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Chronic amphetamine treatment enhances corticotropin-releasing factor-induced serotonin release in the amygdala

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ABSTRACT

Amphetamine use is associated with dysphoric states, including heightened anxiety, that emerge within 24 h of withdrawal from the drug. Corticotropin-releasing factor increases serotonin release in the central nucleus of the amygdala, and this neurochemical circuitry may play a role in mediating fear and anxiety states. We have previously shown that chronic amphetamine treatment increases corticotropin-releasing factor receptor type-2 levels in the serotonergic dorsal raphe nucleus of the rat. Therefore, we hypothesized that chronic amphetamine treatment would enhance the amygdalar serotonergic response to corticotropin-releasing factor infused into the dorsal raphe nucleus. Male rats were injected once-daily with *d*-amphetamine (2.5 mg/kg i.p., or saline) for two weeks. Serotonin release within the central nucleus of the amygdala in response to intra-raphe infusion of corticotropin-releasing factor (100 ng) was measured 24 h after the last treatment in urethane-anesthetized (1.8 mg/kg, i.p.) rats using *in vivo* microdialysis. Rats pretreated with amphetamine showed significantly enhanced serotonin release in the central nucleus of the amygdala in response to corticotropin-releasing factor infusion when compared to saline pretreated rats. Furthermore, this enhanced response was blocked by the corticotropin-releasing factor type-2 receptor antagonist antisauvagine-30 (2 μg) infused into the dorsal raphe nucleus. These results suggest increased sensitivity to corticotropin-releasing factor as mediated by type-2 receptors following chronic amphetamine treatment, which may underlie dysphoric states observed during amphetamine withdrawal.

1. Introduction

Drug addiction is a chronic disorder that is characterized by a cycle of compulsive use and drug seeking, and by a withdrawal syndrome that includes negative affective states (Koob, 2008; Koob and Volkow, 2010). In both humans and rat models, withdrawal from psychostimulant use can lead to increased sensitivity to stress, depressive behavior and increased anxiety that emerges within 24 h of drug abstinence (Harris and Aston-Jones, 1993; Sarnyai et al., 1995, 2001; Basso et al., 1999; Barr and Markou, 2005; Sinha et al., 2006; Perrine et al., 2008; Vuong et al., 2010). Negative affect and increased sensitivity to stress during drug abstinence appear to promote drug seeking and drug relapse (Sarnyai et al., 2001; Weiss, et al., 2001; Koob, 2003, 2008; Sinha et al., 2006). Therefore, the study of neural stress systems following chronic psychostimulant use may identify neural alterations that underlie dysphoric states during drug withdrawal.

Central corticotropin-releasing factor (CRF) mediates stress-related and anxiety-like behaviors, and also regulates serotonin (5-HT) limbic systems that are involved in stress and anxiety states (Heinrichs et al., 1992; Takahashi, 2001; Bale, 2005; Lowry et al., 2005; Forster et al., 2006; 2008; Lukkes et al., 2009b). The dorsal raphe nucleus (dRN) provides serotonergic innervation to the limbic system including the central nucleus of the amygdala (CeA) (Petrov et al., 1994). Both CRF type 1 and type 2 receptors are found within the dRN (Day et al., 2004; Waselus et al., 2009) and have opposing effects on 5-HT release (Lukkes et al., 2008). Infusion of CRF or CRF-like peptides increase 5-HT release in limbic regions including the CeA, and excitatory CRF responses on 5-HT activity are mediated by CRF_2 receptors in the dRN (Amat et al., 2004; Pernar et al., 2004; Forster et al., 2006, 2008; Lukkes et al., 2008). Furthermore, restraint stress increases 5-HT release in the CeA, which is inhibited by an introcerebroventricular injection of a $CRF_{1/2}$ antagonist (Mo et al., 2008). Increased 5-HT activity in the CeA stimulates release of circulating glucocorticoids via activation of the hypothalamic-pituitary-adrenal axis (Feldman and Weidenfeld, 1998, Feldman et al., 2000), and both 5-HT and glucocorticoids in the CeA contribute to anxiety states (Shepard et al., 2000; Myers et al., 2005). Likewise, increased serotonergic tone is believed to be an important mediator of the amygdala hyper-reactivity that is observed in affective disorders (Fisher et al., 2006). Alterations to CRF-5-HT interactions within the amygdala could also be related to dysphoric states during drug withdrawal.

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Recently we have found that CRF_2 receptors are increased in the dRN 20 h following chronic amphetamine treatment and remain elevated up to 6 weeks of withdrawal (Pringle et al., 2008). Furthermore, CRF_2 receptor antagonism in the dRN reduces anxiety-like behavior during amphetamine withdrawal (Vuong et al., 2010). Given that CRF_2 receptors in the dRN have excitatory effects on limbic 5-HT release (Amat et al., 2004; Forster et al., 2006, 2008; Lukkes et al., 2008) we hypothesized that chronic amphetamine treatment enhances CRF-induced 5-HT responses in the CeA via CRF_2 receptors in the dRN.

2. Materials and methods

2.1. Animals

Male Sprague–Dawley rats (n = 56; 3 weeks old) were purchased from the University of South Dakota Animal Resource Center. Rats were pair-housed in a climate-controlled holding room maintained at 22 °C, on a reverse 12 h light/12 h dark cycle with free access to food and water. Rats were used in the subsequent experiments when they reached early adulthood at 8 weeks old. 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 use and suffering of animals.

