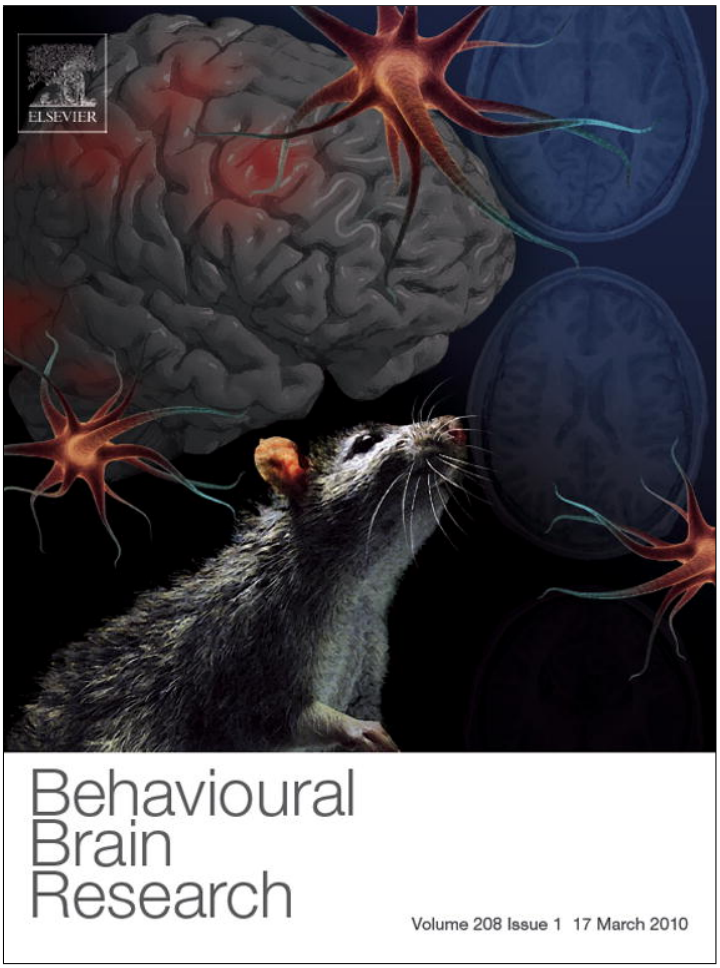

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Short communication

Increased anxiety-like behavior of rats during amphetamine withdrawal is reversed by CRF_2 receptor antagonism

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ABSTRACT

Withdrawal from psychostimulants increases anxiety states, and amphetamine-treated rats show increased CRF_2 receptors in the serotonergic cell body region, the dorsal raphe nucleus (dRN). In the current study, amphetamine (2.5 mg/kg, i.p., 14 days) pre-treated rats spent less time in open arms of the elevated plus maze compared saline pre-treated rats at both 24 h or 2 weeks of withdrawal, and CRF_2 receptor antagonism (ASV-30; 2 μg/0.5 μl) within the dRN reversed the effects of amphetamine withdrawal on anxiety-like behavior. Overall, results suggest that CRF_2 receptor antagonism may be a novel pharmacological target for anxiety states during drug withdrawal.

Transition from drug use to addiction may result, in part, from the need to alleviate dysphoric and anxiety states that emerge during drug abstinence [8]. In animal models, short-term (24–48 h) withdrawal from cocaine increases anxiety-like behaviors [3,7,15,18] which are thought to drive drug craving and relapse [8,20]. However, an animal model of heightened anxiety states during protracted psychostimulant withdrawal has not been described, but is necessary to examine the neurobiology underlying anxiety during withdrawal.

