Behavioural Brain Research 217 (2011) 472–476

Contents lists available at ScienceDirect

Behavioural Brain Research

journal homepage: www.elsevier.com/locate/bbr

Short communication

Central injections of noradrenaline induce reinstatement of cocaine seeking and increase c-fos mRNA expression in the extended amygdala

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ARTICLE INFO

Article history:
Received 4 August 2010
Received in revised form
21 September 2010
Accepted 22 September 2010
Available online 7 October 2010

Keywords:
Noradrenaline
Reinstatement
Self-administration
c-fos
In situ hybridization
Central nucleus of amygdala
Bed nucleus of stria terminalis
Cocaine

ABSTRACT

We recently reported that central injections of noradrenaline (NA) induce reinstatement of cocaine seeking in rats. Here, we replicate and extend our finding to an additional dose of NA and show that it is associated with the induction of c-fos mRNA expression (a marker of neuronal activation) in functionally relevant brain regions, including the bed nucleus of the stria terminalis and central nucleus of the amygdala.

It is well established that in rats with a prior history of drug self-administration, exposure to intermittent and unpredictable footshock stress can serve as a powerful trigger for reinstatement of extinguished drug seeking [1,2]. Moreover, it is known that the stress-related neurotransmitter, noradrenaline (NA), plays a critical role in mediating this reinstatement. For example, systemic or intracerebroventricular (i.c.v.) pretreatment with α_2-adrenoceptor agonists (e.g., clonidine, lofexidine), administered at doses known to act at adrenergic autoreceptors to inhibit NA cell firing and release, block footshock-induced reinstatement of cocaine and heroin seeking [3,4].

Several clusters of NA cell bodies in the brainstem project in two major ascending pathways. The dorsal pathway projects diffusely from the locus coeruleus to the frontal cortex, thalamus, cerebellum, and limbic system (hippocampus and amygdala [5]). The ventral pathway, originating in the lateral tegmental nuclei, provides abundant innervation of the hypothalamus and limbic forebrain (amygdala and bed nucleus of the stria terminalis [BNST] [5–7]).

The ventral NA pathway has been found to play a selective role in mediating footshock-induced reinstatement of heroin seeking [3] and footshock-induced reactivation of a morphine conditioned place preference [8]; in contrast, a role for the dorsal pathway in each of these effects has been ruled out [3,8]. Moreover, the effects of NA on footshock-induced reinstatement of drug seeking have been localized to the central nucleus of the amygdala (CeA) and BNST, where bilateral injections of β_1/β_2-adrenoceptor antagonists attenuate the footshock-induced reinstatement of cocaine seeking [9].

These region-specific effects of NA on footshock-induced reinstatement of drug seeking are consistent with the known region-specific effects of another stress-related neurotransmitter, corticotropin releasing factor (CRF), on this reinstatement. For example, bilateral injections of the CRF receptor antagonist, D-Phe CRF_{12-41}, into BNST, block footshock-induced reinstatement of cocaine seeking [10], and functional inactivation of the CRF-containing pathway from CeA to BNST [11] attenuates footshock-induced reinstatement [12]. Furthermore, there is neuroanatomical evidence that NA terminals and CRF-containing cell bodies interact in the ventral BNST [13,14], and that CRF-containing

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doi:10.1016/j.bbr.2010.09.025

cells in CeA express β_1-adrenergic receptors which regulate CRF mRNA expression in the region [15]; thus, BNST and CeA may represent points of convergence between NA and CRF systems in mediating footshock-induced reinstatement of drug seeking. In fact, consistent with this idea, we recently reported that i.c.v. injections of NA induce the reinstatement of cocaine seeking, and that i.c.v. pretreatment with the CRF-receptor antagonist, D-Phe CRF_{12-41}, interferes in this reinstatement [16].

The present study was comprised of two experiments. In Experiment 1, we replicated our recent finding that i.c.v. injections of NA reliably induce the reinstatement of cocaine seeking in rats, and extended our finding to an additional dose of NA. In Experiment 2, we studied the effects of i.c.v. NA, within the dose range found to produce reliable reinstatement, on neuronal activation within brain nuclei that we would expect to underlie the effects of NA on reinstatement responding. To this end, we used *in situ* hybridization to assess gene expression of c-fos, a generalized marker of neuronal activation [17], within CeA and two subregions of the lateral BNST. In addition, and by way of comparison, we also assessed c-fos mRNA expression in the paraventricular nucleus (PVN) of the hypothalamus. The primary objective of this experiment, which was carried out in drug naive rats, was to verify that doses of NA associated with reliable reinstatement of drug seeking were sufficient to induce neuronal activation in regions of interest.

A total of 51 male long Evans rats (275–300 g), individually housed on a reverse light-dark cycle (lights on 19:00–07:00) with free access to food and water, were used in the two experiments. All procedures received university ethics approval and were performed in accordance with the guidelines of the Canadian Council of Animal Care.

All animals were surgically implanted with a guide cannula (18 mm, 23 gauge; Plastics One, Roanoke, VA, USA) in the left lateral ventricle (AP: −1.0 mm, ML: +1.4 mm, DV: −3.7 mm) under gas anesthesia (3–5% isoflurane; Benson Medical, Markham, ON, Canada). At the time of surgery, rats for Experiment 1 were also implanted with an intravenous silastic catheter (Dow Corning, Midland, MI, USA) into the right jugular vein; the catheter exited at the top of the skull into a cannula (modified 22-gauge; Plastics One). Surgical procedures have been described in detail elsewhere [16]. In both experiments, microinjections of NA bitartrate (Sigma–Aldrich, Oakville, ON, Canada) and its vehicle (physiological saline) were given in a volume of volume of 4 μl, using a 10 μl Hamilton syringe connected to a 30-gauge injector (Plastics One). Injections were made over 2 min, and the injectors were left in place for an additional 1 min.

