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Corticotropin-releasing factor 1 and 2 receptors in the dorsal raphé differentially affect serotonin release in the nucleus accumbens

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Abstract

Corticotropin-releasing factor (CRF) is a neurohormone that mediates stress, anxiety, and affects serotonergic activity. Studies have shown that CRF has dose-dependent opposing effects on serotonergic activity. This effect has been hypothesized to be differentially mediated by CRF₁ and CRF₂ receptors in the dorsal raphé nucleus. We directly tested this hypothesis by using *in vivo* microdialysis to determine the effects of CRF and CRF antagonists in the dorsal raphé nucleus on serotonin (5-HT) release in the nucleus accumbens, a brain region implicated in the neuropathology of stress-related psychiatric disorders. Male urethane-anesthetized rats were implanted with a microdialysis probe into the nucleus accumbens, and CRF (0, 100 or 500 ng) was infused into the dorsal raphé. Infusion of CRF into the dorsal raphé nucleus had dose-dependent opposite effects, with 100 ng of CRF significantly decreasing 5-HT levels in the nucleus accumbens and 500 ng CRF significantly increasing accumbal 5-HT levels. In subsequent experiments, the raphé was pre-treated with the CRF₁ receptor antagonist antalarmin (0.25 µg) or the CRF₂ receptor antagonist antisauvagine-30 (ASV-30; 2 µg) prior to CRF infusion. Antagonism of CRF₁ receptors in the dorsal raphé nucleus abolished the decrease in accumbal 5-HT levels elicited by 100 ng CRF, and CRF₂ receptor antagonism in the raphé blocked the increase in accumbal 5-HT levels elicited by 500 ng CRF. These results suggest that the opposing effects of dorsal raphé CRF on 5-HT release in the nucleus accumbens are dependent on differential activation of CRF₁ and CRF₂ receptors in the dorsal raphé nucleus.

Keywords

microdialysis; antalarmin; antisauvagine-30; CRF receptors; stress

1. Introduction

The nucleus accumbens has been the focus of numerous studies on reward and motivation, and exhibits increased dopaminergic activity during both rewarding and aversive conditions.

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However, these dopaminergic changes in the nucleus accumbens are modulated by other transmitter systems including the serotonergic and corticotropin-releasing factor (CRF) systems (Amato et al., 2006; Bland et al., 2004; Izzo et al., 2005). For instance, CRF in the nucleus accumbens has been shown to be involved in arousal and amplifying motivated behaviors (Holahan et al., 1997; Pecina et al., 2006). Serotonergic innervation of the nucleus accumbens originates from the lateral wings of the dorsal raphé nucleus (Van Bockstaele et al., 1993). Serotonin in the nucleus accumbens is influenced by stress and social interaction (Summers et al., 2003b) and decreased activity of 5-HT in the nucleus accumbens may be associated with impulsivity (Coccaro, 1992; Winstanley et al., 2005). Thus, examining the effects of CRF in the dorsal raphé nucleus on 5-HT release in the nucleus accumbens may help elucidate the roles of CRF and 5-HT in the primary accumbal functions such as reward, motivation, impulsivity, and their psychopathologies.

Corticotropin-releasing factor plays an important role in integrating multiple components of the stress response. Exposure to a stressor increases central CRF levels (Merali et al., 1998; Merlo et al., 1995), while the behavioral effects of administering CRF to rats resemble those induced by stressors (Dunn and Berridge, 1990a; Dunn and Berridge, 1990b; Harvey and Hennessy, 1995; Koob and Bloom, 1985). The behavioral effects induced by CRF are thought to be mediated, in part, by CRF actions on 5-HT systems within the brain (Forster et al., 2006; Hammack et al., 2002; Kirby et al., 2000), via activation of CRF₁ and CRF₂ receptors (Radulovic et al., 1998; Radulovic et al., 1999). Experimental evidence suggests that activation of the CRF₁ receptor subtype is involved in the initiation of HPA responses to stress (Bale et al., 2002; Risbrough et al., 2003; Smith et al., 1998). Blockade of these receptors results in a reduction of the CRF-potentiated acoustic startle response along with startle elicited by fear-inducing stimuli (Schulz et al., 1996). In addition, CRF₂ receptors appear to contribute to some of the anxiogenic functions of CRF, because antagonism of CRF₂ receptors decreases conditioned freezing and acoustic startle behaviors (Bakshi et al., 2002; Takahashi et al., 2001). Furthermore, CRF-mediated increases in conditioned freezing appear to be mediated by CRF₂ receptors located in the 5-HT cell body region of the dorsal raphé nucleus (Hammack et al., 2003b).

The dorsal and median raphé nuclei are major sources of 5-HT innervation of forebrain and limbic regions (Jacobs and Azmitia, 1992), and receive CRF projections from the amygdala (Gray, 1993). Serotonin is involved in neuronal processes related to conflict behavior, inhibitory control and impulsivity as well as reward-related mechanisms (Evenden and Ryan, 1996; LeMarquand et al., 1994a; LeMarquand et al., 1994b; Roy and Linnoila, 1988; Soubrie, 1986). Changes in limbic 5-HT activity are also associated with stressors, and decreased central 5-HT function is implicated in the symptoms of affective disorders (Carrasco and Van de Kar, 2003; Deakin, 1998; Millan, 2003). Corticotropin-releasing factor in the dorsal raphé nucleus is known to affect serotonergic activity (Forster et al., 2006; Kirby et al., 2000; Price et al., 1998; Price and Lucki, 2001; Valentino et al., 2001). Previous studies have shown that both CRF₁ and CRF₂ receptors have been detected in the dorsal raphé nucleus (Commons et al., 2003; Funk et al., 2003; Van Pett et al., 2000) and may have opposing roles for 5-HT release (Kirby et al., 2000; Pernar et al., 2004; Valentino et al., 2001). Corticotropin-releasing factor has a higher affinity for CRF₁ receptors when compared to CRF₂ receptors (Grigoriadis et al., 1996a; Grigoriadis et al., 1996b), and activation of these receptors normally inhibits serotonergic activity in the dorsal raphé (Hammack et al., 2003b; Pernar et al., 2004). In contrast, higher levels of CRF are believed to be required for CRF₂ receptor activation, and activation of these receptors normally facilitates serotonergic activity in dorsal raphé (Kirby et al., 2000; Pernar et al., 2004). Combined, these studies suggest that CRF has opposing effects in the dorsal raphé nucleus that depend on CRF₁ and CRF₂ receptor activation based on the dosage of CRF. If this is the case, then dose-dependent CRF activation of CRF₁ and CRF₂ receptors in the dorsal raphé nucleus should influence 5-HT release in limbic terminal sites,

such as the nucleus accumbens, and thereby influence behavior. This hypothesis has not been directly tested in relation to the nucleus accumbens.