2.2. Experiment 1: Role of dRN-mediated CRF-induced 5-HT increases in the CeA

The first experiments tested whether CRF (100 ng) infused into the dRN of rats that did not receive saline or amphetamine pretreatment (termed "untreated") would elicit a similar increase in CeA 5-HT release in urethane-anesthetized rats as we had demonstrated in freely-moving rats (Forster et al., 2006). The use of urethane-anesthetized rats was necessary in subsequent experiments for dual cannula studies to elucidate receptor mechanisms, similar to procedures we have performed previously in urethane-anesthetized rats (e.g. Lukkes et al., 2008; Forster et al., 2008). Urethane has minimal effect on neurotransmitter release and neuronal firing rates (Maggi and Meli, 1986), and both baseline and CRF-elicited 5-HT release are similar between freely-moving and urethane-anesthetized rats (Forster et al., 2006; 2008).

2.2.1. Surgery

Eleven 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). A stainless-steel guide cannula (26 gauge, laboratory-made) was stereotaxically implanted so the guide cannula was positioned 2 mm above the dRN (AP: −8.1 mm from bregma, ML: +2.8 mm from midline at a 26° angle lateral to medial; DV: −3.8 mm from dura; Paxinos and Watson, 1998). A microdialysis probe (2.5 mm exposed membrane length, 5000 MW cut-off, typical recovery 20%, laboratory-made; Forster et al., 2006) was directly inserted into the CeA (AP: −2.3 mm from bregma; ML: −4.0 mm from midline; −8.7 mm from dura; Paxinos and Watson, 1998). Following probe insertion, artificial cerebral spinal 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).

2.2.2. Microdialysis and dRN infusion

Silica cannula (197 μm o.d., cut 2 mm longer than guide; Polymicro Technologies, Phoenix, AZ, USA) were attached to PE20 tubing and back-loaded with either drug or vehicle and connected to a 10 μl Hamilton syringe. A cannula was inserted into the dRN through the cannula guide 3 h following probe insertion. After 1 h, dialysate samples were collected from the CeA microdialysis probe at 20 min intervals and injected immediately into a high-performance liquid chromatography (HPLC) with electrochemical detection (see details below) to measure 5-HT. Following collection of 3 baseline samples, a 0.5 μl microinfusion of either vehicle (aCSF; n = 4) or CRF (100 ng in aCSF; n = 7) was made into the dRN. All drug infusions were made at a rate of 0.5 μl/min using a microinfusion pump (Stoelting, Wood Dale, IL, USA). Dialysate samples were collected until CeA 5-HT returned to baseline levels, or if no change in 5-HT was detected, 8 post-infusion samples were collected.

2.3. Experiment 2: Effects of amphetamine pretreatment on CRF-induced 5-HT release in the CeA-mediation by CRF_2 receptors in the dRN

These experiments tested whether chronic amphetamine treatment results in increased CRF-induced 5-HT release in the CeA of urethane-anesthetized rats. Given that CRF_2 receptors are up-regulated in amphetamine pretreated rats (Pringle et al., 2008), the role of CRF_2 receptors was assessed by pretreatment of the dRN with the selective CRF_2 receptor antagonist antisauvagine-30 (ASV-30; Lukkes et al., 2008; Forster et al., 2008). This antagonist has 1000–10,000 fold selectivity for the vertebrate CRF_2 receptor over the CRF_1 receptor (Higelin et al., 2001), and does not affect CRF_1 receptor mediated 5-HT release at this concentration infused into the dRN (2 μg; Lukkes et al., 2008).

2.3.1. Amphetamine treatment

Using a separate group of rats from Experiment 1, amphetamine (2.5 mg/kg, i.p. n = 31) or saline (n = 14) was injected into each rat once-daily for 14 days during the dark (active) phase of the light cycle. This amphetamine treatment regime produces heightened anxiety states upon withdrawal of the drug (Vuong et al., 2010) and results in increased CRF_2 receptors in the dRN (Pringle et al., 2008). Following injections, rats were returned to their home cage. Rats were anesthetized for stereotaxic surgery the morning following the last day of injections.

2.3.2. Surgery

Rats were anesthetized with urethane (1.5 g/kg, i.p.) and placed within a small mammal stereotaxic frame as described in Experiment 1. Stainless steel dual guide cannulae (26 gauge, laboratory-made) were made so that the tips of the guides were adjacent to ensure infusions in the same region of the dRN. The dual guide cannulae were stereotaxically implanted so the guides were positioned 2 mm above the dRN (AP: −8.1 mm from bregma, ML: +2.8 mm from midline at a 26° angle lateral to medial; DV: −3.8 mm from dura; Paxinos and Watson, 1998). A microdialysis probe was inserted into the CeA as described in Experiment 1.

2.3.3. Microdialysis and dRN infusion

Two silica cannulae were inserted into the dRN through the cannula guides 3 h following probe insertion and dialysis samples were collected as described in Experiment 1. Following collection of 3 baseline samples, one of four dual infusions was made into the dRN (as described for Experiment 1) with 10 min separating each infusion (see Table 1 for specific infusions made). The concentration of the CRF_2 receptor antagonist ASV-30 used by the current study completely reverses anxiety states in amphetamine treated rats when infused into the dRN without affecting control rat behavior or general activity (Vuong et al., 2010). The 2% ethanol vehicle for ASV has no effect on 5-HT release (Forster et al., 2008; Lukkes et al., 2008). Furthermore, we have utilized this double dRN infusion procedure in the past and it does not result in infusion damage within the dRN (and

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Table 1
Design of amphetamine pretreatment experiments.

