

Prominent Activation of Brainstem and Pallidal Afferents of the Ventral Tegmental Area by Cocaine

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Blockade of monoamine transporters by cocaine should not necessarily lead to certain observed consequences of cocaine administration, including increased firing of ventral mesencephalic dopamine (DA) neurons and accompanying impulse-stimulated release of DA in the forebrain and cortex. Accordingly, we hypothesize that the dopaminergic-activating effect of cocaine requires stimulation of the dopaminergic neurons by afferents of the ventral tegmental area (VTA). We sought to determine if afferents of the VTA are activated following cocaine administration. Rats were injected in the VTA with retrogradely transported Fluoro-Gold and, after 1 week, were allowed to self-administer cocaine or saline via jugular catheters for 2 h on 6 consecutive days. Other rats received a similar amount of investigator-administered cocaine through jugular catheters. Afterward, the rats were killed and the brains processed immunohistochemically for retrogradely transported tracer and Fos, the protein product of the neuronal activation-associated immediate early gene, *c-fos*. Forebrain neurons exhibiting both Fos and tracer immunoreactivity were enriched in both cocaine groups relative to the controls only in the globus pallidus and ventral pallidum, which, together, represented a minor part of total forebrain retrogradely labeled neurons. In contrast, both modes of cocaine administration strongly increased double-labeling relative to the controls in the brainstem, specifically in the caudal ventromedial mesencephalon and rostromedial pontine tegmentum. It is concluded that a previously unappreciated activation of pallidal and brainstem afferents may contribute to the modulation of dopaminergic neuronal activity following cocaine administration.

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INTRODUCTION

The high abuse liability of the psychostimulant drug cocaine (Johanson and Fischman, 1989) is thought to stem mainly from its capacity to block the re-uptake of dopamine (DA) by the DA transporter (Koe, 1976; Ritz *et al*, 1987; Woolverton, 1987; Einhorn *et al*, 1988; Balster, 1990; Vanover *et al*, 1992; Howell and Byrd, 1995; Roberts *et al*, 1999; Wee and Woolverton, 2006). Indeed, the acute reinforcing and locomotor activating effects of cocaine are linked to sustained increases in extracellular concentrations of DA in structures that are innervated by DA transporter-enriched DAergic projections (Carboni *et al*, 1989; Staley *et al*, 1995; Freed *et al*, 1995), such as the nucleus accumbens and prefrontal cortex (Moghaddam and Bunney, 1989; Kalivas and Duffy, 1990; Maisonneuve *et al*, 1990; Sorg

and Kalivas, 1993). This DA overflow, in turn, especially when chronically repeated, leads to long-lasting neuroadaptations (Downs and Eddy, 1932; Kalivas and Duffy, 1990; Kalivas and Stewart, 1991; Hope *et al*, 1992; Pierce and Kalivas, 1997; White and Kalivas, 1998; McFarland and Kalivas, 2001; Nestler, 2001; Borgland *et al*, 2004; Bowers *et al*, 2004; Kelz *et al*, 1999; Jones and Bonci, 2005; Moran *et al*, 2005; Olausson *et al*, 2006; Sun and Rebec, 2006) that are thought to underlie drug dependence (Koob and Bloom, 1988; Kreek and Koob, 1998; White and Kalivas, 1998; Nestler, 2001; Jones and Bonci, 2005; Shaham and Hope, 2005).

It is pertinent, however, that the effect of cocaine on DA turnover is not due to stimulation of the release of DA but rather to a blockade of its re-uptake (references cited above). Thus, the resulting increased extracellular concentrations of DA might be expected to be offset by the inhibitory effect of DA acting at autoreceptors, which, by inactivating DA neurons (Bunney *et al*, 1973; Aghajanian and Bunney, 1977), would tend to reduce rather than increase extracellular DA concentrations. In this regard, White and co-workers (Einhorn *et al*, 1988; White, 1990) reported that the anticipated autoreceptor-mediated inactivation of ventral tegmental DA neurons is less complete and more short-lived than expected considering the binding

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properties of cocaine, which suggests the existence of offsetting mechanisms that maintain the activity of dopaminergic neurons in the presence of cocaine (see also Cameron and Williams, 1994; Shi *et al*, 2004).