Since CRF in the dorsal raphe nucleus is thought to affect 5-HT activity in an opposing manner, we hypothesized that infusion of high and low doses of rat-human CRF into the dorsal raphe nucleus would have contrasting effects on 5-HT release in the nucleus accumbens.

Furthermore, we hypothesized that the opposing effects of CRF on accumbal 5-HT release would be a function of opposing effects of CRF₁ and CRF₂ receptor activation in the dorsal raphe nucleus.

2. Materials and methods

2.1. Animals

Ninety-six male Sprague-Dawley rats (University of South Dakota Laboratory Animal Services, Vermillion, SD, USA) weighing between 250 and 350 g were housed in pairs and maintained at a constant room temperature (22 °C, 60% relative humidity) and a reverse 12h light: 12h dark cycle. Food and water were available *ad libitum*. The following 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. All efforts were made to minimize the number of animals used and their suffering.

2.2. Microdialysis and pharmacological experiments

Rats were anesthetized with urethane (1.8 g/kg i.p; Sigma, St. Louis, MO) and placed in a stereotaxic frame (David Kopf Institute, CA, USA) with the incisor bar set at -3.3 mm. Body temperature was maintained at $37 \pm 0.5^\circ\text{C}$ with a temperature regulated heating pad (CMA, North Chelmsford, MA). A concentric microdialysis probe (membrane length 2 mm, with MW cut-off 5000; (Hoffman et al., 2002)) was inserted into the nucleus accumbens (AP: +1.2 from bregma; ML: -1.4 from midline; DV: -8.1 from dura; (Paxinos and Watson, 1997)). For CRF alone and control infusions (CRF receptor antagonists and vehicles for each antagonist alone) into the dorsal raphe nucleus, a single stainless-steel infusion guide cannula (26 gauge) was implanted 2 mm above the dorsal raphe nucleus (AP: -7.4 from bregma; ML: +2.8 from midline; DV: -4.6 from dura) at a 26° lateral to medial angle to avoid the cerebral aqueduct (Forster et al., 2006; Paxinos and Watson, 1997). For the CRF antagonist with CRF infusion experiments, two 26 gauge stainless steel guide cannulae were fixed together in such a way that the one cannula was immediately behind the other and these cannulae were implanted into the dorsal raphe nucleus as described for the single guide cannula.

Artificial cerebrospinal fluid (aCSF; Moghaddam and Bunney, 1989) was perfused continuously through the probe at a rate of 0.4 $\mu\text{l}/\text{min}$ with a microinfusion pump (CMA) via PE-20 tubing connected to a 1 ml syringe. Three hours after probe insertion, silica cannulae (194 μm od, 2 mm longer than guides; Polymicro Technologies, Phoenix, AZ) were lowered through cannulae. Drug cannulae were fixed to PE-20 tubing connected to a 10 μl Hamilton syringe, and the drug or vehicle was back-loaded into the cannula before implantation as described previously (Forster and Blaha, 2000). Microdialysis sampling began 4 hours after the implantation of the probe (Forster et al., 2006), with perfusates collected at 20 min intervals and analyzed for 5-HT. For the first experiment, following at least 3 stable baseline 5-HT samples, either aCSF (0.5 μl ; vehicle for CRF) or rat-human CRF (100 or 500 ng; 0.5 μl ; Sigma-Aldrich, St. Louis, MO) was infused through a single cannula directed at the dorsal raphe nucleus. As the natural ligand for rat CRF receptors was used for these experiments, i.e. r/hCRF, from this point we will refer to this form of the neuromodulator as CRF, indicating oCRF for comparison to studies using ovine CRF.

Treatment for the second experiment consisted of (1) infusing antalarmin (0.25 µg/0.5 µl; Sigma-Aldrich), a CRF₁ receptor antagonist, into the dorsal raphé nucleus alone or 10 minutes prior to infusion of CRF (100 ng or 500 ng/0.5 µl) into the dorsal raphé nucleus; or (2) infusing antisauvagine-30 (2 µg/0.5 µl; Sigma-Aldrich), a CRF₂ receptor antagonist, into the dorsal raphé nucleus alone or ten minutes before CRF (100 ng or 500 ng/0.5 µl) into the dorsal raphé nucleus. Vehicle treatments for the second experiment consisted of infusing (1) 5% ethanol and 5% camphor in aCSF (0.5 µl; vehicle for antalarmin) into the dorsal raphé nucleus either alone or 10 minutes prior to the infusion of 100 ng/0.5 µl CRF into the dorsal raphé nucleus; or (2) 2% ethanol in aCSF (0.5 µl; vehicle for antisauvagine-30) either alone or ten minutes before 500 ng/0.5 µl CRF into the dorsal raphé nucleus. All drugs and vehicle solutions were delivered at a rate of 0.5 µl/min using a microinfusion pump (Stoelting, Wood Dale, IL). The doses of CRF were determined from previous studies demonstrating CRF effects on 5-HT activity (Amat et al., 2004; Forster et al., 2006; Kirby et al., 2000; Price et al., 1998). Doses of receptor antagonists were derived from studies demonstrating effective antagonism of CRF receptors as indicated by the drug induced blockade of CRF-dependent effects (Amat et al., 2004; Hammack et al., 2003b; Hammack et al., 2003a; Le et al., 2002). Sampling of 5-HT in perfusates was continued until 5-HT activity returned to pretreatment levels.