All behavioral procedures utilized in Experiment 1 were carried out in self-administration chambers (Med Associates, St Albans, VT, USA) equipped with two retractable levers. Responding on one lever ("active" lever) activated an infusion pump, while responding on the other lever ("inactive" lever) was without consequence. All responding was automatically recorded using a computer interface and software (Med Associates).

The details of the procedures used in Experiment 1 were based on those described in a recent and related study by our group (see [16]). Briefly, rats were trained to self-administer cocaine HCl (Medisca Pharmaceuticals; St Laurent, QC, Canada), during once daily 3-h sessions on a fixed ratio 1 schedule of reinforcement. Self-administration conditions were in place for 8–10 days, after which they were left undisturbed in their home cages for 7 days. Subsequently, extinction training began, during which all conditions that were present during training were maintained, except that lever presses were no long reinforced with drug. Extinction training occurred during four 60-min sessions on each of 3 consecutive days.

In the subsequent 3 days, animals were given 3 consecutive tests for reinstatement. At the start of each test day, one 60-min extinction session was given, in order to ensure and/or re-establish a low baseline level of responding prior to testing. Twenty-five min after the end of this extinction session, animals were challenged with an i.c.v. injection of NA bitartrate (10 or 20 μg) or its vehicle and 5 min later were given a 3-h test for reinstatement. Tests for reinstatement occurred under extinction conditions. Each animal was tested with both doses of NA and vehicle, on consecutive days and in a counterbalanced order.

Fig. 1 shows the mean (±SEM) number of responses on the active (A) and inactive (B) levers in each hour of the 3-h tests for reinstatement. It can be seen that most responding that occurred on the previously active lever (Fig. 1A) occurred in the first hour of testing and that, during this time, animals responded more after administration of 10 μg or 20 μg of NA than after vehicle. These observations were confirmed by a repeated measures ANOVA which revealed significant main effects of Test Condition ($F[2,14] = 6.9, p < 0.01$) and Hour of Testing ($F[2,14] = 25.6, p < 0.001$), and a significant Test Condition by Hour of Testing interaction ($F[4,28] = 8.5, p < 0.001$). Moreover, subsequent repeated measures ANOVAs for each hour revealed that the two-way interaction was attributable to a significant effect of Test Condition only in the first hour of testing ($F[1,6] = 10.9, p < 0.02$), and that in this first hour, both doses of NA (10 and 20 μg) relative to vehicle were associated with a significantly greater number of responses on the previously active lever.

Inspection of Fig. 1B shows that responding on the inactive lever was very low during all test sessions. Although a repeated measures ANOVA for responses on this lever revealed a significant main effect of hour ($F[1,7] = 14.98, p < 0.01$), reflecting slightly higher responding in the first than second or third hours of testing, there was no effect of Test Condition, and no interaction between the factors (p's > 0.1).

Having established an effective dose range for inducing reinstatement of cocaine seeking by i.c.v. NA, Experiment 2 was carried out to determine whether similar doses of NA would correspond to increased neuronal activation in brain regions thought to mediate the effects of NA on stress-induced reinstatement of drug seeking. Accordingly, in this experiment, rats were challenged with an i.c.v. injection of 10 or 20 μg NA or its vehicle and, 45 or 90 min later, were exposed briefly (30–45 s) to isoflurane vapors before being killed by decapitation. The brain was rapidly removed, flash-frozen in isopentane (−35 to −40 °C) and stored at −86 °C until processing for *in situ* hybridization.

Coronal brain sections (12 μm) were collected at −20 °C through all levels of the BNST, CeA, and PVN. Sections were thaw mounted onto glass slides and stored at −86 °C. Subsequently, the sections were thawed and fixed in 4% paraformaldehyde for 5 min, at room temperature. Sections were then rinsed twice in 1× PBS (5 min per wash), and were then treated with 0.1 M triethanolamine-HCl for 5 min followed by 0.1 M triethanolamine-HCl containing 0.25% acetic anhydride for 10 min. Finally, sections were rinsed twice in 2× SSC (5 min per wash) and were then dehydrated in graded ethanol. Hybridization was performed using a ^{35}S-UTP labeled riboprobe, which was prepared by in vitro transcription using the Maxiscript kit (Ambion) and cDNA defined by primers complementary to bases 479–498 (5′-gggagtggtgaagaccatgt-3′) and bases 961–942 (5′-ctgaaggctgaacctttga-3′; GenBank no. NM_022197.2). The probe was diluted to 18,000 cpm/μl in hybridization solution containing 50% formamide, 35% Denhardt's solution, 10% dextran sulfate, 0.1× SSC, salmon sperm DNA (300 μg/ml), yeast tRNA (100 μg/ml), and dithiothreitol (40 μM). The probe was subsequently applied to the sections and the slides were incubated overnight at 60 °C. The next day, sections were rinsed with agitation using decreasing concentrations of SSC dipped in Milli-Q water and dehydrated in 70% ethanol. The slides were exposed to Kodak BioMax film at 4 °C for 7 days.