Corticotropin-releasing factor (CRF) is released from the amygdala in response to stressors [12], and increases anxiety-like behaviors in rodents [11]. Likewise, psychostimulants activate CRF neurons [17], and alterations to CRF levels throughout the limbic system have been observed during psychostimulant withdrawal [19]. Alterations in CRF systems may represent a compensatory neuroadaptation to restore homeostatic function in response to the presence of the drug, but result in emotional dysfunction in the absence of the drug [8]. In support, intracerebroventricular (icv) administration of CRF antiserum or a CRF receptor antagonist reverses the heightened anxiety behavior observed at 48 h after cocaine withdrawal in rats [3,18].

The serotonin (5-HT) neurons of the dorsal raphe nucleus (dRN) project throughout the limbic system and 5-HT is important in the regulation of anxiety states [13]. Both CRF_1 and CRF_2 receptors are found in the dRN [4] and have opposing effects on 5-HT release [9]. Activation of CRF_2 receptors in the dRN increases 5-HT release in limbic brain regions [1,6,9]. Importantly, amphetamine treatment of rats daily for 14 days increased CRF_2 receptors in the dRN, which persisted for at least 6 weeks of withdrawal [16].

While activation of central CRF_1 receptors is associated with increased anxiety-like behavior in animal models, the exact role of CRF_2 receptors has not been clear [21]. Studies of CRF_2 knockout mice suggest that CRF_2 receptor activity may be associated with reduced anxiety-like behaviors [2]. However, icv administration of specific CRF_2 receptor agonists or antagonists increase or decrease rodent anxiety-like behaviors respectively, suggesting an anxiogenic effect of CRF_2 receptor activity [21]. Given that CRF_2 receptors are elevated in the dRN of amphetamine-treated rats during protracted withdrawal [16], we hypothesized that amphetamine-treated rats would show heightened anxiety-like behavior during long-term withdrawal, and that administration of a specific CRF_2 receptor antagonist into the dRN would reverse this anxiety-like behavior.

Sixty-eight male Sprague–Dawley rats were acquired from the University of South Dakota Laboratory Animal Services (Vermillion, SD, USA) and housed in pairs at 22 °C (60% relative humidity) on reverse 12 h light/dark cycle (dark cycle started at 10:00 a.m.) with food and water freely accessible. The procedures were approved by the IACUC of the University of South Dakota, and were carried out in accordance with the NIH Guide for the Care and Use of Laboratory Animals. Adult male rats were injected with amphetamine (2.5 mg/kg, i.p.) or saline daily within their holding room for 14

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consecutive days [16]. Injections began 1 h after the start of the dark cycle and rats were returned to their home cages following injections.

To determine whether increased anxiety-like behavior emerges immediately upon amphetamine withdrawal or during protracted withdrawal, rats were allocated to a 24-h or 2-week withdrawal group ($n = 10$ per treatment group at each time period). The 24-h period represents the shortest withdrawal period that can be easily distinguished and is the time point that reflects expectation of a subsequent injection. Rats were tested on the elevated plus maze (EPM) at 24 h or 2 weeks after the last amphetamine or saline injection, and testing took place 1 h after the onset of the dark phase of the light cycle in a dark room under red lighting. Each arm of the EPM was 50 cm × 11 cm, and the maze was elevated 1 meter from the ground with a camera positioned overhead (Noldus Information Technology, Wageningen, The Netherlands). Rats were introduced to the center of the EPM facing an open arm and allowed to explore the maze for 5 min. Latency to enter an open arm (s), time spent on open arms (s), and total distance traveled (cm) were measured by Ethovision 3.1 (Noldus Information Technologies).