2.3. Serotonin analysis

Analysis of 5-HT in dialysates was accomplished by using high-performance liquid chromatography (HPLC) with electrochemical detection (Bradberry et al., 1991; Hoffman et al., 2002). Samples were injected into a chromatographic system with a 5 µl loop. The mobile phase used for 5-HT separation (0.3 g EDTA, 0.432 g of 1-octanesulfonic acid, 4.8 g NaH₂PO₄, 120 ml acetonitrile, 200 µl triethylamine per 1 L, pH 5.35) was pumped through a UniJet 3 µm C₁₈ silica column (Bioanalytical Systems, West Lafayette, IN) under nitrogen gas pressure (2000 psi). The collection rate of 0.4 µl/min resulted in approximately 8 µl of dialysate to insure 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) maintained at +0.5 V with respect to the Ag/AgCl₂ reference electrode with a LC-4C potentiostat (Bioanalytical Systems). The voltage output was recorded by CSW32 v1.4 Chromatography Station for Windows (DataApex, Prague, Czech Republic). Serotonin 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 0.08 ± 0.04 pg.

2.4. Histology

At the conclusion of these experiments, rats were administered a lethal dose of Fatal-plus (Vortech, Dearborn, MI, 0.5 ml, ip.). Brains were removed and fixed in 10% formalin. Sections (60 µm) were cut using a Leica Jung CM1800 cryostat (Wetzlar, Germany) at -12 °C, and analyzed under a light microscope to confirm appropriate placements of microdialysis probes and drug infusion cannulae. Only data from rats with correct probe and cannulae placements were included in the data analyses (n=4-9 per group).

2.5. Data Analysis

Serotonin levels are expressed as a percentage change (±SEM) from baseline levels where the baseline level is determined by averaging the 3 baseline measurements. Significance levels for all statistical tests were set at P<0.05 (SigmaStat v2.03). The effects of CRF or CRF receptor antagonists in the dorsal raphé nucleus on NAc 5-HT concentrations were analyzed using a two-way ANOVA (treatment × time) with repeated measures where the repeated factor was time. In analyses where a significant treatment × time interaction was present, effects of treatment were analyzed further using one-way repeated measures ANOVA. Comparisons between drug and control treatments at a given time were analyzed using Student Newman-

Keuls posthoc analysis. The effects of treatments across time were analyzed using one-way repeated measures ANOVA in analyses where a significant treatment \times time interaction was present. The effects of treatment over time were then compared using the Dunnett's test where the sample immediately preceding treatment was used as the control 5-HT value.

3. Results

3.1. Probe and injection placement

The placement of microdialysis probes ensured that the 2 mm length of dialysis membrane sampled 5-HT from both the nucleus accumbens shell and core (Figs. 1A, 1C, and 1E). Drug infusion cannulae were located in the mid to posterior aspect of the dorsal raphe nucleus (Figs. 1B, 1D, and 1E). The area of the dorsal raphe nucleus infused has been shown to provide 5-HT innervation to the nucleus accumbens (Van Bockstaele et al., 1993). Although infusions into the dorsal raphe nucleus were close to the cerebral aqueduct, cannulae were placed on a lateral to medial angle to minimize the possibility of diffusion into the cerebral aqueduct (Forster et al., 2006). Probe placements outside of the dorsal raphe nucleus were used for anatomical control (Figs. 1B).

3.2. Effects of CRF infusions into the dorsal raphe on 5-HT levels in nucleus accumbens

Infusion of CRF into the dorsal raphe nucleus had opposing effects depending on dose on extracellular 5-HT levels in the nucleus accumbens (Fig. 2A). There was a significant effect of CRF treatment ($F_{2, 165} = 3.53, P < 0.05$) and a significant treatment \times time interaction ($F_{16, 165} = 8.85, P < 0.001$). Infusion of 100 ng of CRF produced a significant effect on accumbal 5-HT over time ($F_{8, 55} = 6.605, P < 0.001$). Serotonin decreased in the nucleus accumbens in the first twenty minutes following 100 ng CRF infusion into the dorsal raphe nucleus when compared to pretreatment 5-HT levels (Dunnett's $q_{55, 9} = 5.86, P < 0.05$). There was also a significant effect on 5-HT in the nucleus accumbens in response to the infusion of 500 ng CRF into the dorsal raphe nucleus ($F_{8, 55} = 7.378, P < 0.001$). In the first sample collected from the nucleus accumbens following the infusion of 500 ng CRF into the dorsal raphe nucleus, 5-HT was increased approximately 55% over baseline values (Dunnett's $q_{55, 9} = 5.78, P < 0.05$). When CRF treatments were compared within each time point, a significant effect was found at 20 min post treatment ($F_{2, 21} = 21.45, P < 0.001$). The decrease in accumbal 5-HT levels observed after the infusion of 100 ng into the dorsal raphe nucleus was significant when compared to both aCSF (SNK $P = 0.02$) and 500 ng (SNK $P < 0.001$) CRF infusions. In contrast, when 500 ng CRF was infused into the dorsal raphe, a significant increase was evident at 20 min when compared to aCSF (SNK $P = 0.001$).

A few cannulae for infusion of CRF did miss the dorsal raphe nucleus, resulting in CRF delivery to regions outside, but close to, the serotonergic cells of interest (Figs. 1B). Infusion of CRF outside the dorsal raphe nucleus did not affect accumbal 5-HT release (Fig. 2B). This suggests that although the CRF delivered to the dorsal raphe nucleus is contained in a large volume (0.05 μ l), the effects are specific to the dorsal raphe as no influence of CRF on 5-HT overflow were observed when CRF was infused outside the dorsal raphe nucleus.

