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Inactivation of the dorsal raphé nucleus reduces the anxiogenic response of rats running an alley for intravenous cocaine

Aaron Ettenberg *, Oren A. Ofer, Carl L. Mueller, Stephanie Waldroup, Ami Cohen, Osnat Ben-Shahar

Behavioral Pharmacology Laboratory, Department of Psychology, University of California, Santa Barbara, United States

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ABSTRACT

Rats traversing a straight alley once a day for delivery of a single i.v. injection of cocaine develop over trials an ambivalence about entering the goal box. This ambivalence is characterized by the increasing occurrence of "retreat behaviors" where animals leave the start box and run quickly to the goal box, but then stop at the entry point and "retreat" back toward the start box. This unique pattern of retreat behavior has been shown to reflect a form of "approach–avoidance conflict" that stems from the animals' concurrent positive (cocaine reward) and negative (cocaine-induced anxiety) associations with the goal box. Cocaine blocks reuptake of the serotonergic (5-HT) transporter and serotonin has been implicated in the modulation of anxiety. It was therefore of interest to determine whether inactivation of the serotonergic cell bodies residing in the dorsal raphé nucleus (DRN) and projecting to brain areas critical for the modulation of anxiety, would alter the anxiogenic state exhibited by rats running an alley for single daily i.v. injections of 1.0 mg/kg cocaine. Reversible inactivation of the DRN was accomplished by intracranial application of a mixed solution of the GABA agonists baclofen and muscimol. While DRN inactivation had no impact on the subjects' motivation to initiate responding (i.e., latencies to leave the start box were unaffected) it reliably reduced the frequency of approach–avoidance retreat behaviors (conflict behavior). These data suggest that inactivation of the dorsal raphé reduces the conflict/anxiety otherwise present in experienced cocaine-seeking animals.

1. Introduction

In addition to cocaine's well-known positive/rewarding properties, the drug produces a host of negative and/or aversive effects. So while cocaine is readily self-administered by both humans and animals (e.g., Comer et al., 2008; Fischman and Schuster, 1982; Haney, 2009; Porrino et al., 2004; Wolverton, 1992), produces preferences for places associated with its administration in animals (Aguilar et al., 2009; Bardo et al., 1995; Carr et al., 1989) and is described as having rewarding or euphoric properties by human users (Lynch et al., 2006; Rotheram-Fuller et al., 2007; Singha et al., 1999), the "crash" that follows its administration in human cocaine users is characterized by feelings of anxiety, agitation and craving for more drug (Gawin, 1991; Gawin and Kleber, 1986; Resnick and Resnick, 1984; Rohsenow et al., 2007; Williamson et al., 1997). Similarly, while animals develop preferences for places associated with the immediate positive effects of cocaine, they exhibit place aversions for locations paired with the effects present 15 min *after* an i.v. injection (Ettenberg and Bernardi, 2007; Ettenberg et al., 1999; Knackstedt et al., 2002). Cocaine has also been reported to enhance the anxiogenic response of animals to the presentation of a variety of learned and unlearned aversive stimuli (e.g., Costall et al., 1989; Dworkin et al., 1989; Hayase et al., 2005; Paine et al., 2002; Simon et al., 1994).

The mixed positive and negative properties of cocaine can be assessed concurrently in the same animal during the same trial. In our laboratory, rats trained to run a straight alley once each day for an i.v. injection of cocaine develop over trials a unique "retreat behavior" in which the subjects rapidly approach the goal, but then stop at the goal-box threshold, and turn and retreat back toward the start box (Ettenberg and Geist, 1991). This unique pattern of runway behavior closely resembles that of animals approaching a goal box with known positive and negative associations (e.g., food + foot-shock; Cohen et al., 2009; Geist and Ettenberg, 1997; Miller, 1944). Retreats are therefore thought to reflect a form of "approach–avoidance conflict" that stem from cocaine's immediate positive (cocaine reward) and delayed negative (cocaine-induced anxiety) properties (Ettenberg, 2004, 2009). Indeed, like other forms of conflict behavior, retreat frequency is dose-dependently attenuated by pretreatment with anxiolytic drugs (Ettenberg and Geist, 1991; Guzman and Ettenberg, 2004; Knackstedt and Ettenberg, 2005).

Given the growing literature implicating 5-HT circuitry in the modulation of anxiety-related states (e.g., Abrams et al., 2004; Begg et al., 2005; Chaouloff, 2000; Hammack et al., 2009; Sena et al., 2003), it was of interest to investigate the role of 5-HT function in the anxiogenic response to self-administered cocaine. Toward that end, we have shown that the 5-HT_{1A} partial agonist buspirone (Eison and

* Corresponding author. Department of Psychology, University of California, Santa Barbara, CA 93109-9660, United States. Tel.: +1 805 893 3682; fax: +1 805 893 4303.
E-mail address: Ettenberg@psych.ucsb.edu (A. Ettenberg).