Pretreatment	Dorsal raphe pre-infusion	Dorsal raphe infusion	N	Figure
Amphetamine	Vehicle (2% ethanol/aCSF)	CRF (100 ng/0.5 μl)	8	3A
Saline	Vehicle (2% ethanol/aCSF)	CRF (100 ng/0.5 μl)	6	3A
Amphetamine	Vehicle (2% ethanol/aCSF)	Vehicle (aCSF)	5	3B
Saline	Vehicle (2% ethanol/aCSF)	Vehicle (aCSF)	6	3B
Amphetamine	ASV-30 (2 μg/0.5 μl)	Vehicle (aCSF)	7	5
Amphetamine	ASV-30 (2 μg/0.5 μl)	CRF (100 ng/0.5 μl)	7	5

see Fig. 1D) (Lukkes et al., 2008; Forster et al., 2008). Dialysate samples were collected until CeA 5-HT returned to baseline levels, or if no change in 5-HT was detected, 8 post-infusion samples were collected.

2.4. HPLC measurement of 5-HT

Detection of 5-HT in dialysates was accomplished using HPLC with electrochemical detection (Bradberry et al., 1991; Forster et al., 2008). Mobile phase (300 mg EDTA, 432 mg sodium octanesulfonate, 4.8 g NaH_2PO_4, 300 μl triethylamine and 122 ml acetonitrile per liter, 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 perfusion rate through the probe of 0.4 μl/min resulted in the collection of approximately 8 μl of dialysate/20 min to ensure that when the sample was injected into the HPLC, the loop was overfilled. 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 a LC-4C potentiostat (Bioanalytical Systems). The voltage output was recorded by Clarity v2.4 Chromatography Station for Windows (DataApex, Prague, Czech Republic).

2.5. Histology

Following completion of experiments, rats were killed by overdose with sodium pentobarbital (0.5 ml Fatal Plus, i.p.; Vortech, Dearborn, MI, USA). The brains were removed and fixed in 10% buffered formalin (Fisher Scientific). After fixation, the brains were frozen and sectioned at 60 μm on a microtome; the series of sections encompassing the probe or the cannulae was stained with cresyl violet and was examined under a light microscope by two experimenters that were blind to treatment. Correct placement was determined for both the infusion cannulae and the microdialysis probe.

2.6. Data analysis

Three baseline samples were collected, and the height of the 5-HT peaks averaged. All post-drug 5-HT peak heights were calculated as a percent change from the average baseline level for each animal to account for potential variance in 5-HT recovery between microdialysis probes (e.g. Hoffman et al., 2002; Forster et al., 2008; Li et al., 2010; Navailles et al., 2010). For each experiment, the 5-HT levels were analyzed with a two-way ANOVA with one repeated measure (time). A one-way ANOVA was used when there was a significant effect of treatment, to compare the groups at each separate time point. Significant effects of treatment at a given time point were further analyzed 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. Significant time points were then identified by Dunnett's *post-hoc* test for multiple comparisons, where the sample collected immediately prior to the first drug infusion served as the control sample.

2.7. Drugs

Amphetamine, urethane, corticotropin-releasing factor (CRF) and antisauvagine-30 trifluoroacetate salt (ASV-30) were purchased from Sigma. CRF was dissolved in aCSF (pH 7.4) and amphetamine was dissolved in sterile physiological (0.9%) saline. Urethane was dissolved in nanopure water to a concentration of 0.345 g/ml, whereas ASV-30 was dissolved in a 2% ethanol/aCSF vehicle (Forster et al., 2008).

3. Results

3.1. Experiment 1: Role of dRN-mediated CRF-induced 5-HT increases in the CeA

Microdialysis probe membranes were located within the CeA from −2.12 to −3.3 mm posterior from bregma (Paxinos and Watson, 1998) (Fig. 1A). The tips of the dRN cannula were located between −7.64 and −8.72 mm posterior from bregma (Paxinos and Watson, 1998) with the majority of the infusions made at −8.0 from bregma (Fig. 1A). Drug infusions were made on an angle to avoid the cerebral aqueduct and we have previously demonstrated that infusion of 0.5 μl CRF on this angle provides a specific infusion to the dRN without functional diffusion to the ventricle or surrounding periaqueductal gray (e.g. Forster et al., 2006; 2008; Lukkes et al., 2008; and see Fig. 4).

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 0.09 ± 0.01 pg, and the mean ± S.E.M. baseline level of CeA 5-HT for untreated rats was 0.31 ± 0.04 pg/5 μl, for amphetamine treated rats was 0.57 ± 0.16 pg/5 μl, and for saline treated rats was 0.32 ± 0.05 pg/5 μl (uncorrected for recovery). There was no statistical difference in baseline levels between the three groups ($F_{(2,42)} = 0.980$, $P = 0.384$). Furthermore, these 5-HT baseline values are in line with those obtained from the CeA of awake rats 0.32 ± 0.11 pg/5 μl (e.g. Mo et al., 2008).