3.3. Effects of CRF receptor antagonists in dorsal raphe on accumbal 5-HT

Prior infusion of the CRF₁ receptor antagonist, antalarmin, into the dorsal raphe nucleus blocked the 100 ng CRF-induced decrease in accumbal 5-HT levels (Fig. 3). There was a significant effect of time ($F_{9, 216} = 3.85, P < 0.001$) and treatment \times time interaction ($F_{45, 216} = 2.61, P < 0.001$). Consistent with the results from experiment one, infusion of 100 ng CRF in the dorsal raphe following infusion of the vehicle, resulted in a significant decrease in accumbal 5-HT levels at 20 min ($F_{9, 45} = 5.76, P < 0.001$; Dunnett's $q_{45, 10} = 5.97, P < 0.05$). Similarly, infusion of 100 ng CRF into the dorsal raphe nucleus following infusion of the

CRF₂ receptor antagonist resulted in a significant decrease in accumbal 5-HT levels at 20 min ($F_{9,36} = 36.24$, $P < 0.001$; Dunnett's $q_{36, 10} = 13.11$, $P < 0.05$). In the group treated with the CRF₁ receptor antagonist antalarmin, there was a trend towards an effect across time ($F_{9, 45} = 5.76$, $P = 0.07$). Furthermore, the comparison of the CRF response elicited with antalarmin compared to vehicle at 20 min post infusion showed antalarmin pretreatment effectively blocked the 100 ng CRF effect on 5-HT in the nucleus accumbens ($P = 0.004$). When comparing between groups within each time point, there were significant differences between treatment groups at post 20 ($F_{5, 23} = 7.034$, $P < 0.001$), post 100 ($F_{5, 23} = 3.753$, $P = 0.012$), and post 120 ($F_{5, 23} = 5.398$, $P = 0.002$). At 20 min post infusion, antalarmin alone (SNK=0.007), vehicle for antalarmin alone (SNK = 0.012), aCSF (SNK= 0.008), and infusion of antalarmin 10 min prior to the infusion of 100 ng CRF (SNK= 0.005) were all significantly different from the infusion of either vehicle or ASV-30 prior to the infusion of 100 ng CRF. There was a gradual, unexplained decline in baseline after the infusion of antalarmin 10 min prior to the infusion of 100 ng CRF into the dorsal raphé, resulting in significant differences at post 100 and post 120 from all other treatment groups (SNK $P < 0.05$). No effect on accumbal 5-HT levels over time were observed after the infusion of antalarmin ($F_{8, 24} = 1.57$, $p = 0.187$) or the infusion of vehicle for antalarmin ($F_{8, 32} = 1.013$, $p = 0.447$) without the presence of CRF into the dorsal raphé nucleus.

Similarly, prior infusion of the CRF₂ receptor antagonist, ASV-30, into the dorsal raphé nucleus blocked the 500 ng CRF-induced increase in accumbal 5-HT levels ($F_{9, 72} = 3.579$; Fig. 4). There was a significant effect of treatment ($F_{5, 252} = 3.047$, $P = 0.026$), time ($F_{9, 252} = 4.892$, $P < 0.001$), and treatment \times time interaction ($F_{45, 252} = 2.984$, $P < 0.001$). As in the first experiment, infusion of 500 ng CRF preceded by infusion of the vehicle for the CRF₂ receptor antagonist, stimulated a significant increase at 20 min ($F_{9, 54} = 4.38$, $P < 0.001$; Dunnett's $q_{54, 10} = 4.23$, $P < 0.05$). Similarly, infusion of 500 ng CRF in the dorsal raphé nucleus following infusion of the CRF₁ receptor antagonist (antalarmin) resulted in a significant increase in accumbal 5-HT levels at 20 min ($F_{9, 45} = 26.514$, $P < 0.001$; Dunnett's $q_{45, 10} = 12.158$, $P < 0.05$). There was however, a gradual but significant ($F_{9, 72} = 3.579$, $P < 0.001$) decline of baseline at the final sampling time points (Post 100 min Dunnett's $q_{72, 10} = 3.107$, $P < 0.05$; Post 120 min Dunnett's $q_{72, 10} = 3.169$, $P < 0.05$) in the ASV-30 with CRF group, which were not significantly different from the same time points when compared to the ASV-30 alone or vehicle treated groups. Due to the gradual decline in baseline observed in the ASV-30 prior to the infusion of 500 ng group, a significant difference was observed at time points post 100 (SNK $P = 0.041$) and post 120 (SNK $P = 0.022$) when compared to prior infusion of antalarmin into the dorsal raphé nucleus. No significant effects on accumbal 5-HT levels across time were observed when vehicle (SNK $P = 0.061$) or ASV-30 (SNK $P = 0.589$) were infused alone into the dorsal raphé nucleus in the absence of CRF.

4. Discussion

Our results demonstrate for the first time that CRF infused into the dorsal raphé nucleus alters 5-HT release in the nucleus accumbens. Infusion of 100 ng CRF into the dorsal raphé decreases extracellular 5-HT concentrations in the nucleus accumbens (Fig. 2). In contrast, a higher dose of 500 ng CRF infused into the dorsal raphé resulted in an increase in accumbal 5-HT. Furthermore, the results suggest that the decrease in accumbal 5-HT release induced by 100 ng CRF is dependent on CRF₁ receptors in the dorsal raphé nucleus while the increase in 5-HT release in the nucleus accumbens following 500 ng CRF infusions into the dorsal raphé appears to be mediated by CRF₂ activation.

The observation that CRF has receptor-dependent opposite effects on 5-HT release may explain seemingly inconsistent findings from other studies that infused CRF into the dorsal raphé nucleus and measured 5-HT neuronal activity (Kirby et al., 2000; Lowry et al., 2000) or 5-HT

release and activity in terminal sites (Kirby et al., 2000; Kirby et al., 2003; Pernar et al., 2004; Price et al., 1998; Price and Lucki, 2001; Thomas et al., 2003). Previous studies suggest that lower doses of CRF (intra-dorsal raphé 3–10 ng; icv 0.1–1 µg; Kirby et al., 2000; Price et al., 1998) inhibit 5-HT release in the lateral septum and striatum, whereas higher doses were shown to either increase (intra-dorsal raphé 1µg CRF; Price and Lucki, 2001) or have no effect on 5-HT concentrations. Our results combined with those of previous studies suggest that the opposing effects of CRF on 5-HT terminal release are probably due to variable concentrations of CRF acting differentially on CRF₁ and CRF₂ receptors to inhibit or excite 5-HT neuronal cell firing.