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Temple, 1986; Jahanshahi et al., 2010) can reduce the approach–avoidance conflict of rats running an alley for i.v. cocaine (Ettenberg and Bernardi, 2006) and block the delayed aversive effects of cocaine as measured in a conditioned place preference test (Ettenberg and Bernardi, 2007). 5-HT_{1A} receptors are typically coupled to inhibitory G-proteins whose activation produces a hyperpolarization of cell membranes that results in an inhibition of neuronal activity (Aghajanian, 1995; Albert et al., 1997), thus the effects of buspirone could be accounted for by an inhibition of 5-HT neurotransmission. However, since buspirone has been shown to exert effects on multiple neurotransmitter systems (e.g., Eison and Temple, 1986; Jahanshahi et al., 2010), the current study adopted a more direct approach by examining the impact on cocaine-induced approach–avoidance conflict behavior of inactivating the dorsal raphé nucleus — the primary source of 5-HT in the rat forebrain (Azmitia and Segal, 1978; Vertes, 1991).

2. Methods

2.1. Subjects

The subjects were 16 male Sprague Dawley rats obtained from Charles River Laboratories (Wilmington, MA, USA). The animals were housed individually in plastic cages located within a secure and temperature-controlled vivarium (23 °C). Subjects were gentled each day during the week prior to surgery and provided *ad libitum* access to food and water throughout the study. All aspects of the experimental protocol were reviewed and approved by the University of California at Santa Barbara's Institutional Animal Care and Use Committee (IACUC) for compliance with the National Institutes of Health *Guide for the Care and Use of Laboratory Animals*. Over the course of the experiment, six animals were removed from the study either due to i.v. catheter failure, misplaced intracranial cannulae, or the subject's failure to acquire the operant runway response (i.e., no improvement in start latencies and/or development of retreat behaviors as trials progressed). Ten animals successfully completed all aspects of the within-group experimental protocol (as described below).

2.2. Surgery

Each animal (300–350 g at the time of surgery) was surgically implanted with a chronic silastic jugular catheter and intracranial guide cannula under deep isoflurane-induced anesthesia administered continuously via inhalation (4% for induction, 1.5–2.5% for maintenance). During surgery, animals received an injection of atropine sulfate (0.04 mg/kg; IM) to prevent respiratory congestion and a non-opioid analgesic, flunixin meglumine (2.0 mg/kg; SC; FluMeglumine; Phoenix Pharmaceuticals, Belmont, California, USA), to alleviate post-surgical pain. The i.v. catheters each consisted of a 13 cm segment of polyethylene tubing (PE 20) attached to a threaded 22 gauge guide cannula (Plastics One; Roanoke, Virginia, USA) that exited the animal's back from a 3 mm biopsy-punch opening. Each cannula was fastened with dental cement to a 2.0 cm square of surgical Mersilene mesh (Ethicon Co, Sommerville, NJ, USA) that was laid flat subdermally on the animal's back. The opposite end of the PE catheter was inserted into the rat's jugular vein where it was sutured in place. Immediately following catheterization, each subject was administered 50 mg of the antibiotic ticarcillin disodium/clavulanate potassium (Timentin) dissolved in 0.25 ml of 0.9% physiological saline through the implanted catheter to prevent infection, followed by the anticoagulant heparin (66 IU/ in 0.1 ml of saline) to maintain catheter patency.

Following jugular catheterization and during the same surgical session, each rat was placed in a stereotaxic instrument and implanted with a single 22 gauge guide cannula (Plastics One, Roanoke, Virginia, USA) aimed at the mid-line (straddling both hemispheres) of the dorsal raphé nucleus using the coordinates derived from the rat brain atlas of Paxinos and Watson (2007). The coordinates were: −7.8 mm posterior to bregma, −1.6 mm from the midline, and −5.6 mm from the skull surface at a 14° angle to bypass the sagittal sinus. The injection cannulae used for drug infusion (described below) protruded 1 mm beneath the tip of the guide cannulae. The cannulae were secured to the skull with the use of dental cement and four stainless steel screws. An obdurator was inserted into each guide cannula to seal the opening and thereby maintain cannula patency and reduce the risk of infection. Finally, before returning animals to their home cages for recovery, each subject was injected (SC) with 3.0 ml of 0.9% physiological saline to prevent dehydration. Animals were permitted to recover from surgery for at least 7 days prior to the initiation of behavioral testing.

Each day following surgery, animals were injected through the i.v. catheter with a 0.1 ml solution of heparin (66 IU) and Timentin (20 mg/infusion during the 7-day recovery period and 10 mg each day thereafter) prepared in a vehicle of 0.9% physiological saline. Prior to the start of the experiment, after each week of testing, and upon completion of the experiment, catheter patency was confirmed by observing the loss of each subjects' righting reflex after an i.v. injection of the fast-acting barbiturate, Brevital (2.0 mg/kg/0.1 ml).