To determine whether antagonism of CRF_2 receptors in the dRN could reverse increased anxiety-like behavior exhibited by amphetamine-treated rats in withdrawal, rats were tested for anxiety-like behavior 2 weeks following the last treatment. Seven days after last injection, saline and amphetamine-treated rats ($n = 14$ per treatment group) underwent aseptic stereotaxic surgery for implantation of guide cannula into the dRN as described by [10]. Rats were anesthetized with a ketamine (80 mg/kg, i.p.; Met-Vet, Libertyville, IL) xylazine (16 mg/kg, i.p.; Met-Vet) mixture and placed into a small mammal stereotaxic frame (David Kopf Institute, CA, USA). A 22-gauge, 5 mm long stainless steel guide cannula (Plastics One, Roanoke, VA) was stereotaxically implanted 1 mm above the dRN (AP: −7.8 mm from bregma; ML: −2.6 from midline [14]) at a 23° lateral to medial angle, so to avoid the cerebral aqueduct and to ensure a bilateral infusion [10]. At the conclusion of surgery, the analgesic Ketoprofen (5 mg/kg, i.m.; Met-Vet) was administered. All rats were allowed 3 days of recovery before being acclimated to the dRN infusion procedure.

Following 3 days of acclimation to the infusion procedures as detailed by [10], rats were infused with either vehicle (2% ethanol) or the selective CRF_2 receptor antagonist antisauvagine-30 (ASV-30; 2 μg/0.5 μl; Sigma [9]) using a 30-gauge stainless steel infusion cannula (1 mm longer than the guide) inserted into the guide cannula. This vehicle has no effect on 5-HT release when infused into the dRN, and the dose of ASV-30 used is sufficient to completely block CRF_2 receptor-mediated effects in the dRN [6,9]. Furthermore, this volume infused into the dRN is thought to provide a specific bilateral effect, with little effective diffusion outside the dRN [5,10]. Twenty minutes after dRN infusion, rats were introduced to the EPM and behaviors measured as described above.

Rats were euthanized with Fatal Plus (0.5 ml, i.p.; Vortech, Dearborn, MI, USA), brains were removed and fixed in 10% formalin. Brains were sectioned (60 μm) on frozen, stained with cresyl violet, and analyzed under light microscope by two experimenters blind to treatment. Only data from rats with cannulae placements in the dRN were included in the following data analyses ($n = 7$ amphetamine or saline pre-treated rats per dRN infusion group).

Behaviors were compared between saline and amphetamine pre-treatment groups at 24 h and 2 weeks following the last treatment, or between pre-treatment groups that received vehicle or ASV-30 infusions, using separate two-way ANOVAs. Significant effects were analyzed further using Student–Newman–Keuls post-hoc test for multiple pair-wise comparisons. Significance levels for all statistical tests were set at $P \leq 0.05$ (SigmaStat v2.03, SPSS Inc., Point Richmond, CA, USA).