Opposing actions by varying doses of CRF suggest mediation by different CRF receptors in the dorsal raphé nucleus (Hammack et al., 2003b; Hammack et al., 2003a; Kirby et al., 2000; Pernar et al., 2004). The dorsal raphé nucleus contains an unusually high density of CRF₁ and CRF₂ receptors. Decreased accumbal 5-HT release elicited by a lower CRF dose was dependent upon CRF₁ receptors, demonstrated by the CRF₁ receptor antagonist (antalarmin) blocking the effect of 100 ng CRF delivered to the dorsal raphé, whereas the CRF₂ receptor antagonist had no effect (Fig. 3). Similarly, dorsal raphé firing rates inhibited by CRF, were reversed by a selective CRF₁ receptor antagonist (Kirby et al., 2000). Furthermore, a higher dose of CRF in the dorsal raphé nucleus stimulated increased 5-HT release in the nucleus accumbens as a result of CRF₂ activation (Fig. 4). That is, the stimulatory effect of 500 ng CRF infused into the dorsal raphé nucleus on accumbal 5-HT release was inhibited by a CRF₂ receptor antagonist (ASV-30) but was not affected by a CRF₁ receptor antagonist.

It is possible that the divergent effects of CRF we observed are mediated by neuroanatomical heterogeneity of CRF₁ and CRF₂ receptor subtypes associated with regional specificity of function of the raphé (Lowry et al., 2000; Lowry, 2002; Summers et al., 2003a; Valentino et al., 2001). Regional specificity in raphé may yield direct control of terminal 5-HT output, such as from the lateral wings of the dorsal raphé nucleus to the nucleus accumbens (Van Bockstaele et al., 1993). Of course, in our model, presumably CRF binds to both CRF₁ and CRF₂ receptors regardless of the quantity released or the dosage delivered, and the effect is then modulated by the affinity of the receptor type, which is greater for CRF binding to the type 1 receptor (Grigoriadis et al., 1996a; Grigoriadis et al., 1996b). At lower concentrations, primarily CRF₁ receptors are activated by CRF due to their higher affinity for CRF. Higher concentrations of CRF are required to bind and stimulate CRF₂ receptor activity, and activation of CRF₂ receptors may mask CRF₁ receptor function by some unidentified mechanism. The possible mechanisms could include inhibitory receptor-to-receptor interactions or perhaps more likely inhibitory effects from inhibition by other neurotransmitter systems within the raphé. The raphé includes numerous GABAergic interneurons, particularly in the lateral wings, and also catecholaminergic input (Day et al., 2004). These inhibitory systems may have reduced accumbal 5-HT release observed in the slightly declining baseline of receptor antagonist treated preparations. However, only animals treated with receptor antagonist followed by CRF showed this decline suggesting an inhibitory feedback mechanism. In addition, the raphé also produces CRF binding proteins (Peto et al., 1999; Potter et al., 1992). These binding proteins may be involved in modulating cellular events that occur following CRF receptor binding, such as processing and degradation of CRF and/or ligand-receptor complexes (Peto et al., 1999). Therefore, direct or indirect mechanisms may be operational when concentration differences of CRF influence the output of 5-HT.

A decrease in serotonergic activity in the nucleus accumbens has been associated with increased aggression (Giacalone et al., 1968; Haney et al., 1990; Welch and Welch, 1968) and impulsivity (Cardinal et al., 2001; Tobin and Logue, 1994). In contrast, an increase in serotonergic activity in the nucleus accumbens is associated with stressful conditions (Petty et al., 1994; Rueter and Jacobs, 1996; Wilkinson et al., 1996), such as in the forced swim test

(Amat et al., 1998a; Amat et al., 1998b; Kirby et al., 1997). In addition behavioral states or processes mediated by increased accumbal dopamine include salient events such as reward, memory, motivation, and hedonia (Ikemoto and Panksepp, 1999; Kalivas, 1993; Pennartz et al., 1994; Robinson and Berridge, 1993; Zangen et al., 2001), that may also be influenced by 5-HT. The nucleus accumbens has been shown to regulate impulsive choice through interactions between dopamine and 5-HT (Winstanley et al., 2005). Therefore, CRF effects on the raphé serotonergic output to nucleus accumbens has the potential for modifying a suite of behaviors that involve anticipation, impulsivity, motivation, reward, aggression and/or depression.

The results of our experiments suggest that different concentrations of CRF yield differential CRF receptor binding in the dorsal raphé nucleus. This implies that CRF may act with a dual potential to elicit functional modulation of 5-HT in nucleus accumbens, thereby modifying behavior. Similarities between the effects of centrally administered CRF and the behavioral and physiological symptoms of human affective disorders, suggest an important role for CRF (Nemeroff, 1996; Nemeroff, 1998). Depressed individuals show elevated CRF levels and abnormal CRF functioning (Dunn and Berridge, 1990a; Dunn and Berridge, 1990b; Gold and Chrousos, 1999; Nemeroff, 1996; Nemeroff, 1998; Plotsky et al., 1998; Wong et al., 2000), as well as altered serotonergic functioning in the nucleus accumbens (Avgustinovich et al., 2004; Dremencov et al., 2005) which normalize in CSF after antidepressant treatment (Arborelius et al., 1999). The production of anxiety appears to be influenced by CRF₂ receptors in the dorsal raphé via 5-HT projections from the dorsal raphé nucleus (Graeff et al., 1996) during anxiety-arousing circumstances such as exposure to uncontrollable stressors (Bakshi et al., 2002; Hammack et al., 2003b; Ho et al., 2001; Takahashi et al., 2001). The importance of our results is to suggest that CRF₁ and CRF₂ receptors can be differentially activated simply by controlling the level of CRF neuronal output. If this is the case, then neurotransmitter activity modified by CRF, such as 5-HT in terminal regions like the nucleus accumbens, can be up- or down-regulated by modulation of the dorsal raphé nucleus through changes in the activity of CRF projections from cell body regions such as the central amygdala to affect or fine tune behavior.