One such mechanism could involve stimulation of the ventral tegmental area (VTA), which contains DA neurons that project to the accumbens and prefrontal cortex (Beckstead *et al*, 1979; Swanson, 1982), by inputs arising in other parts of the brain. Indeed, neurons projecting to the VTA are numerous, occupying many differentiated structures and intervening brain areas aligned along the medial forebrain bundle from the prefrontal cortex to the medulla oblongata (Phillipson, 1979; Geisler and Zahm, 2005). A large proportion of these VTA afferents possess neurotransmitter/modulator substances that excite DA neurons (White, 1996; Mathon *et al*, 2003; Fields *et al*, 2007), including glutamate (Geisler *et al*, 2007), neurotensin (Geisler and Zahm, 2006), and cocaine and amphetamine-related transcript (Philpot *et al*, 2005).

It was with a view to these considerations that the present study was carried out in the rat to determine whether afferents of the VTA are activated by cocaine administration. To accomplish this, VTA-projecting neurons were prelabeled with retrogradely transported Fluoro-Gold (FG) a week before when groups of rats began self-administering cocaine or saline or received investigator-administered infusions of cocaine. They were killed for analysis after the sixth consecutive daily self-administration session. Neuronal activation was assessed by immunohistochemical detection of Fos protein, the product of the immediate-early gene, *c-fos*.

MATERIALS AND METHODS

Twenty-seven male Sprague–Dawley rats (Harlan, Indianapolis, IN, USA) weighing 225–250 g at the start of the study were used in accordance with guidelines mandated in the National Institutes of Health Guide for the Care and Use of Laboratory Animals. The rats were singly housed under a 12 h reverse light–dark cycle and given food and water *ad libitum*. Unless stated otherwise, chemicals were purchased from Sigma (St Louis, MO).

Implantation of Self-Administration Catheters and Tracer Injections

The rats were deeply anesthetized by intraperitoneal injections of a cocktail consisting of 45% ketamine (100 mg/ml), 35% xylazine (20 mg/ml), and 20% saline at a dose of 0.16 ml/100 g of body weight. A silastic catheter (20 μ l dead volume) was inserted via the external jugular vein into the right atrium of the heart, passed under the skin, and fixed in the midscapular region. Immediately after the catheters were implanted, the rats were placed into a Kopf stereotaxic instrument and the retrograde tracer Fluoro-Gold (FG; Fluorochrome Inc., Englewood, CO; 1% in 0.1 M cacodylate buffer, pH 7.4) was injected iontophoretically into the VTA through 1.0 mm filament-containing glass pipettes pulled to tip diameters of 15–20 μ m using 1 μ A positive pulses (7 s on and 7 s off for 15 min). After surgery, the rats were kept warm until they awakened.

Subsequently, the catheters were flushed daily with sterile saline to prevent clogging.

Self-Administration of Cocaine and Vehicle; Investigator-Administered Cocaine

Immediately following surgery and daily thereafter, the rats were gently handled to flush the catheters with saline. One week after surgery, the animals were placed in a self-administration chamber (Med Associates, St Albans, VT) and allowed to self-administer cocaine (SAC group; $n = 9$; 500 μ g/kg per 30 μ l infusion) or saline (SAS group; $n = 9$) for 6 consecutive days (2 h/day, during the dark part of the dark/light cycle) as described previously (Marinelli *et al*, 2003). Briefly, nose poking in the active hole delivered an infusion (cocaine or saline) and, for a period of 30 s, illuminated the hole with an LED light, which, in and of itself, is reported to sustain a moderate level of responding (Marinelli *et al*, 2003). Nose poking in the inactive hole had no consequences. Self-administration was regarded as established when nose pokes were significantly more numerous in the active than in the inactive hole. Rats given investigator-administered infusions of cocaine (YAC group; $n = 9$) were placed in separate chambers located in the same environment as the SAC and SAS rats. These rats received infusions administered by the investigator in numbers and at time intervals reflecting the averages delivered on the same day to the rats in the SAC group. Catheters were to be regarded as having remained patent throughout the study if 200 μ l of the ketamine/xylazine mixture could be delivered through them on the last day of the experiment. Thus, rats that did not succumb within 3–5 s following this treatment were to be removed from the study. All of the lines remained open in the present study. Self-administration cages were also equipped with two photocell beams located in the long axis of the cage to monitor locomotor activity.