Infusion of CRF (100 ng/0.5 μl) into the dRN resulted in an immediate and transient increase in CeA 5-HT in rats that were not previously treated with amphetamine or saline (Fig. 2). There was a significant effect of treatment ($F_{(1,9)} = 8.930$, $P = 0.015$) as well as a significant effect of time ($F_{(11,91)} = 2.022$, $P = 0.035$) but no significant interaction between treatment and time ($F_{(11,91)} = 1.340$, $P = 0.216$, Fig. 2). Infusion of CRF into the dRN caused an increase in CeA 5-HT at 20 min (SNK, $P<0.001$) and 40 min (SNK, $P = 0.050$) post-infusion compared to infusion of vehicle. Separate one-way ANOVA showed that rats receiving a microinfusion of CRF exhibited a significant increase in CeA 5-HT over time ($F_{(11,61)} = 2.514$; $P = 0.011$, Fig 2). Serotonin levels were significantly different from pre-infusion levels at 20 min post-infusion (Dunnett's, $P<0.05$). Rats receiving a microinfusion of vehicle into the dRN exhibited a significant change in CeA 5-HT over time ($F_{(11,30)} = 2.701$; $P = 0.015$) at 120 and 160 min post-infusion (Dunnett's, $P<0.05$, Fig 2), representing a slight decline in 5-HT levels over the testing period.

3.2. Experiment 2: Effects of amphetamine pretreatment on dRN-mediated CRF-induced 5-HT release in the CeA-mediation by CRF_2 receptors in the dRN

Microdialysis probe membranes were located within the CeA from −2.56 to 3.3 mm posterior from bregma for saline pretreated rats (Paxinos and Watson, 1998) (Fig. 1B). The tips of the dRN cannula for saline pretreated rats were located between −6.72 and −8.3 mm posterior from bregma (Paxinos and Watson, 1998) with the majority of the infusions made at −8.0 from bregma (Fig. 1B). Similarly,

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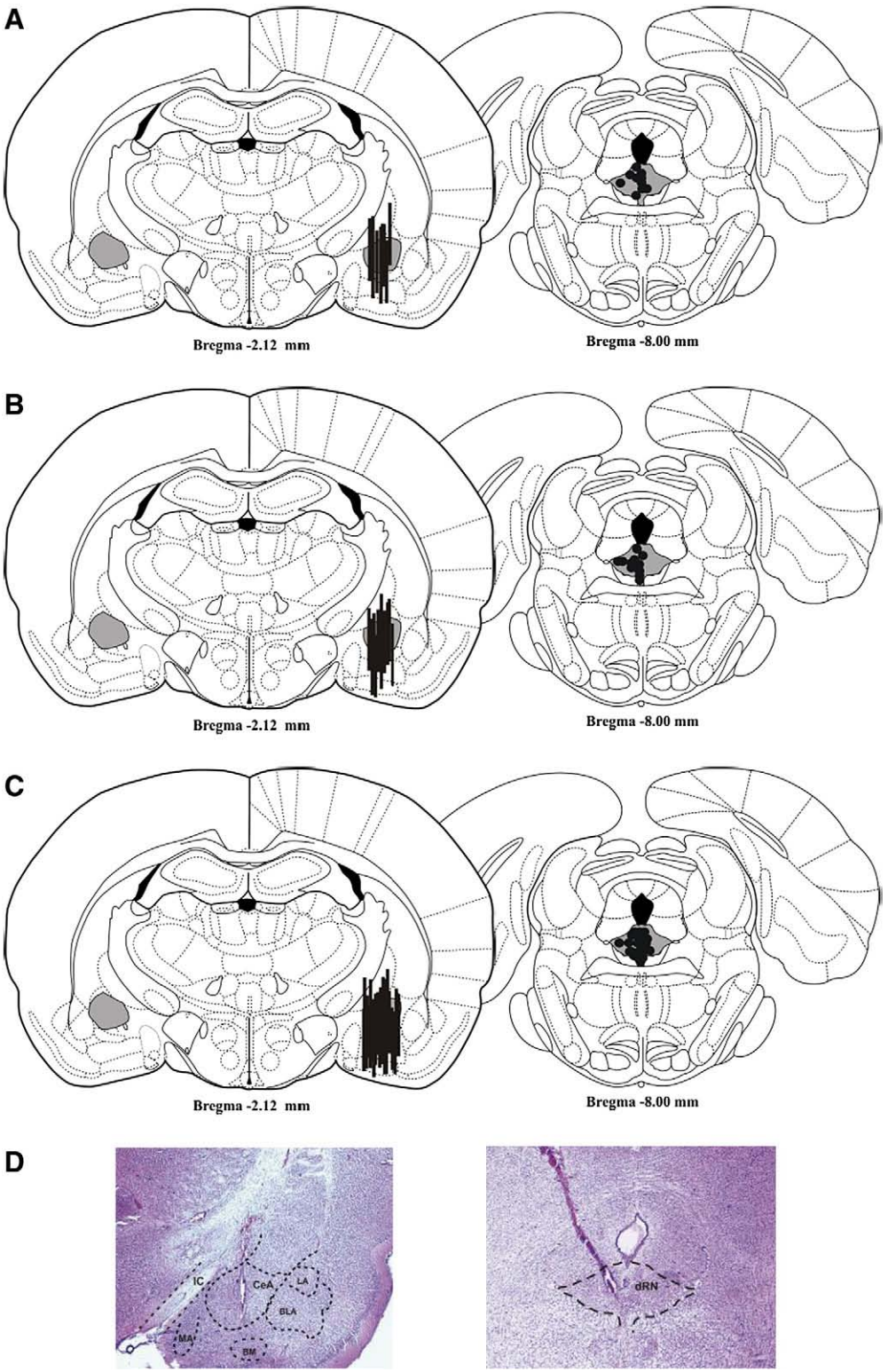