2.3. Drug administration

Cocaine hydrochloride (provided by the National Institute of Drug Abuse) was dissolved in a vehicle solution of 0.9% physiological saline and administered i.v. in a dose of 1.0 mg/kg via a 10 ml syringe seated in a motorized syringe pump (Razel Scientific Instruments, St Albans, Vermont, USA). The cocaine dose was selected because it produced optimal running in previous work (e.g., Raven et al., 2000). To achieve temporary inactivation of the dorsal raphé nucleus, the region was infused with a mixed solution of the GABA-A agonist muscimol and the GABA-B agonist baclofen (Sigma Aldrich, USA) prepared in a vehicle solution of 0.9% physiological saline. Each drug was initially prepared in a separate solution of 500 ng/μl so that the combination of the two drugs produced a single solution in which both drugs were at a concentration of 250 ng/μl. An injection volume of 0.3 μl/side was used resulting in an infusion of 75 ng of drug solution. This means that reversible inactivation has been successfully employed by us and by others in investigations of the functional significance of a number of brain structures including the dorsal raphé nucleus (e.g., Floresco et al., 2006, 2008; McFarland and Kalivas, 2001; Moscarello et al., 2010; Tao and Auerbach, 2002).

2.4. Runway apparatus

All behavioral testing was conducted in a single straight-arm runway measuring 155 cm long × 15 cm wide × 40 cm high. A start box and goal box of equal dimensions (24 × 25 × 40 cm) were connected at opposite ends of the alley. The floor of the apparatus consisted of 3 mm diameter steel rods laid in parallel 1.2 cm apart and oriented perpendicular to the sidewalls of the runway. Thirteen infrared photocell emitter–detector pairs were distributed approximately 15 cm apart along the inside walls of the long axis of the runway 2.5 cm above the floor. These infrared sensors were wired to a custom interface that was in turn connected to a desktop computer running ANY-maze® software through a Stoelting Co. (Wood Dale, IL, USA) I/O AMi interface. The custom software recorded the precise location of the animal in the runway in real time throughout each trial. The computer also controlled the opening of the start box door, the closing of the goal box door, and the delivery of the drug reinforcer (i.e., activation of the syringe pump).

Suspended above the alley were two long bar magnets aligned in parallel along the entire length of the apparatus. Positioned between the magnetic rails was a liquid swivel (model 375-22PS, Instech Laboratories Inc., Plymouth Meeting, PA, USA) that connected the

guide cannula on each animal's back to the 10-ml drug filled syringe (seated in the Razel pump) via polyethyne-20 tubing. The swivel was seated in the center of a plastic collar or disk that prevented it from falling through the gap between the rails. A pot magnet was attached to the underside of the plastic disk with the polarity arranged to repel the charge of the magnetic rails. The resulting magnetic repulsion between the swivel assembly and the rails permitted the swivel to float slightly above the tracks. Thus, this system served as a low-friction and low-resistance mechanism that allowed the rat to move freely throughout the alley, pulling the swivel assembly along behind and above it as it moved (for a more complete description of the runway apparatus, see Geist and Ettenberg, 1990).

2.5. Procedure

On the day prior to the initiation of runway training, each rat was placed into the runway with the start door open and allowed to acclimate to the apparatus during a single 10 min session. The goal door was closed during this single acclimation session. Runway testing was then initiated on the next day and continued, one trial per day, for 18 days. On each trial, the subject was connected to the cocaine-delivery system by connecting PE tubing emanating from the swivel to the guide cannula mounted on the animal's back. The animal was then placed into the start box where, after 5 s, the start door was opened and the trial initiated. The subject was then free to travel the length of the alley until it entered the goal box whereupon the goal door was automatically closed (restricting the animal to the goal box) and a single i.v. infusion of cocaine (1.0 mg/kg) was administered. Although uncommon (i.e., occurring on less than 5% of trials), on occasions where an animal had not entered enter the goal box within 10 min, it was manually placed inside the goal box whereupon the cocaine was administered. Each animal was removed from the goal box 5 min post-injection, disconnected from the drug delivery system, and returned to its home cage.

The 18-day training period was intended for subjects to form a strong association between the goal box and the effects of cocaine (as confirmed by fast latencies to initiate responding and the development of approach–avoidance retreat behaviors by the end of the second week; e.g., see review by Ettenberg and Geist, 1991). The formal testing then occurred over two consecutive days/sessions. Immediately prior to each session, animals were fitted with an injection cannula (that protruded 1 mm beyond the tip of the guide cannula) and slowly infused with 0.3 µl of vehicle or baclofen/muscimol solution directly into the dorsal raphé nucleus over a 3 min period. After each injection, an additional minute was allowed for the solution to diffuse away from the cannulae tip, after which time the injection cannula was removed, the obdurator replaced, the animals put into a holding cage for 5 min, and then placed into the start box of the runway for a single cocaine-reinforced runway trial. Each animal received both the vehicle and the baclofen/muscimol treatment on different days in a counterbalanced order. So for the first session (trial 19), half the animals experienced a cocaine runway trial with a function intact and half with an inactivated dorsal raphé nucleus, with these conditions reversed for each animal on the next day/session (trial 20). A third and final trial was conducted on the 21st day of the protocol with no intracranial infusions to assess whether the preceding days' treatments had produced any long-lasting effects.