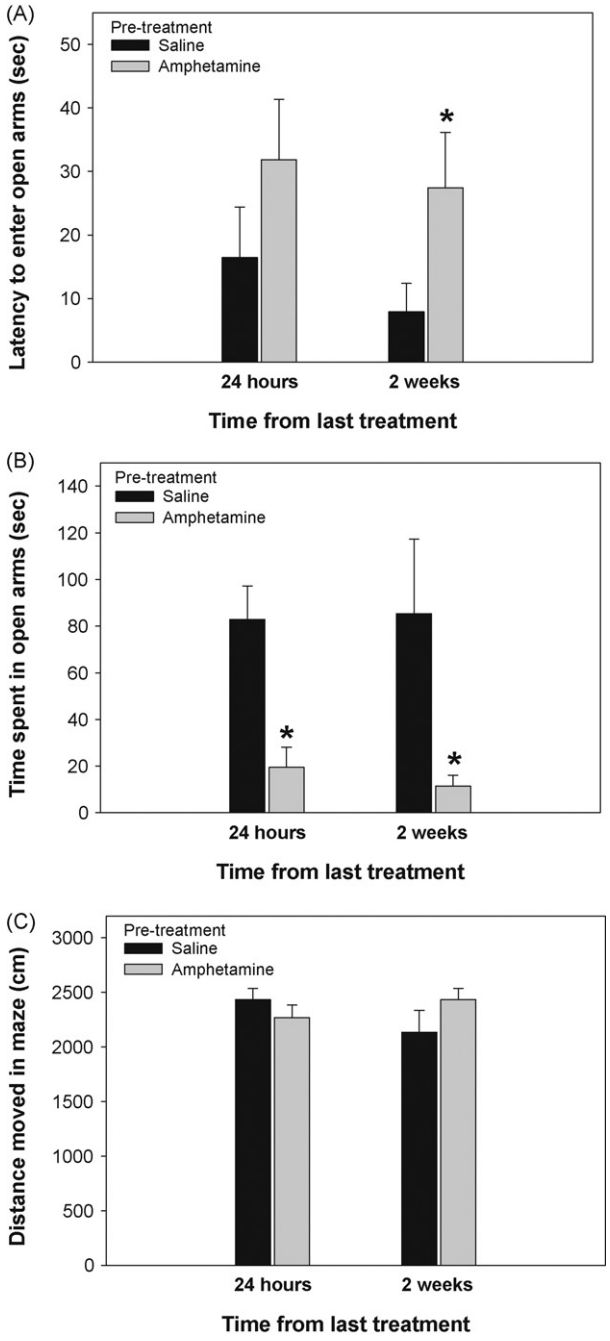

Fig. 1. (A) Latency to enter open arms, (B) time spent in open arms and (C) total distance moved in the elevated plus maze for amphetamine and saline pre-treated rats at 24 h and 2 weeks withdrawal. Data represent mean ± S.E.M. $N = 10$ per treatment at each time point. *Significant differences between saline and amphetamine pre-treated rats within the one time point.

When anxiety-like behavior during amphetamine withdrawal was assessed, latency to enter open arms was significantly affected by pre-treatment treatment ($F_{1,33} = 8.338$, $P = 0.007$), but there was no effect of withdrawal period ($F_{1,33} = 0.173$, $P = 0.680$), nor an interaction between withdrawal period and pre-treatment ($F_{1,33} = 0.665$, $P = 0.421$). A significant increase in latency was observed between treatment groups only within the 2-week withdrawal group (SNK $P < 0.05$; Fig. 1A). Similarly, time spent in open arms was significantly different between pre-treatment groups ($F_{1,33} = 25.850$, $P < 0.001$) but was not affected by withdrawal period ($F_{1,33} = 0.0421$, $P = 0.839$), and an interaction between pre-