Fixation of Brains and Immunocytochemistry

One and one-half hours after the beginning of the sixth daily self-administration session, the rats were deeply anesthetized and perfused transaortically with buffered (0.1 M. Sorensen's phosphate buffer, pH 7.4-SPB) aldehydes and the brains were sectioned and processed for immunohistochemistry as has been described (see Geisler and Zahm (2005) for details). Five adjacent series of sections were collected, each representing an entire brain from the frontal pole to the caudal medulla at intervals of 250 μ m. Each series of sections was stored in a separate glass vial at -20°C in a cryoprotectant consisting of SPB containing 30% sucrose and 30% ethylene glycol.

One series of sections from each case was immersed in SPB containing 0.1% Triton X-100 (SPB-t) and a rabbit polyclonal antibody raised against a synthetic peptide corresponding to amino acids 4–17 of human c-Fos at a dilution of 1:5000 (anti-c-Fos (Ab-5) (4–17) rabbit pAb, formerly from Oncogene Science, Cambridge MA, now from Calbiochem, San Diego, CA). The following day, after thorough rinsing in SPB-t, the sections were immersed in SPB-t containing a biotinylated antibody made in donkey against rabbit IgGs at a dilution of 1:200 (Jackson ImmunoResearch Laboratories Inc., West Grove, PA) for

an hour. Afterward, the sections were rinsed in SPB and then immersed in SPB containing avidin–biotin–peroxidase complex (ABC) at a dilution of 1:200 (Vector Laboratories, Burlingame, CA) for an hour. After thorough rinsing in SPB, the sections were reacted in 0.025 M Tris buffer (pH 8.0) containing 0.015% 3,3'-diaminobenzidine (DAB), 0.4% nickel ammonium sulfate, and 0.003% hydrogen peroxide, which generates an insoluble black reaction product.

The sections were then subjected to additional rinsing in SPB and then immersed in SPB-t containing an antibody raised in rabbit against FG and used at a dilution of 1:5000 (Chemicon, Temecula, CA, USA). The following morning, the sections were rinsed in SPB-t and immersed for 1 h in SPB-t containing a donkey antibody against rabbit IgGs used at a dilution of 1:200 (Jackson). Following further rinsing in SPB, the sections were immersed for 1 h in SPB containing rabbit peroxidase-anti-peroxidase (PAP) at a dilution of 1:3000 (ICN Biomedicals Inc., Aurora, OH), after which they were again rinsed thoroughly. Then the sections were immersed for 20–30 min in 0.05 M SPB (pH 7.4) containing 0.05% DAB, 0.04% ammonium chloride, 0.2% β -D-glucose, and 0.0004% glucose oxidase, which generates an insoluble brown reaction product. The sections were then mounted onto gelatin-coated slides, dehydrated through a series of ascending concentrations of ethanol, transferred into xylene, and coverslipped with Permount (Fisher, Pittsburgh, PA). Staining for Fos and FG was absent when the relevant primary or secondary antibodies, ABC or PAP reagents were omitted.

Additional sections were prepared to exhibit single labeling for tyrosine hydroxylase (TH)-ir (Chemicon), for the evaluation of FG injection sites, and for double-labeling with Fos-ir and TH-ir or Fos-ir and nitric oxide synthase (NOS)-ir (Sigma), to determine if Fos-ir was expressed in dopaminergic and cholinergic neurons, respectively. Protocols to accomplish this were carried out as described above using appropriate second antibodies, except that for Fos/TH double-labeling the second antibodies were conjugated to the fluorescent compounds fluorescein and Texas Red and examined with epifluorescence illumination (Jackson ImmunoResearch Labs). All of the antibodies used in the study have been well characterized by the manufacturers and were evaluated in our hands with appropriate omission controls.