Fig. 1. Representative coronal diagrams for microdialysis probe and drug infusion cannula placements. Microdialysis probe membrane placement (black bars) in the central nucleus of the amygdala (CeA) and location of drug infusion cannula tips in the dorsal raphe nucleus (dRN; black circles) and cannula tip placements outside the dRN (black stars) of (A) untreated rats, (B) saline pretreated rats and (C) amphetamine pretreated rats. (D) Representative stained sections of CeA microdialysis probe placement and dRN cannula tip placement.
Figures were adapted from Paxinos and Watson (1998).

microdialysis probe membranes in amphetamine pretreated rats were located within the CeA from −1.6 to −3.3 mm posterior from bregma (Fig. 1C) and the tips of the dRN cannula for amphetamine pretreated rats were located between −6.72 and −8.72 mm posterior from bregma (Fig. 1C). The distribution of microdialysis probes and drug cannulae was similar for the different pharmacological treatment groups. Representative stained sections of probe membrane and drug cannula tracks are depicted in Fig. 1D.

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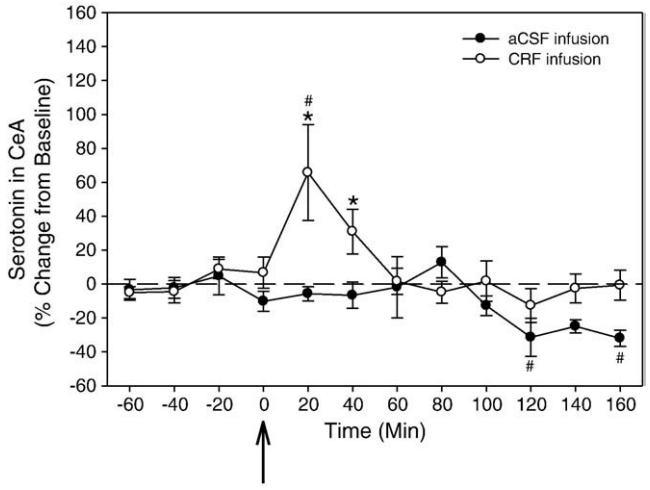

Fig. 2. Effects of corticotropin-releasing factor in the dorsal raphe nucleus on serotonin release in the central nucleus of the amygdala in rats with no previous amphetamine/saline exposure. Corticotropin-releasing factor (CRF; 100 ng/0.5 μl) infused into the dorsal raphe nucleus resulted in an immediate and transient increase in serotonin release in the central nucleus of the amygdala (CeA). Vehicle (aCSF, 0.5 μl) infused into the dRN had no effect on serotonin in the CeA. Data represent mean ± S.E.M. # = significantly different from pre-infusion levels, * = significant differences between treatment groups, $p<0.05$. Arrow represents time of dRN infusion.

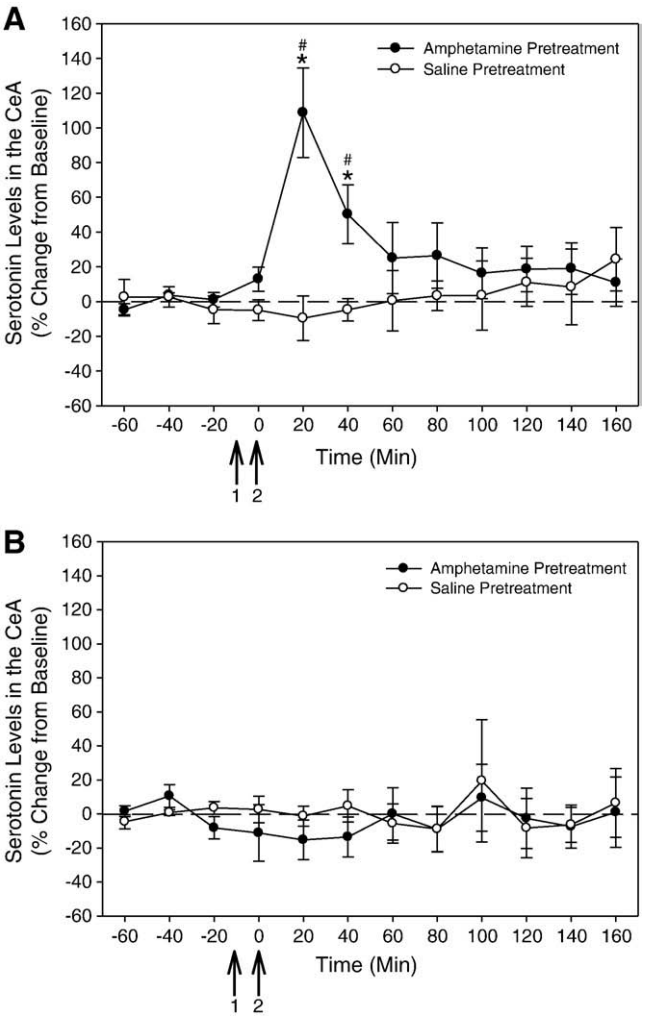

Fig. 3. Effects of corticotropin-releasing factor in the dorsal raphe nucleus on serotonin release in the central nucleus of the amygdala in amphetamine and saline pretreated rats. (A) Corticotropin-releasing factor (CRF; 100 ng/0.5 μl) infused into the dorsal raphe nucleus of rats previously treated with amphetamine (2.5 mg/kg, i.p.) caused an immediate and transient increase in serotonin release in the central nucleus of the amygdala (CeA), but had no effect on rats previously treated with saline. (B) Vehicle (aCSF, 0.5 μl) infused into the dRN had no effect on serotonin release in the CeA in either pretreatment group. Data represent mean ± S.E.M. # = significantly different from pre-infusion levels, * = significant differences between treatment groups, $p<0.05$. Arrows indicate time of infusion, 1 = vehicle for ASV-30 and 2 = CRF (A) or aCSF (B).