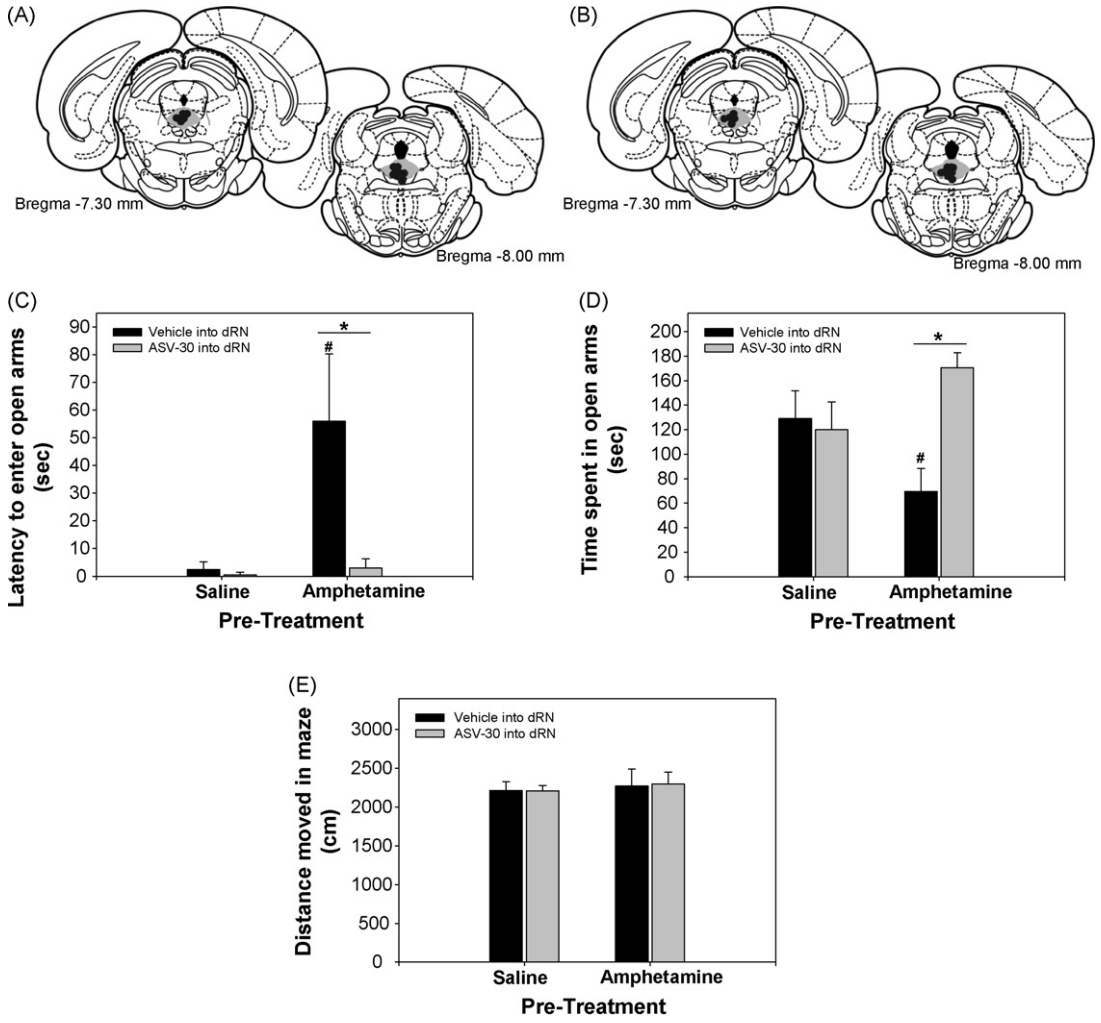

Fig. 2. Schematic representations (adapted from [14]) illustrating the location of dorsal raphe nucleus (dRN) infusions for (A) saline pre-treated and (B) amphetamine pre-treated rats. (C) Latency to enter open arms, (D) time spent in open arms and (E) total distance moved in the elevated plus maze for amphetamine and saline pre-treated rats infused with either vehicle or ASV-30 into the dRN. Data represent mean ± S.E.M. $N = 7$ per treatment group. *Significant differences between ASV-30 and vehicle within the same pre-treatment group. #Significant differences between saline and amphetamine pre-treated rats within the same dRN treatment group.

treatment and withdrawal period was not observed ($F_{1,33} = 0.152$, $P = 0.699$). Time spent in the open arms was significantly decreased in the amphetamine treatment groups compared to saline-treated rats within both the 24-h and 2-week withdrawal periods (SNK $P < 0.05$; Fig. 1B). There was no effect of treatment ($F_{1,33} = 0.586$, $P = 0.449$), withdrawal ($F_{1,33} = 3.561$, $P = 0.068$), nor an interaction between treatment and withdrawal ($F_{1,33} = 0.362$, $P = 0.551$) on the total distance traveled in the maze (Fig. 1C).