2.6. Dependent measures

Three dependent measures were collected on each trial: *Start Latency* — the time required for the animal to enter the runway once the start door had opened; *Run Time* — the time required for the animal to traverse the alley and enter the goal box once it had left the start box; and *Retreat Frequency* — the number of times that an animal advanced to the goal box but then stopped, turned around, and "retreated" backwards toward the start box. Each retreat required that the rat stops its forward locomotion and returns toward the start box through a distance spanning two of the infrared photoemitter–detector pairs (i.e., approximately 30 cm). Upon completion of each trial, the software generated a "spatiotemporal record" that provided a graphic representation of the path that the animal took to the goal on that particular trial.

2.7. Histology

After completion of behavioral testing, all animals were deeply anesthetized by an i.v. injection of sodium pentobarbital (50 mg in a volume of 0.1 ml) and transcardially perfused with a 10% formalin solution. Brains were removed and sliced in 40-µm frozen sections prior to being mounted and stained with Cresyl Violet. Cannula placements were verified by magnified visual inspection of the slides using Paxinos and Watson (2007) as a guide.

3. Results

Histological analyses confirmed the location of the cannulae in the dorsal raphé nucleus in eight of the ten subjects (see Fig. 1). A problem with the freezer component of our cryostat rendered two of the samples unreadable and no confirmation of cannula location could be determined for these animals. The data from these two subjects were nevertheless included in the data analyses since their behavioral responses during both the vehicle and baclofen/muscimol trials were comparable to those of the other members of the group. Histological analyses identified no significant tissue trauma in the region where the cannula tips were located and indeed runway behavior returned to "normal" on the day following the intracranial infusions (see below).

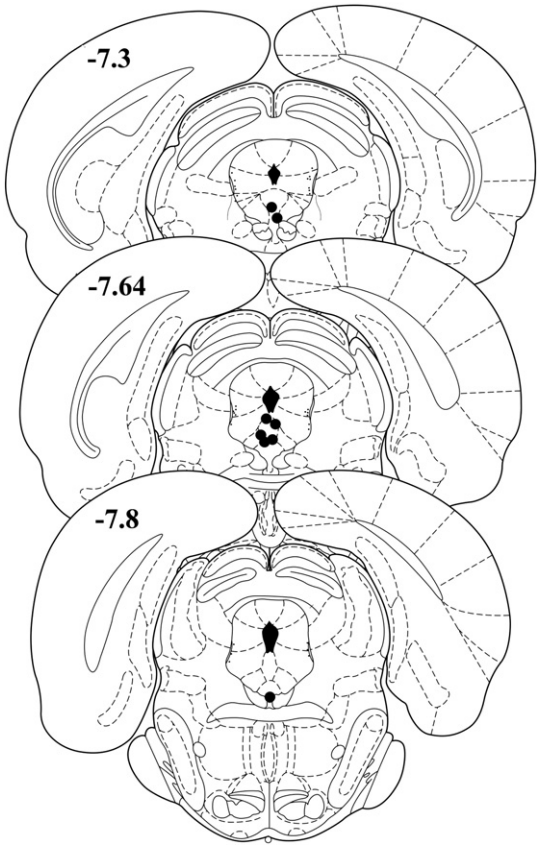

Fig. 1. Locations of injection cannula placements within the dorsal raphé nucleus. Numbers represent mm from bregma. Redrawn from Paxinos and Watson (2007).

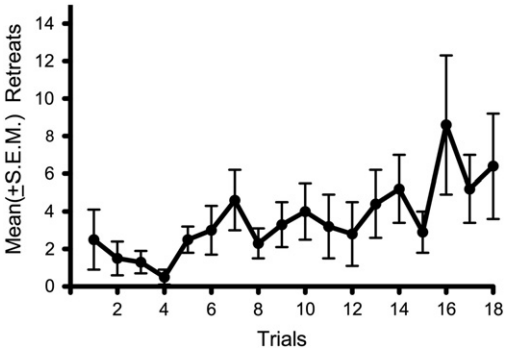

Fig. 2. The development of approach–avoidance conflict (i.e. retreat behaviors) in a group of ten rats running a straight alley for single daily injections of 1.0 mg/kg i.v. cocaine. The data are expressed as the means (±S.E.M.) frequency of retreat behaviors per trial.