Infusion of CRF (100 ng/0.5 μl) to the dRN resulted in a transient increase in 5-HT release in the CeA in rats pretreated with amphetamine (2.5 mg/kg, i.p.) but not saline (Fig 3A). There was a significant effect of time ($F_{(11,117)}=3.583$; $P<0.001$) and a significant interaction between pretreatment and time ($F_{(11,117)}=4.805$; $P<0.001$), but no significant effect of pretreatment alone ($F_{(1,12)}=3.792$, $P=0.075$) on CeA 5-HT levels. Corticotropin-releasing factor infused into the dRN of amphetamine pretreated rats caused an increase in CeA 5-HT levels which was significant at 20 and 40 min post-infusion compared to saline pretreated rats receiving CRF into the dRN (SNK, $P<0.001$ at 20 min and $P=0.015$ at 40 min). Separate one-way ANOVA showed that amphetamine pretreated rats receiving a microinfusion of CRF exhibited a significant increase in CeA 5-HT over time ($F_{(11,69)}=8.667$; $P<0.001$, Fig 3A), which was significant at 20 and 40 min post-infusion (Dunnett's, $P<0.05$ for each time point), compared to pre-infusion levels. Rats pretreated with saline receiving microinfusions of CRF exhibited no significant effect over time ($F_{(5,48)}=0.468$; $P=0.914$, Fig 3A).

Infusion of vehicle (aCSF, 0.5 μl) into the dRN of rats pretreated with amphetamine (2.5 mg/kg, i.p.) or saline had no effect on 5-HT levels in the CeA (Fig. 3B), suggesting that the 2% ethanol vehicle did not alter 5-HT release. For vehicle-infused rats, there was no significance between pretreatment group ($F_{(1,10)}=0.0536$, $P=0.822$), across time ($F_{(11,101)}=0.893$, $P=0.550$) nor was there a significant interaction between pretreatment and time ($F_{(11,101)}=0.311$, $P=0.982$; Fig. 3B).

Infusion of CRF (100 ng/0.5 μl) outside but adjacent to the dRN (Fig. 1B–C; $n=6$) in rats previously treated with amphetamine or saline had no effect on CRF-induced 5-HT release in the CeA (Fig. 4). Serotonin levels in the CeA of rats pretreated with amphetamine (2.5 mg/kg, i.p) or saline were not significantly different between pretreatment group ($F_{(1,4)}=0.0849$, $P=0.785$) or across time ($F_{(11,39)}=0.974$, $P=0.486$) nor was there a significant interaction between pretreatment and time ($F_{(11,39)}=0.194$, $P=0.997$; Fig. 4) when CRF was infused adjacent to the dRN.

In order to confirm that the augmented CRF-induced 5-HT release in the CeA of amphetamine pretreated rats was mediated by CRF_2 receptors in the dRN, the selective CRF_2 receptor antagonist ASV-30 was infused into the dRN prior to CRF or vehicle infusion (Table 1).

Infusion of ASV-30 (2 μg/0.5 μl) into the dRN 10 min prior to an infusion of CRF (100 ng/0.5 μl) into the dRN of amphetamine pretreated (2.5 mg/kg, i.p.) rats blocked the effect of CRF on 5-HT release in the CeA (Fig. 5). This was revealed by no significant effect of infusion ($F_{(1,12)}=0.0651$, $P=0.803$) or time ($F_{(11,119)}=1.216$, $P=0.284$) nor was there a significant interaction between infusion and time ($F_{(11,119)}=0.742$, $P=0.697$; Fig. 5) on 5-HT levels in the CeA of amphetamine pretreated rats when ASV-30 was infused into the dRN prior to CRF or vehicle (aCSF).

4. Discussion

Microinfusion of 100 ng of CRF into the dRN of untreated (no saline or amphetamine pretreatment) urethane-anesthetized rats produced an immediate and transient increase in CeA 5-HT release. This effect (maximum increase at 20 min post-infusion) on 5-HT release shown here is similar to the effect found in freely-moving rats also treated with a higher (500 ng) concentration of CRF (Forster et al., 2006). The effects of CRF in the dRN on CeA 5-HT release are also similar to those

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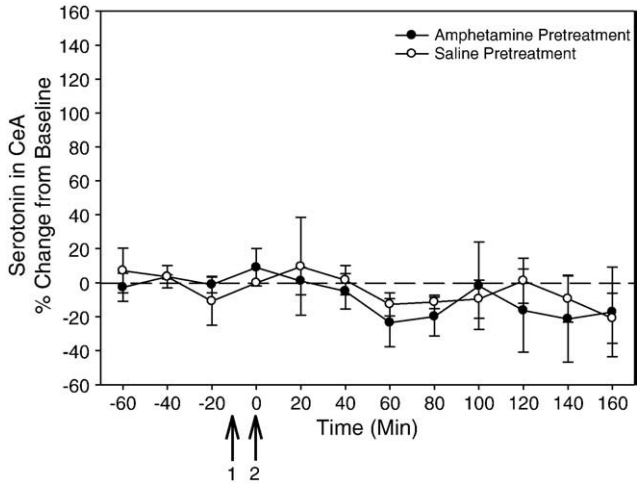