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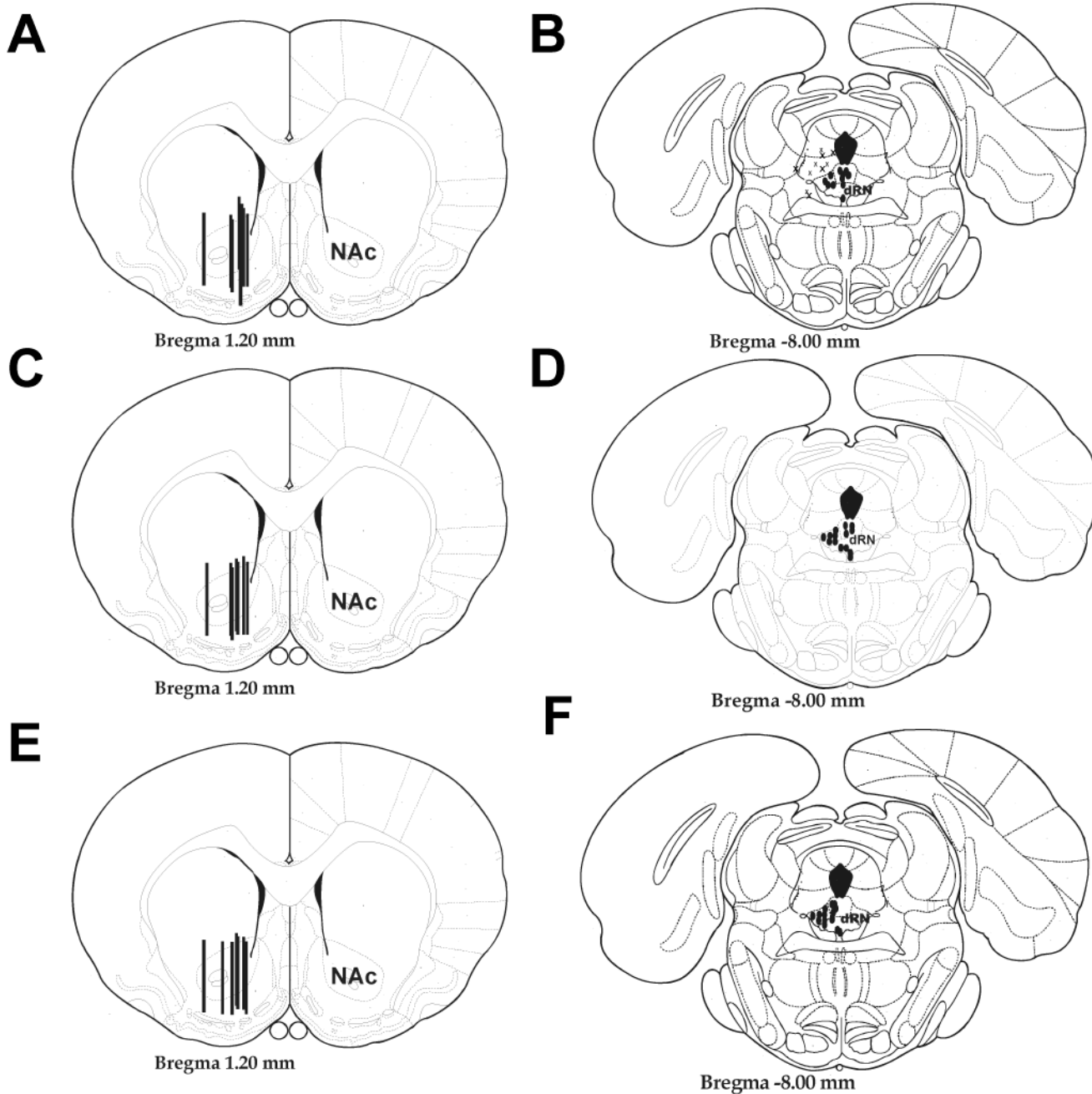


Figure 1. Probe and Cannula Placements in the Nucleus Accumbens and dorsal Raphé
 Schematic representations of microdialysis probe (A,C,E) and dorsal raphé nucleus drug infusion cannulae (B, D, F) for CRF dose-response experiments (A and B), CRF₁ receptor antagonist experiments (C and D), and CRF₂ receptor antagonist experiments (E and F). Microdialysis probes (black bars which represent more than one placement) were placed in the nucleus accumbens (NAc) with a 2 mm membrane to record from both the shell and the core (A, C, E; Figures adapted from Paxinos and Watson (1997); AP: +1.2 from bregma; ML: -1.4 from midline; DV: 8.1 from dura). Drug cannulae (black circles) were placed in regions encompassing areas of the dorsal raphé nucleus (AP: -7.4 from bregma; ML: +2.8 from

midline; DV: -4.6 from dura) that project to the nucleus accumbens (B, D, and F). Figure B also illustrates cannula placements outside the dorsal raphe nucleus (crosses).