In previous work (reviewed in Ettenberg, 2004, 2009), the subjects' motivation to seek cocaine was reflected by a progressive decrease in start latencies as trials progress, while the mixed positive + negative attributes of cocaine were reflected in the animals' growing ambivalence about goal box entry (i.e., increased retreat frequency) and the consequent decrease in Run Times. These results were confirmed in the current study: start latencies over the first three trials averaged 25.4 s and fell to 1.8 s over the final three trials of the 18-day training period. Conversely, Run Times increased over trials (as one would expect if animals are exhibiting more and more retreats per trial) although only modestly; average run times over the first three trials were 215 s increasing to 271 s over the final three trials. Repeated measures one-way analyses of variance (ANOVA) computed on data derived over the 18 days/trials of runway training confirmed a statistically significant reduction in Start Latencies ($F(17,153) = 1.33$, $p = .04$) but no reliable change in Run Times ($F(17,153) = 1.33$, $p > .05$) as the experiment progressed.

Of course, since our intent was to firmly establish retreat behaviors (a putative index of approach–avoidance conflict) against which the effects of dorsal raphé nucleus (DRN) inactivation would be assessed, retreat frequency represents the most critical dependent measure in the current study. The development of retreat behaviors is depicted in Fig. 2. The ANOVA computed on these data confirmed a highly significant increase in retreat frequency as trials progressed ($F(17,153) = 2.13$, $p = .01$). There was also a concomitant change in the qualitative nature of retreat behaviors as testing continued. During the first three runway trials 16% of the retreats were emitted in close proximity to the goal (i.e., at the entryway), compared to 91% during the final three trials. Hence as animals learn the association between the goal box and cocaine, retreat behaviors increase in frequency and become restricted to the vicinity of the goal box entryway (see also Fig 4).

The mean (+SEM) Start Latencies, Run Times, and Retreats of animals running the alley for i.v. cocaine on the two test days is illustrated in Fig. 3. The open bars represent the scores of animals pretreated with the intracranial vehicle solution ("DRN intact") and the hashed bars depict the same animals' performance during dorsal raphé nucleus inactivation ("DRN inactivated"). Comparisons of the baclofen/muscimol results with vehicle results were determined by two-tailed repeated measures *t*-tests. As suggested by the data depicted in the figure, inactivation of the dorsal raphé nucleus produced: a) no change in the subjects' start latencies ($t(9) = 0.17$, $p > .05$); b) a significant reduction in run times (the animals entered the goal box sooner; $t(9) = 3.44$, $p = .007$); and c) significantly fewer retreats during DRN inactivation compared to vehicle baseline ($t(9) = 2.57$, $p = .03$). Note that on the day after the DRN inactivation trial, all measures returned back to "normal" and were no longer different than vehicle-day ("intact") performance ($p > .05$).

A representative spatiotemporal record illustrating the difference in the pattern of runway behavior exhibited by the same subject during the "DRN intact" and "DRN inactivated" trials is provided as Fig. 4. In the figure, the numbers on the Y-axis refer to the locations of the photocells within the apparatus with "1" representing a location inside the start box, "2" a location just outside the start box in the alleyway of the apparatus, and "12" a location in the alley just outside the entry to the goal box. The X-axis represents time in seconds. Hence the curve depicts the path that the animal took to the goal in real time during the trial. In the "DRN intact" condition (on the left) this animal exhibited multiple approach and avoidance behaviors (i.e., retreats) as represented by the "peaks" in the chart and did not in fact enter the goal box by the 600 s (10 min) mark. In contrast, with the "DRN inactivated" (right side) this same subject exhibited very few retreats and entered the goal box within 250 s.

4. Discussion

The present results build upon and extend our prior work with the 5-HT_{1A} partial agonist, buspirone, and lend further support to the growing consensus that 5-HT cells emanating from the dorsal raphé nucleus (DRN) play an important role in the modulation of anxiety-related states (e.g., Abrams et al., 2004; Begg et al., 2005; Chaouloff, 2000; Eison and Eison, 1994; Graeff, 2002; Hammack et al., 2009; Sena et al., 2003). In previous work we reported that buspirone dose-

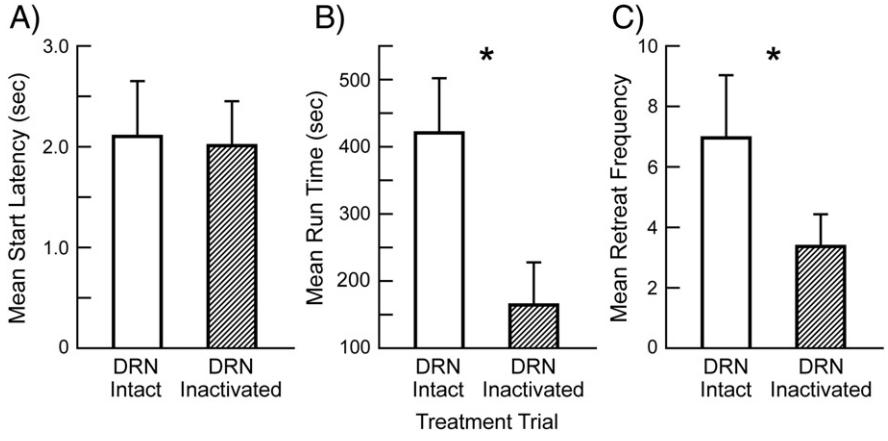

Fig. 3. Effects of dorsal raphé nucleus (DRN) inactivation on the start latencies (panel A), run times (panel B) and retreat frequency (panel C) of animals running an alley for a single i.v. injection of 1.0 mg/kg cocaine. Each animal was tested under two experimental conditions on two consecutive days in a counterbalanced order — the shaded bars represent performance following an intra-DRN infusion of baclofen/muscimol ("DRN inactivated") and the open bars represent the performance of the same animals following an intracranial infusion of vehicle ("DRN intact"). * indicates a statistically significant difference between "intact" and "inactivated" runway performance ($p < .05$).