For experiments that tested CRF_2 receptor mediation of amphetamine-induced anxiety-like behavior, placement of infusion cannulae into the dRN did not differ between saline and amphetamine pre-treated rats (Fig. 2A and B). A significant effect of pre-treatment ($F_{1,24} = 6.155$, $P = 0.021$), dRN infusion ($F_{1,24} = 5.900$, $P = 0.023$), and a significant interaction between pre-treatment and dRN infusion ($F_{1,24} = 4.916$, $P = 0.036$) was observed in the latency to enter open arms. Amphetamine pre-treated rats infused with vehicle showed greater latency to enter open arms compared with saline pre-treated rats and amphetamine pre-treated rats infused with ASV-30 (SNK $P < 0.05$; Fig. 2C). For time spent in opens arms, there was an effect of dRN infusion ($F_{1,24} = 6.715$, $P = 0.016$) and an interaction between drug pre-treatment and dRN infusion ($F_{1,24} = 9.686$, $P = 0.005$). Amphetamine pre-treated rats infused with vehicle showed reduced time in open arms compared with saline pre-treated rats and amphetamine pre-treated rats infused with ASV-30 (SNK $P < 0.05$; Fig. 2D). There was no significant effect of pre-treatment ($F_{1,24} = 0.296$, $P = 0.592$), dRN infusion ($F_{1,24} = 0.00752$, $P = 0.932$), nor an interaction of pre-treatment and dRN infusion ($F_{1,24} = 0.0108$, $P = 0.918$) on total distance traveled in the maze (Fig. 2E).

Overall, our results show that rats exhibited heightened anxiety-like behaviors both 24 h and 2 weeks following the last repeated injection of amphetamine. The distance traveled in the EPM was nearly identical between saline- and amphetamine-treated groups at both time points measured, suggesting that differences observed in anxiety-like measures were not due to differences in the activity between the treatment groups. Therefore, these findings suggest that chronic amphetamine treatment increases anxiety states, which persist during protracted drug withdrawal.

Infusion of a CRF_2 receptor antagonist directly into the dRN reduced anxiety-like behaviors exhibited by amphetamine pre-treated rats at 2 weeks of withdrawal, with no effect on locomotion within the maze. These results extend those of Pringle et al. [16] showing increased CRF_2 receptors in the dRN up to 6 weeks following 14 days of amphetamine treatment, and of Sarnyai et al. [18] and Basso et al. [3] demonstrating increased anxiety-like behaviors of rats at 48 h of withdrawal following 14 days of cocaine treatment, which were ameliorated by icv CRF antiserum or a $CRF_{1/2}$ receptor antagonist. Combined, these findings suggest that

one of the mechanisms by which chronic psychostimulant treatment and withdrawal results in elevated anxiety states may be increased CRF_2 receptor activity in the dRN. Given that CRF_2 receptor activation within the dRN leads to increases in 5-HT release in limbic regions such as the amygdala and nucleus accumbens [1,9], heightened CRF_2 receptor activity in the dRN during amphetamine withdrawal may amplify serotonergic activity within the limbic system to result in increased anxiety states.

Interestingly, ASV-30 infusion in the dRN of saline pre-treated rats had no effect on anxiety-like behaviors. This result may be due to the saline pre-treated group exhibiting relatively low levels of anxiety-like behaviors (when compared to saline pre-treated rat of the first experiment that were not acclimated to handling prior to EPM testing). Our result is consistent with a number of studies showing that general CRF receptor antagonists administered intracranially or icv have little effect on the behavior of non-stressed animals (see [10]). However, in some studies, icv administration of ASV-30 decreases anxiety-like behavior of rats in the EPM without prior exposure to stress or drugs [21]. The discrepancy in findings may be due to the site of infusion, with CRF_2 receptor antagonism specific to the dRN only effective in rats that show heightened CRF_2 receptor levels in this region. This implies that chronic exposure to amphetamine results in dRN CRF_2 receptor regulation of behavior that is not typically mediated by the activity of these receptors in drug naive rats.

In summary, the current data suggest that chronic amphetamine treatment of rats results in an increased anxiety state which persists following drug abstinence, and that CRF_2 receptor antagonism within the dRN reverses this anxiety state. Future research should confirm these findings using different anxiety paradigms with more global antagonism of central CRF_2 receptors. Such future studies may suggest CRF_2 receptor antagonism as a possible direction for therapeutic treatment to reduce anxiety states during amphetamine withdrawal.

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