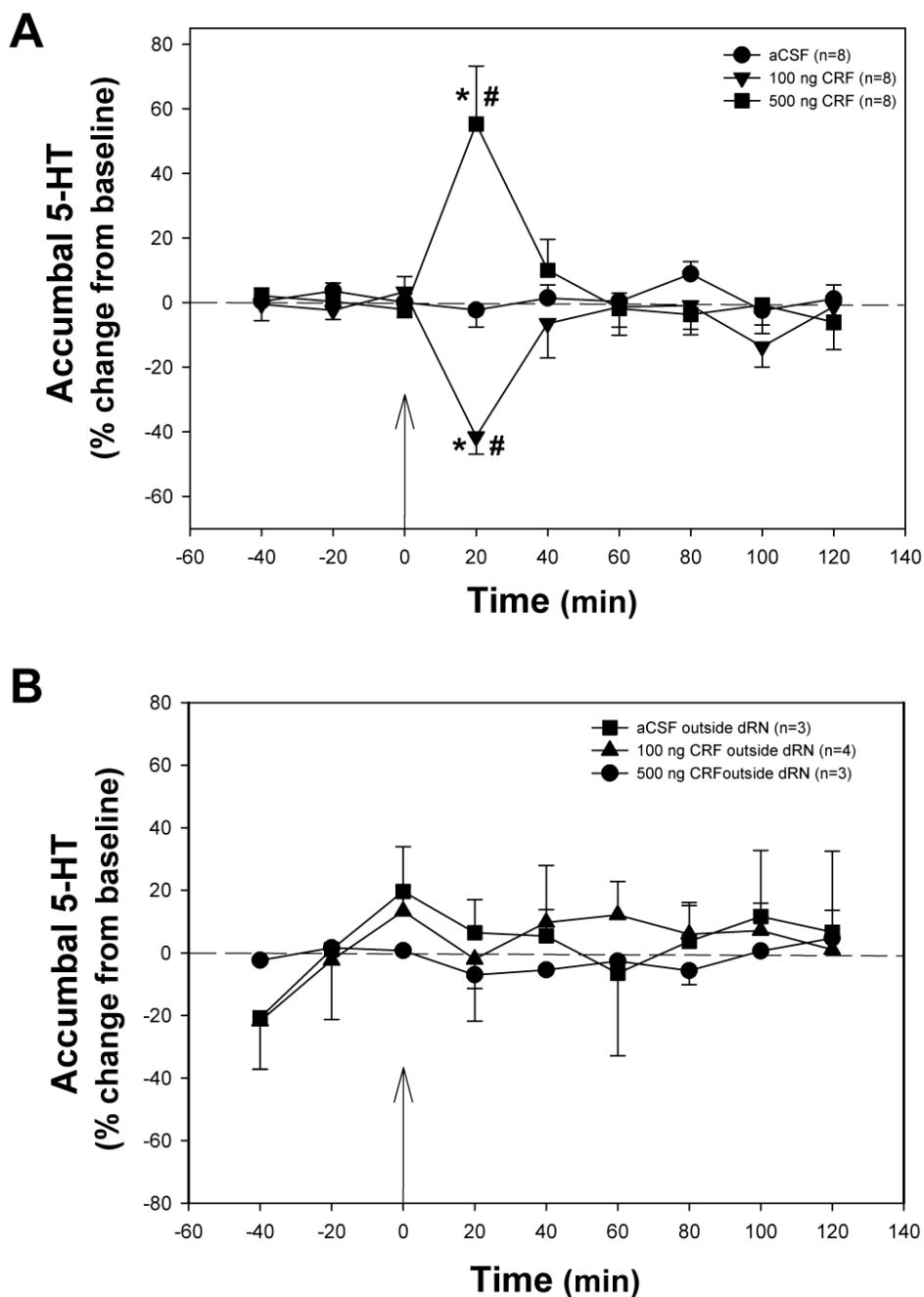


Figure 2. Effects of CRF Infusion into the dorsal Raphé nucleus on Accumbal 5-HT levels
 Infusion of 100 ng CRF into the dorsal raphé nucleus (A) resulted in a significant decrease in accumbal 5-HT when compared to CSF (A; *significant difference in accumbal 5-HT levels as compared to aCSF infusion, $P < 0.05$), and compared to pre-infusion baseline 5-HT overflow (# significant difference over time in accumbal 5-HT levels, $P < 0.05$). Arrow indicates time of intra-dorsal raphé injection. In contrast, infusion of 500 ng into the dorsal raphé nucleus (A) resulted in a significant increase in accumbal 5-HT levels when compared to controls (A). When cannulae placements were outside the dorsal raphé nucleus (B), no change in 5-HT overflow was recorded, regardless of the treatment.