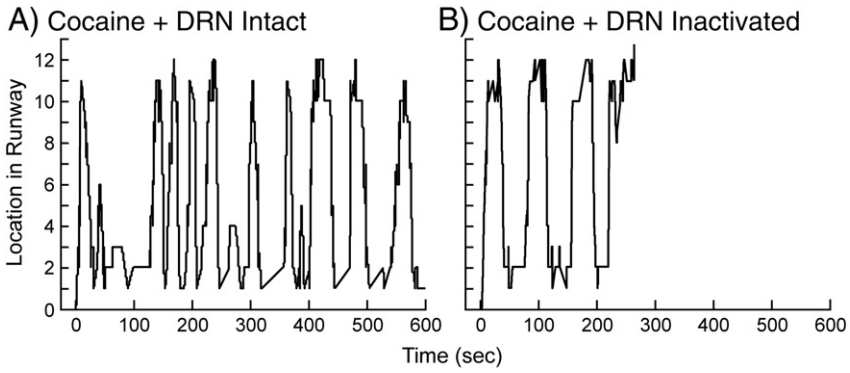

Fig. 4. Two spatiotemporal records depicting the runway behavior of a single representative animal during control (panel A) and DRN-inactivated (panel B) conditions. The *x*-axis indicates time (in seconds) and the *y*-axis represents locations within the runway with the number "2" being just outside the start box door, number "12" being at the threshold of the goal door, and number "13" being inside the goal box (and resulting in the closure of the goal door and the delivery of the i.v. cocaine reinforcer). Thus the curves reflect the path and speed of the animal's behavior from start to finish during each of the two trials. Retreat behaviors (a measure of approach–avoidance conflict about goal-box entry) are represented by the peaks in the records. Dorsal raphé inactivation produced a clearly distinguishable pattern of responding that involved a reduction in the frequency of retreat behaviors and a shorter run time (i.e., the rat entered the goal box sooner) compared to the vehicle "intact" trial.

dependently reduced the frequency of approach–avoidance retreat behaviors observed in animals running a straight alley for i.v. cocaine (Ettenberg and Bernardi, 2006). As reviewed in the Introduction to this paper, the occurrence of "retreats" reflects a subject's ambivalence about entering a goal box associated with the mixed positive (rewarding) and negative (anxiogenic) properties of i.v. cocaine administration (see review by Ettenberg, 2004). In this situation buspirone behaved comparably to diazepam (Ettenberg and Geist, 1991), suggesting that it can exert a strong anxiolytic profile in this novel model of approach–avoidance conflict. Since buspirone also impacts the neurotransmission of non-serotonergic systems (e.g., Eison and Temple, 1986; Jahanshahi et al., 2010), it was of interest to assess the impact on cocaine-induced retreat behaviors of selectively inactivating the cells of origin of the primary source of forebrain 5-HT (i.e., in the dorsal raphé nucleus). The results of these manipulations in the current study suggest that 5-HT inactivation significantly reduces the anxiogenic response of animals approaching a goal box associated with cocaine administration. More specifically, rats running a straight alley for i.v. cocaine during DRN inactivation made significantly fewer approach–avoidance retreat behaviors than did those same animals when the DRN was left intact (i.e., following intracranial vehicle administration).

The conclusion that the retreat behaviors reflect a form of approach–avoidance conflict is based upon the results from a large number of empirical studies conducted over the past decade (see reviews by Ettenberg, 2004, 2009). For example, the pattern and frequency of cocaine-induced retreat behaviors closely resemble that of food-deprived animals running to a goal-box where both food and shock are co-delivered (e.g., Cohen et al., 2009; Geist and Ettenberg, 1997; Miller, 1944). Hence goal boxes associated with mixed positive and negative events produced mixed approach and avoidance responses. Additionally, the spatial distribution of retreats in the current and prior studies was not random throughout the runway. Rather, the animals tended to stop and turn back toward the start box only when in close proximity to the goal box entryway (see Fig 4; and Ettenberg and Geist, 1991, 1992). Such data are consistent with classic animal behavior studies demonstrating that measures of conflict increase in magnitude and/or frequency with proximity to the target stimulus (e.g., Brown, 1942; Miller, 1944). Alternatively, one might suggest that the progressive increase in retreats observed in the current study stems not from any mixed positive–negative properties of the cocaine, but rather because cocaine simply reinforced the occurrence of spontaneously occurring retreat behaviors. Such an explanation would of course predict that any positive reinforcing goal box event would produce the same effects — and yet after literally dozens of studies conducted in our laboratory using the same apparatus with a wide variety of natural and drug reinforcers, only cocaine or the combination of food + shock have produced the frequency and pattern of retreats observed here (see Ettenberg, 2009). Finally, we note that like other behavioral measures of conflict, cocaine-induced retreats are dose-dependently attenuated by treatment with anxiolytic agents, such as diazepam and alcohol (Ettenberg and Geist, 1991; Guzman and Ettenberg, 2004). Hence the authors feel confident in concluding that the retreats observed in animals running an alley for i.v. cocaine reflect the presence of mixed positive and negative associations with the goal box that in turn stem from the drug's dual and opposing affective properties.