Analysis of Fos-Ir in VTA Afferents

The analysis was carried out with the aid of the MDPlot5 hardware–software platform (Accustage, Shoreview, MN), which was used to generate digitized 2-D representations of the outlines of the sections, ventricles, and major white matter bundles, as well as all FG-ir (retrogradely labeled) and double-labeled (FG-ir plus Fos-ir) neurons. For the forebrain, a series of 17 consecutive sections spaced at 250 μ m intervals, beginning at the rostral tip of the accumbens and extending to include the rostral one-third of the lateral hypothalamus, was analyzed. The lateral habenula was evaluated in a separate series of sections. For the brainstem, a similarly spaced series of six sections, beginning at the midpoint of the interpeduncular nucleus and extending caudalward to the level of the laterodorsal tegmental (LDTg) nucleus, was evaluated. Structures of interest containing retrogradely labeled neurons were thus

equivalently represented in all of the evaluated cases. FG-ir and double-labeled neurons were counted with the aid of an MDPlot5 software feature and double-labeled neurons were expressed as percent of retrogradely labeled neurons. Means reflecting four cases for each of the three experimental groups (SAS, SAC, and YAC) were tested with a one-way ANOVA followed as indicated by Fisher's LSD *post hoc* test.

RESULTS

FG Injection Sites

Four cases with FG injection sites well constrained within the boundaries of the VTA were selected from the SAS, SAC, and YAC administration groups for quantitation. The injection sites were located fairly uniformly in the lateral part of the subnucleus parabrachialis pigmentosus at the midrostocaudal level of the VTA (Figure 1).

Afferents of the VTA

Consistent with reports cited in Introduction, FG-labeled descending projections to the VTA were observed in the medial prefrontal cortex, medial and lateral septum, accumbens, ventral parts of the bed nucleus of stria terminalis, ventral pallidum, medial preoptic area, lateral habenula, and the lateral preoptic region and its continuation into the lateral hypothalamus. Owing to the lateral placement of FG injections in the VTA, retrogradely labeled neurons also were observed consistently in the ventral and medial parts of the globus pallidus (Figures 2–4). Most of the labeling in the forebrain was ipsilateral to the injections, but significant contralateral labeling was present in the preoptic regions and lateral hypothalamus. In the brainstem, retrogradely labeled neurons were abundant on both sides of the brain within and between numerous structures, including the periaqueductal gray, dorsal raphe, pedunculopontine tegmental (PPTg) nucleus, LDTg nucleus, parabrachial nucleus, locus ceruleus, median and paramedian raphe, deep mesencephalic nucleus, and the mesopontine reticular formation.

Fos-ir and Double-Labeling

Fos-ir neurons were abundant throughout the entire brain, albeit more so in the brains from the SAC and YAC groups as compared to the SAS group (Zahm, unpublished data). Neurons containing both FG-ir and Fos-ir, that is, double-labeled neurons (Figure 2), were observed in most structures that project to the VTA. In the forebrain, almost all structures exhibited similar numbers of double-labeled neurons in the three treatment groups (Table 1, Figure 3). Significantly greater numbers of double-labeled forebrain neurons were observed in the SAC and YAC brains only in the ventral pallidum and globus pallidus (Figures 2–4). Retrogradely labeled neurons were located predominantly medially in the globus pallidus and sublentiform ventral pallidum (Figure 4b), but they were spread more uniformly through the subcommissural ventral pallidum (Figure 4a). Double-labeling as a percent of numbers of retrogradely labeled neurons was significantly greater