Fig. 4. Effects of corticotropin-releasing factor outside the dorsal raphe nucleus on serotonin release in the central nucleus of the amygdala in amphetamine and saline pretreated rats. Corticotropin-releasing factor (CRF 100 ng/0.5 μl) infused outside but adjacent to the dorsal raphe nucleus of rats previously treated with amphetamine (2.5 mg/kg, i.p.) or saline had no effect on the release of serotonin in central nucleus of the amygdala (CeA). Data represent mean ± S.E.M. # = significantly different from pre-infusion levels. Arrows indicate time of infusion, 1 = vehicle for ASV-30 and 2 = CRF.

elicited by restraint stress, which is also known to be CRF dependent (Mo et al., 2008). Thus, while the levels of stress-induced or amphetamine-induced CRF in the dRN are not known, 100–500 ng of CRF infused into the dRN appears to produce a similar effect on 5-HT as stress. Furthermore, given the similar pattern and time-course of CRF-elicited effects on 5-HT release in the CeA between anesthetized and non-anesthetized rats, the use of anesthetized rats can serve as a useful protocol to examine the mechanisms underlying CRF-mediation of CeA 5-HT release, as we have demonstrated earlier for other limbic brain regions (e.g. Forster et al., 2008; Lukkes et al., 2008).

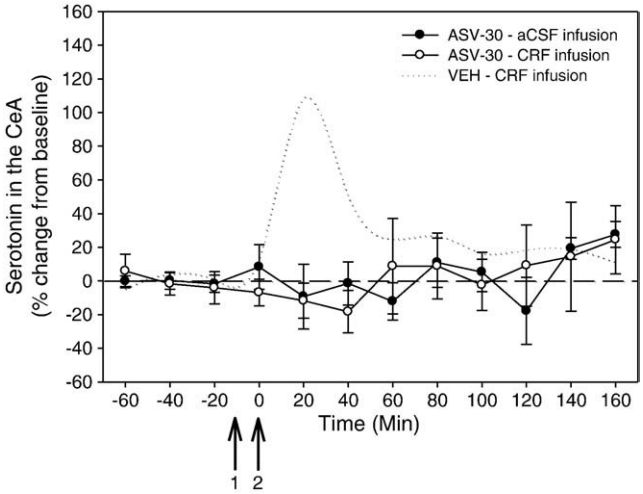

Fig. 5. Effects of corticotropin-releasing factor receptor 2 antagonism in the dorsal raphe nucleus on corticotropin-releasing factor elicited serotonin release in the central nucleus of the amygdala in amphetamine pretreated rats. Pre-infusion of the dorsal raphe nucleus (dRN) with the corticotropin-releasing factor (CRF) receptor 2 antagonist antisauvagine-30 (ASV-30, 2 μg/0.5 μl) blocked the stimulatory effect of intra-dRN CRF (100 ng/0.5 μl) on central nucleus of the amygdala (CeA) serotonin levels in rats previously exposed to amphetamine (2.5 mg/kg, i.p.). Data represent mean ± S.E.M. Arrows indicate time of infusion, 1 = ASV-30 and 2 = CRF or aCSF (vehicle). Dashed line represents the positive control for this study (vehicle followed by CRF infusion into the dRN of amphetamine pretreated rats) previously shown in Fig. 3A and plotted here to illustrate the impact of ASV-30 on this CRF-induced response.

It has been previously shown that CRF infused into the dRN of anesthetized rats can alter 5-HT neuronal firing rates and 5-HT release in a biphasic fashion (Price et al., 1998; Kirby et al., 2000; Pernar et al., 2004; Lukkes et al., 2008). To illustrate, Lukkes et al. (2008) demonstrated that a 100 ng infusion of CRF decreased 5-HT release in the nucleus accumbens (NAc) via CRF_1 receptor activation, where a 500 ng infusion resulted in increased NAc 5-HT release via CRF_2 receptor activation. However, the current findings show that 100 ng CRF infused into the dRN of urethane-anesthetized rats, using methods identical to that of Lukkes et al. (2008), actually results in increased 5-HT release in the CeA, as mediated by CRF_2 receptors. The disparate effects are possibly due to differing weighting of CRF_1 and CRF_2 receptor actions on output neurons to the NAc versus the CeA, with CRF_2 receptors perhaps playing a greater role in mediating the activity of neurons projecting to the CeA. A similar hypothesis has been suggested for 5-HT activity in the medial prefrontal cortex, which like the CeA, appears to be predominantly mediated by CRF_2 receptors in the dRN (Forster et al., 2008). While a small number of serotonergic neurons in the dRN send axon collaterals to these regions, the majority do not (van Bockstaele et al., 1993). This raises the interesting possibility that pharmacological differences between the NAc and CeA/medial prefrontal cortex may arise from differential distribution of CRF receptor types in non-collateral dRN neurons or in dRN interneurons that influence these projection neurons.