Of course, even if one accepts that retreats reflect the dual actions of self-administered cocaine, several explanations can be proposed to account for the change in retreat frequency observed during DRN inactivation, independent of a reduction in the subject's level of anxiety. For example, there has been considerable interest in the role of 5-HT systems and the dorsal raphé nucleus in memory formation and/or retrieval (e.g., Adams et al., 2008; Colpaert et al., 2000; Luna-Munguía et al., 2005; Michelsen et al., 2008; Perez-Garcia and Meneses, 2008). In the context of the current results, one might therefore suggest that DRN inactivation prevented the animals from retrieving their prior associations with the goal box and thereby reduced the subjects' ambivalence about entering. However, closer inspection of the entire data set suggests that a dysfunctional memory retrieval explanation for the current results is unlikely. For example, if the subjects' retrieval of prior associations with the goal box was prevented during DRN inactivation, then one would expect to see a reduction in response initiation (start latency) and not just retreat frequency. That is, the subjects should not be motivated to leave the start box quickly and approach a goal box about which they have no prior associative memories. In fact, DRN inactivation had no impact on start latencies (see Fig. 3), suggesting that the initiation of the operant runway response and therefore the motivation to seek the cocaine and approach the goal box were left intact. Additionally, DRN inactivation reduced but did not prevent the expression of approach–avoidance conflict (Figs. 3 and 4) — i.e., the animals still exhibited retreat behaviors (albeit in lower frequency) suggesting that their memories of prior goal-box events were left intact, while the motivational impact of those memories (i.e., the level of ambivalence or anxiety normally associated with those goal box associations) was reduced during DRN inactivation.

Previous research has demonstrated that the 5-HT system plays an important role in modulating the psychostimulant locomotor response to cocaine in rats (Carey et al., 2004, 2005; Szumlinski et al., 2004). Additionally, application of muscimol directly into the DRN has been shown to increase locomotor activity in and of itself (Paris and

Lorens, 1987; Sainati and Lorens, 1982). The argument might therefore be made that the baclofen/muscimol-induced inactivation of the DRN produced an increase in locomotor behavior that might account for the results of the current study. Indeed, such an explanation would be consistent with the observed reductions in run times observed during the DRN inactivation. The hypothesis is weakened, however, by the animals' pattern of responding and the inherent nature of retreat behavior. First and foremost, the effects of DRN inactivation cannot be accounted for by an enhanced reaction to cocaine since the behavioral measures in his study were collected in undrugged animals *prior to* the goal-box delivery of cocaine. A treatment-induced hyperactivity would also be expected to produce a faster response initiation, yet (as described above) start latencies were unaffected by DRN inactivation. There is also no evidence to suggest that the speed with which the animals traversed the runway was different during DRN inactivation compared to baseline. Indeed, an examination of Fig. 2 suggests that animals approached the goal box as quickly during baseline as they did on test day (i.e., the near perpendicular slopes of the curves suggest a very fast approach on both trials). The primary difference in the pattern of responding during the intact versus the inactivated DRN trials, lays in the number of retreats (approach–avoidance responses) that the animal exhibited before entering the goal, and *not* the speed with which the rats approached or avoided the goal. Finally, there is no *a priori* reason to presume that a treatment that alters motoric capacity would affect the animals' "decision" to stop and retreat. Unlike lever-press based measures of conflict behavior, retreat frequency represents a rate-free index of approach–avoidance conflict. Of course, if there had been no retreats observed during DRN inactivation, then one might argue that a strong hyperlocomotor response was propelling the animals into the goal box. However, in reality, the subjects in the current study continued to emit retreat behaviors during DRN inactivation (albeit at a lower frequency) thereby demonstrating that they are fully capable of stopping at the goal box threshold, turning and moving away from the goal box. We conclude that the primary impact of DRN inactivation, then, was not to enhance general locomotor activity, but rather to reduce the frequency of rate-independent approach–avoidance responses.