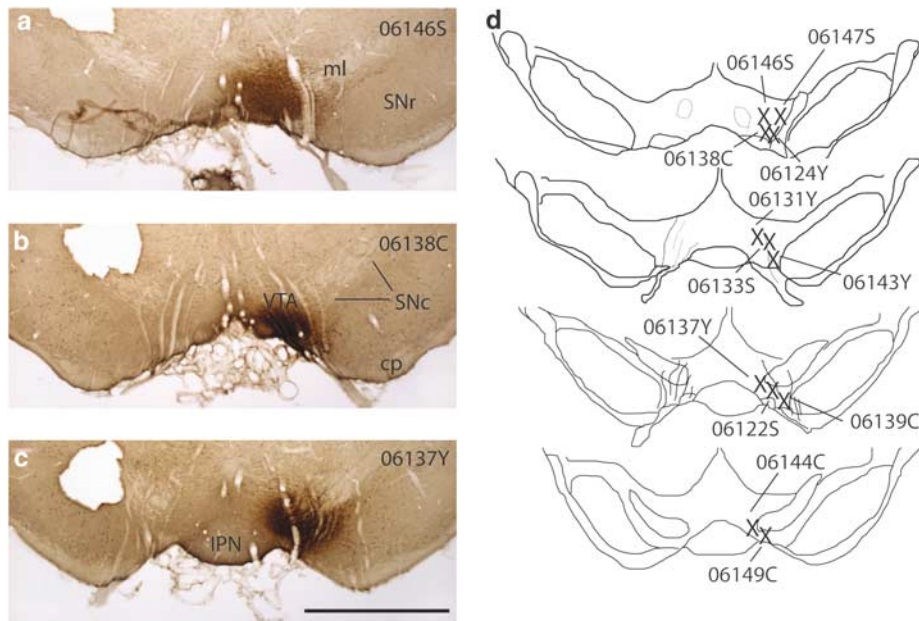


Figure 1 Micrographs illustrating representative Fluoro-Gold injection sites (a–c) and a map (d) showing, in rostrocaudal order from top to bottom, four levels of the VTA with the centers of each of the injection sites from the evaluated cases marked with an X. The case numbers are indicated. S, C, and Y indicate self-administration of saline and cocaine and yoked administration of cocaine, respectively. cp, cerebral peduncle; IPN, interpeduncular nucleus; ml, medial lemniscus; SNc, substantia nigra compacta; SNr, substantia nigra reticulata; VTA, ventral tegmental area. Scale bar: 1 mm.

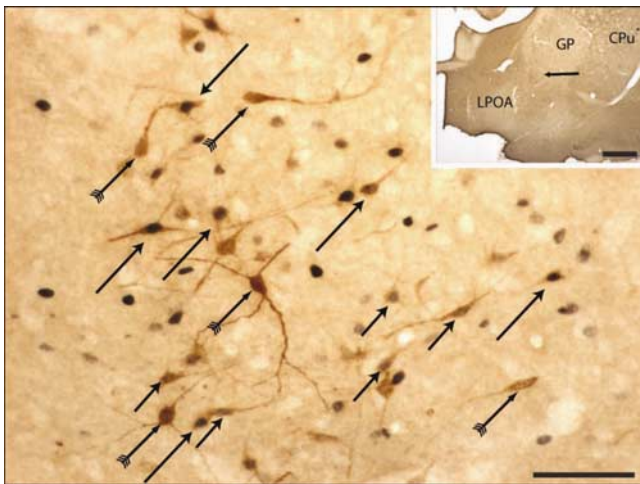


Figure 2 Micrographs illustrating a section from the brain of a rat killed following the last of six cocaine self-administration sessions. The brain was pre-labeled 7 days before the first self-administration session by injecting the retrogradely transported tracer Fluoro-Gold into the mesencephalic VTA. The position of the illustrated retrogradely labeled neurons in the ventromedial part of the globus pallidus (GP) is indicated by the arrow in the inset. Retrogradely labeled neurons (fletched arrows) exhibit a brown reaction product in the neuronal cell body and dendrites. The sections were also processed to exhibit Fos immunoreactivity, which appears as an intense black reaction product restricted to the nucleus of many of the neurons, including some containing FG-ir, which are therefore regarded as double labeled (long arrows). Also shown are double-labeled neurons exhibiting less intense Fos immunoreactivity (short arrows). CPu, caudate-putamen; LPOA, lateral preoptic area. Scale bars: 100 μ m; inset-1 mm.

in the globus pallidus and the sublenticular, as compared to the subcommissural, part of the ventral pallidum (Table 2).