In the current study rats were pretreated with an amphetamine treatment schedule that increases anxiety states and increased CRF_2 but not CRF_1 receptors in the dRN (Pringle et al., 2008; Vuong et al., 2010). Here we show that these rats also exhibit sensitized CRF-induced 5-HT release in the CeA, which was mediated by CRF_2 receptors in the dRN. Thus, the increased levels of CRF_2 receptors in the dRN following chronic amphetamine treatment (Pringle et al., 2008) appear to have functional consequences on 5-HT release, and this effect may not be specific to the CeA given the vast 5-HT projections from the dRN to the limbic brain. Related, Waselus et al. (2009) showed that a swim stressor, which increased trafficking of CRF_2 receptors to the neuronal membrane in the dRN, also increased the excitability of dRN 5-HT neurons. Furthermore, post-weaning social isolation of rats which results in heightened anxiety states and increased CRF_2 receptors in the dRN also results in prolonged CRF-mediated 5-HT release in the NAc (Lukkes et al., 2009a). Thus, there may be common consequences of stressors and long-term amphetamine treatment on CRF-5-HT interactions within the brain.

Activation of CRF_2 receptors in the dRN is thought to increase 5-HT activity and 5-HT release via inhibition of GABAergic input to dRN 5-HT neurons, effectively disinhibiting 5-HT neuronal activity (Pernar et al., 2004). While sensitized CRF-induced 5-HT release in amphetamine pretreated rats is mediated by CRF_2 receptors, alterations to GABAergic function in the dRN could underlie this observation. Therefore, it would be important to determine whether chronic amphetamine treatment reduces GABAergic inhibition of 5-HT neurons in the dRN, which would then facilitate CRF_2-induced disinhibition of 5-HT neuronal activity and increase 5-HT release.

Recently, we have demonstrated the CRF_2 receptor antagonist ASV-30 infused into the dRN, at the same dose as used here, reduces heightened anxiety-like behaviors of amphetamine pretreated rats to control rat levels (Vuong et al., 2010). The effect of ASV-30 in the dRN on anxiety-like behaviors may be related to our current finding that this antagonist abolishes heightened CRF-induced 5-HT responses in the CeA. Mediation of increased anxiety behavior has been linked specifically to the CRF_2 receptor, with antagonism of CRF_2 receptors decreasing anxiety-like behavior in several animal models of anxiety (Takahashi et al., 2001). Importantly, CRF-induced 5-HT release in the CeA is correlated with the induction in fear behavior (Forster et al., 2006) and restraint stress also increases 5-HT release in the CeA via central CRF receptor mechanisms (Mo et al., 2008). Furthermore, inactivation of the CeA results in an anxiolytic-like effect on the

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elevated plus maze (Moreira et al., 2007), suggesting a relationship between the CeA and anxiety/fear behavior. In addition, increased extracellular 5-HT levels elicited by acute administration of selective serotonin reuptake inhibitors are associated with increased anxiety states in both humans and animal models (Handley and McBlane, 1993; File et al., 1999; Amsterdam et al., 1994; Artaiz et al., 1998; Katzman, 2009). Therefore, future work should test whether the augmented CRF-elicited 5-HT activity in the CeA of amphetamine pretreated rats is directly related to heightened anxiety states.

Interestingly, saline pretreated rats exhibited no change in CRF-induced 5-HT release in the CeA, which was in contrast to rats that did not receive any injection pretreatment, who exhibited increased CRF-induced 5-HT release in the CeA. This suggests that the mild stressor of injections/handling prior to CRF infusion may have dampened the CeA serotonergic response to CRF, and this will require confirmation with dose–response studies. A blunted response of CeA 5-HT has been shown in response to restraint stress, where exposure to experimental manipulations (introcerebroventricular injections) prior to a stressor also dampened CRF-mediated stress-induced 5-HT release in the CeA (Mo et al., 2008). Related, repeated exposure to mild predictable stressors reduces anxiety-like and depressive-like behaviors while increasing hippocampus function (Parihar et al., in press). Therefore while speculative at this stage, dampened CRF-induced 5-HT release in the CeA following chronic saline treatment may reflect an adaptation to mild stressors, which is absent in amphetamine pretreated rats.

Overall, our results show that chronic amphetamine treatment heightens CeA serotonergic responses to CRF via CRF_2 receptors in the dRN. This finding may explain, in part, increased stress sensitivity and dysphoric states during psychostimulant withdrawal (e.g. Sarnyai et al., 1995, 2001; Koob, 2008; Shoptaw et al., 2009; Vuong et al., 2010). Interestingly, CRF_1 rather than CRF_2 receptors have more often been implicated in stress- and ethanol- or opiate-induced dysphoric states (e.g. Liebsch et al., 1995; Bale, 2005; Koob, 2009). With relation to stress-induced anxiety states, findings by Henry et al. (2006) suggest that CRF_2 receptors may play a greater role in mediating anxiety states when animals have been exposed to prior stressors. Recently, CRF_1 receptors in the dRN have been implicated in CRF- and stress-induced sensitization of ethanol-induced anxiety behaviors in rats, with CRF_2 receptors in the dRN appearing to play little role in this anxiety state (Huang et al., 2010). Therefore, anxiety states following ethanol administration appear to be mediated by different CRF receptor mechanisms in the dRN as compared to amphetamine treatment. While our study shows that infusion of a selective CRF_2 receptor antagonist to the dRN completely blocks sensitized CRF-induced release of 5-HT in the CeA in amphetamine pretreated rats, a potential role for CRF_1 receptors cannot be ruled out and should be investigated in the future. Furthermore, the relationship between 5-HT activity in the CeA and dysphoric states during amphetamine withdrawal should be explored, in addition to the possible use of the CRF_2 receptor antagonists to alleviate psychostimulant withdrawal symptoms.

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