As indicated above, retreat behaviors were first described in classic studies on approach–avoidance conflict where hungry animals were trained to run a straight alley and enter a goal box previously associated with food + shock (e.g., Brown, 1942; Miller, 1944; see also Cohen et al., 2009; Geist and Ettenberg, 1997). In the early studies, parametric analyses confirmed that changing either the *reinforcer magnitude* or the *motivational state* of the animals (i.e., the level of food deprivation) predictably altered the strength of the resulting conflict behavior (e.g., Brown, 1942). Put simply, giving rats more food or making them hungrier reduced the subjects' conflict about entering a goal box where both food + shock were concurrently presented. In the present study, DRN inactivation altered the animals' runway behavior prior to their entry into the goal box and hence prior to the delivery of cocaine — so the treatment could not have altered the reinforcing properties of the drug itself. However, it might be argued that DRN inactivation reduced retreats by changing the motivational state underlying cocaine-seeking behavior. Consistent with this notion are neuroanatomical studies demonstrating that the DRN projects to numerous brain structures implicated in the neurobiology of motivated behavior including the nucleus accumbens, hippocampus, amygdala, prefrontal cortex and hypothalamus (Lechin et al., 2006; Michelsen et al., 2007) and functional studies have suggested a role for 5-HT pathways as a neuromodulator of reward and motivational systems (e.g., Bromberg-Martin et al., 2010; Kranz et al., 2010; Walsh and Cunningham, 1997). For example, administration of the serotonin reuptake inhibitor, fluoxetine, which produces a short-term potentiation in the neurotransmission at 5-HT synapses, decreased responding and reduced "break points" for self-administered i.v. cocaine in rats working on a progressive ratio schedule of reinforcement (Richardson and Roberts, 1991). Conversely, depletions of forebrain 5-HT increased such "break points" (Loh and Roberts, 1990). Both such effects are consistent with the view that activation of 5-HT circuits can reduce, and inactivation can enhance, the motivation of subjects to work for cocaine.

A role for 5-HT in the modulation of cocaine motivation is also suggested by recent reports that 5-HT depletion, while attenuating one measure of cocaine-seeking (i.e., responding during extinction trials when the reinforcer is no longer available), enhanced the animals' responsiveness to a cocaine prime or to cocaine-paired cues during a test of response reinstatement (Tran-Nguyen et al., 2001). Additionally, when activation of 5-HT systems is produced (by systemic application of agonists at selective 5-HT receptors), both cocaine- and cue-induced response reinstatement of cocaine seeking is attenuated (Neisewander and Acosta, 2007; Pentkowski et al., 2009). Furthermore, this latter effect can be was reversed by administration of the appropriate receptor antagonist (Neisewander and Acosta, 2007). So it would appear that the motivation to seek cocaine upon presentation of a stimulus predictive of cocaine availability (either a cocaine prime or a cocaine-paired cue) is attenuated by 5-HT activation and enhanced by 5-HT depletion. In the context of the current runway procedure, the placement of the animal in the start box could be seen as comparable to providing a cue predictive of cocaine availability, and if DRN inactivation enhanced the motivation to seek that cocaine, then such a treatment would be expected to reduce the frequency of approach–avoidance conflict about goal box entry. Despite the intriguing nature of this argument, the authors again note that there was no change in the start latencies of animals during DRN inactivation — as one would have expected had the subjects' motivation to seek cocaine been enhanced. It would seem that additional research is needed to more clearly elucidate the nature of the role of 5-HT systems in the motivation to seek cocaine, particularly in view of the apparent complexity and differential functionality of 5-HT receptor subtypes in reinforcement and motivational processes (e.g., Burmeister et al., 2004; Kranz et al., 2010).

As noted in our previous work and clearly reflected in Fig. 2, development of cocaine-induced retreat behavior occurs only after repeated drug exposure over several days. This suggests a possible parallel with the development of behavioral sensitization, which also requires repeated administration of cocaine over multiple trials. In fact, several studies have recently reported a role for 5-HT systems in the mediation and/or modulation of cocaine-induced sensitization (e.g., Filip et al., 2010), and the observation that disruption of 5-HT function interferes with the expression of both retreats (the current study) and sensitization (e.g., Zayara et al., in press) lends support to the notion that common underlying mechanisms may be subserving these two behavioral consequences of repeated cocaine exposure. Additional work would again be needed to determine whether or not the neuroadaptations that are thought to underlie the development of sensitization, also play a role in the development of retreat behaviors.

In terms of the current data set, the results clearly suggest that inactivation of the dorsal raphé nucleus produced attenuation in the approach–avoidance conflict of animals running toward a goal box associated with i.v. cocaine. In the authors' view, the most parsimonious explanation for the observed reduction in retreat behavior is that DRN inactivation attenuated the anxiogenic state of the animals — this may have been due to a direct impact on 5-HT substrates involved in anxiety or secondary to an enhanced motivation to seek cocaine. Work is ongoing in our laboratory to try and elucidate the precise nature of serotonin's role in the modulation of cocaine-seeking behavior.

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