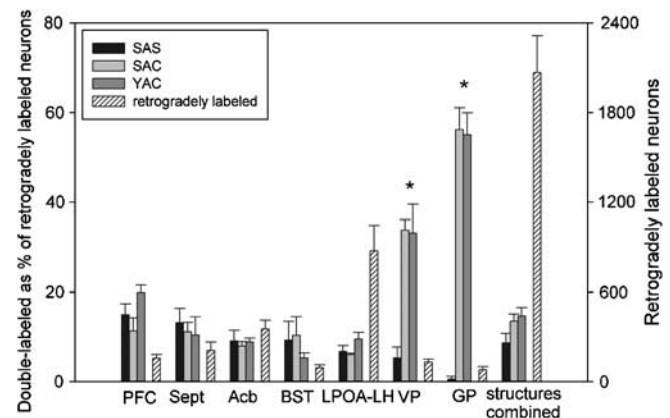


Figure 3 Graph illustrating the means \pm SEM of double-labeled (Fluoro-Gold + Fos) neurons expressed as percent of retrogradely labeled neurons (grouped bars, referenced to left ordinate) and numbers of retrogradely labeled neurons (stand-alone bars, referenced to right ordinate) in structures in the forebrain. Numbers of retrogradely labeled neurons were provided to give the reader an idea of how many retrogradely labeled neurons were counted in each structure and in the entire evaluated series of sections. Means \pm SEM are illustrated for retrogradely labeled neurons for all animals in the three experimental groups (ie $n = 12$). SAS, SAC, and YAC refer to the treatment groups described in the text. One-way ANOVA followed by Fisher's LSD *post hoc* test revealed that the numbers of double-labeled neurons in the SAC and YAC groups differ from the SAS group only in the ventral pallidum (VP) and globus pallidus (GP). Note that the VP and GP contain a minor part of total retrogradely labeled neurons. Acb, accumbens; BST, bed nucleus of stria terminalis; LPOA-LH, lateral preoptic area and rostral lateral hypothalamus; PFC, prefrontal cortex; Sept, septum.

In contrast to the forebrain, double-labeled neurons were enriched throughout the mesencephalon and pons in the brains of rats from the SAC and YAC groups as compared to

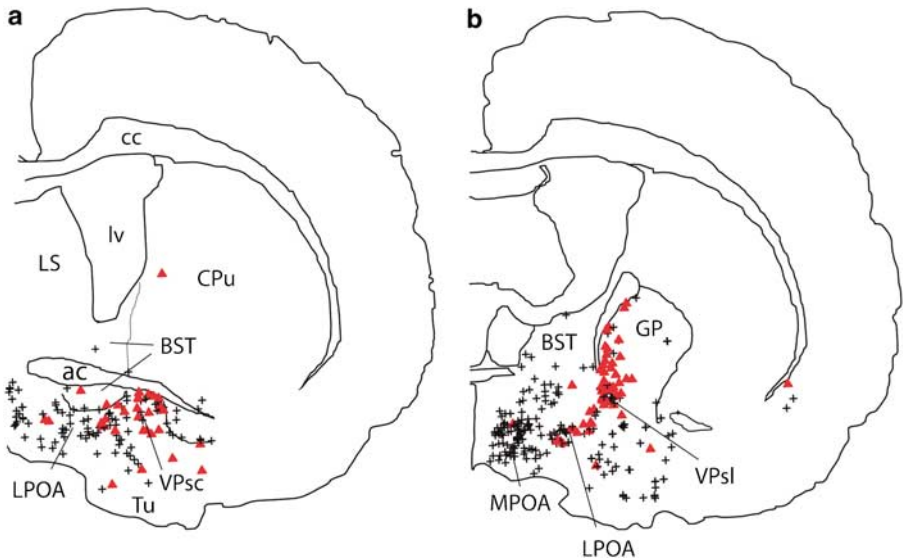


Figure 4 Plot illustrating more rostral (a) and caudal (b) levels of forebrain where Fos immunoreactive VTA afferents were most numerous in rats killed after six daily cocaine administration sessions. The brains were processed to show the retrograde label (Fluoro-Gold-FG) and Fos immunoreactivity. Black crosses indicate neurons exhibiting only FG. Red triangles indicate double-labeled neurons. ac, anterior commissure; BST, bed nucleus of stria terminalis; cc, corpus callosum; CPU, caudate-putamen; GP, globus pallidus; LPOA, lateral preoptic area; LS, lateral septum; LV, lateral ventricle; MPOA, medial preoptic area; Tu, olfactory tubercle; VPsc, subcommissural ventral pallidum; VPsl, sublenticular ventral pallidum.