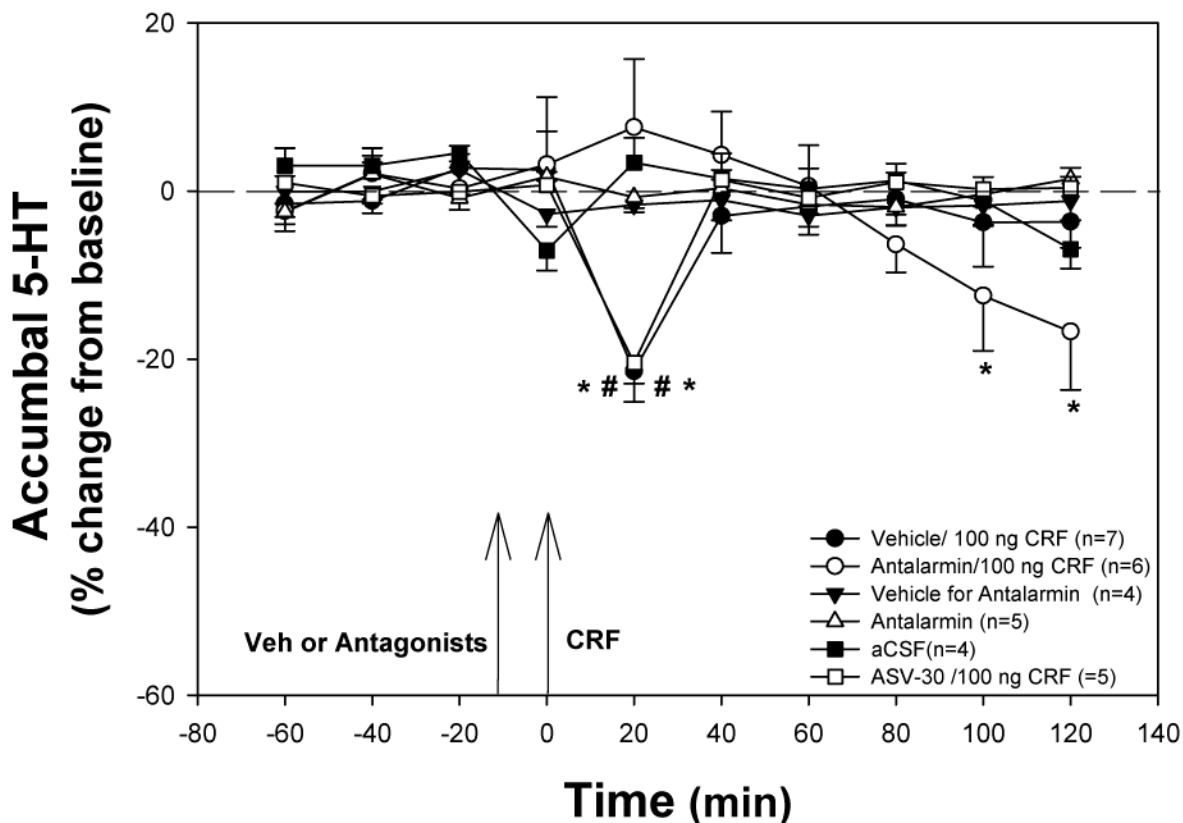


Figure 3. Infusion of a CRF₁ Antagonist into the dorsal Raphé nucleus Blocks the Effects of 100 ng CRF Infusion into the dorsal Raphé

Infusion of 100 ng CRF into the dorsal raphé nucleus (in the absence of the CRF₁ antagonist antalarmin) resulted in a significant 5-HT decrease in the nucleus accumbens (* significant compared to control groups; # significant difference over time; $P < 0.05$). The effect of 100 ng CRF on accumbal 5-HT levels was not affected by infusion of the CRF₂ antagonist (ASV-30) into the dorsal raphé nucleus 10 min prior to the infusion of 100 ng CRF. In contrast, infusion of antalarmin, a CRF₁ receptor antagonist, into the dorsal raphé nucleus completely blocked the 100 ng CRF effect. No effects on accumbal 5-HT levels were observed after infusion of vehicle or antalarmin alone in the absence of CRF. Arrows indicate time of intra-dorsal raphé injection: first arrow represents infusion of receptor antagonist or vehicle, and the second arrow represents infusion of CRF.

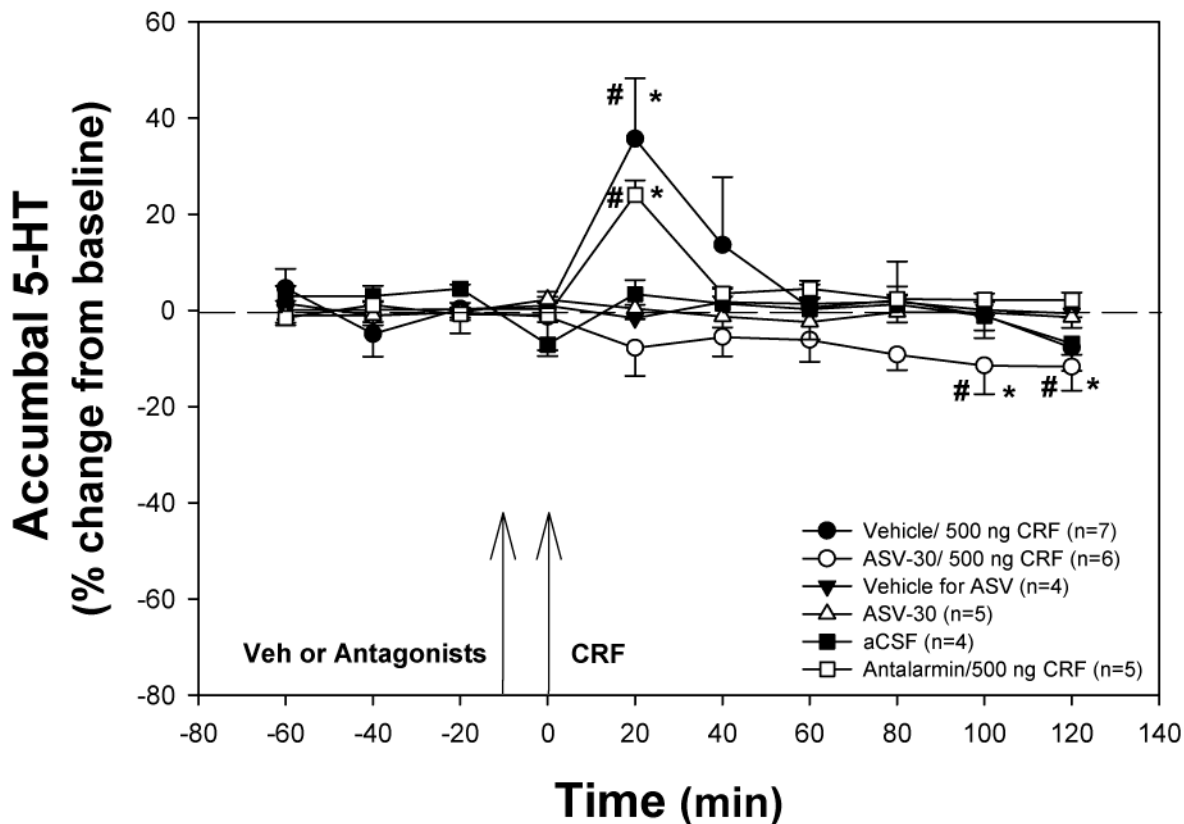


Figure 4. Infusion of a CRF₂ Antagonist into the dorsal Raphé nucleus Blocks the Effects of 500 ng CRF Infused into the dorsal Raphé

Infusion of 500 ng CRF into the dorsal raphé nucleus (in the absence of the CRF₂ antagonist ASV-30) resulted in a significant increase in accumbal 5-HT (* significant compared to control groups; # significant difference over time; $P < 0.05$). The effect of 500 ng CRF on accumbal 5-HT levels was not affected by infusion of the CRF₁ antagonist (antalarmin) into the dorsal raphé nucleus 10 min prior to the infusion of 500 ng CRF. In contrast, infusion of ASV-30, blocked the effects of 500 ng CRF into the dorsal raphé nucleus. No effects on accumbal 5-HT levels were observed after infusion of vehicle or ASV-30 alone in the absence of CRF. Arrows indicate time of intra-dorsal raphé injection: first arrow represents infusion of receptor antagonist or vehicle, and the second arrow represents infusion of CRF.