%	54%	46%	31%	114% ± 32
mPFC (IL) deposit center size (area in μm ²)/layer	379/ IV	497/ V	396/ VI	343/ III	319/ V	354/ III	388/ V	425/ V	
Dorsal Raphe (DR)									
# FS+ cells:									
rostral	32	53	53	19	35	52	42	47	42 ± 4
mid	68	140	134	78	77	72	79	140	99 ± 12
caudal	34	79	42	40	26	36	32	95	48 ± 9
total	134	272	229	137	138	160	153	282	188 ± 22
# FS+ cells expressing c-Fos:									
rostral	10	15	16	5	4	8	10	9	10 ± 1
mid	31	64	54	16	45	18	30	40	37 ± 6
caudal	23	40	25	21	16	16	15	37	24 ± 3
total	64	119	95	42	65	42	55	86	71 ± 10
% labeled DR→mPFC cells activated by CRF:									
rostral	31%	28%	30%	26%	11%	15%	24%	19%	23% ± 3
mid	46%	46%	40%	21%	58%	25%	38%	29%	38% ± 4
caudal	68%	51%	60%	53%	62%	44%	47%	39%	53% ± 3
total	48%	44%	41%	31%	47%	26%	36%	30%	38% ± 3
Median Raphe (MR)									
# FS+ cells:									
rostral	7	23	19	23	21	74	33	32	29 ± 7
mid	39	42	42	82	31	53	73	83	56 ± 7
caudal	13	16	8	35	14	23	20	40	21 ± 4
total	59	81	69	140	66	150	126	155	106 ± 14
# FS+ cells expressing c-Fos:									
rostral	1	2	5	5	7	18	5	4	6 ± 2
mid	13	2	16	23	4	16	10	11	12 ± 2
caudal	6	0	3	14	5	5	5	12	6 ± 2
total	20	4	24	42	16	39	20	27	24 ± 10
% labeled MR→mPFC cells activated by CRF:									
rostral	14%	9%	26%	22%	33%	24%	15%	13%	20% ± 3
mid	33%	5%	38%	28%	13%	30%	14%	13%	22% ± 4
caudal	46%	0%	38%	40%	36%	22%	25%	30%	30% ± 5
total	34%	5%	35%	30%	24%	26%	16%	17%	23% ± 3

^a Animals are ranked by highest to lowest level of CRF-enhanced startle.

^b Values are collapsed across blocks of time and expressed as percent difference from Pre-CRF match.

Table 2

Quantification of FluoSphere-filled cells (FS+) and those double labeled for tryptophan hydroxylase (TrpOH) in the raphe after FS deposits in the mPFC

Brain area	# FS+ cells	# FS+ cells expressing TrpOH	% Raphe→ mPFC cells that contain 5-HT
DR			
rostral	34 ±4	30 ±3	90% ±3
mid-level	75 ±11	67 ±10	90% ±3
caudal	36 ±5	34 ±5	95% ±1
total	144 ±15	131 ±14	92% ±2
MR			
rostral	36 ±8	34 ±8	94% ±3
mid-level	57 ±8	52 ±8	91% ±1
caudal	23 ±4	19 ±5	80% ±8
total	115 ±15	105 ±14	91% ±1

DR, dorsal raphe; MR, median raphe