Table 1 Numbers of Retrogradely (FG) and Double (FG+Fos) Labeled Neurons in Forebrain (Including Rostral Diencephalon, Excluding Pallidum^a) and Brainstem^b in the Three Treatment Groups

Group	Case	FG only		FG+Fos (double-labeled)		% FG+Fos ^c	
		Forebrain	Brainstem	Forebrain	Brainstem	Forebrain	Brainstem
SAS	06122	1457	793	89	33	5.8	4.0
	06133	1120	494	116	18	9.4	3.5
	06146	2644	1249	163	22	5.8	1.7
	06147	2047	1016	352	76	14.7	7.0
	Mean ± SEM	1817 ± 336	888 ± 161	180 ± 59	37 ± 13 ^d	8.9 ± 2.1	4.1 ± 1.1 ^e
SAC	06138	574	268	39	60	6.4	18.3
	06139	1904	833	237	217	11.1	20.7
	06144	1248	1280	145	170	10.4	11.7
	06149	1590	958	147	194	8.5	16.8
	Mean ± SEM	1329 ± 285	835 ± 211	142 ± 40	160 ± 35 ^f	9.1 ± 1.1	16.9 ± 1.9 ^g
YAC	06124	1607	810	248	196	13.4	19.5
	06131	1485	1103	196	241	11.7	17.9
	06137	1394	1011	125	318	8.2	23.9
	06138	3177	1404	241	312	7.1	18.2
	Mean ± SEM	1915 ± 423	1082 ± 124	202 ± 2	267 ± 29	10.1 ± 1.5	19.9 ± 1.4 ^g

^aCounted in 17 sections spaced at 250 μm extending caudalward from the rostral tip of the accumbens. Values for GP and VP, which had significantly more double-labeled (FG+Fos) neurons in the SAC (self-administering cocaine) and YAC (yoked infusions of cocaine) rats, have been omitted in order to provide a more valid comparison of the rest of forebrain/diencephalon. A one-way ANOVA revealed no significant between-group differences in any category for forebrain/diencephalon. Had values for GP and VP been included, the mean % FG+Fos values for the SAS (self-administering saline), SAC, and YAC groups would have been, respectively, 8.6 ± 2.1, 12.7 ± 1.9, and 14.1 ± 1.9 (as in Figure 4—tot), but still lacking significant differences.

^bCounted in five sections spaced at 250 μm extending caudalward from the midpoint of the interpeduncular nucleus as illustrated in Figure 7.

^cFG+Fos (double-labeled)/([FG+Fos]+FG only) × 100.

^dSAS vs SAC—*p* = 0.011; SAS vs YAC—*p* < 0.001.

^eSAS vs SAC and SAS vs YAC—*p* < 0.001. (One-way ANOVA; Fisher LSD *post hoc* test).

^fSAC vs YAC—*p* = 0.022.

^gForebrain vs brainstem—*p* = 0.012 (SAC), *p* = 0.002 (YAC). (Student's *t*-test).

Table 2 Means \pm SEM of Percent of Retrogradely Labeled Neurons Exhibiting Fos-ir in Named Forebrain Structures Following Self Administration of Saline (SAS) and Cocaine (YAC) or Yoked Administration of Cocaine (YAC)

	SAS	SAC	YAC
<i>Pallidum</i>			
Subcommissural ventral pallidum	4.2 \pm 1.4	32.6 \pm 3.5*	30.3 \pm 5.9*
Sublenticular ventral pallidum	1.0 \pm 1.0	50.6 \pm 5.0* #	44.9 \pm 5.6*
Globus pallidus	0.5 \pm 0.3	52.3 \pm 4.2** #	59.4 \pm 8.5** #
<i>Lateral preoptic area</i>			
	7.7 \pm 2.7	6.2 \pm 1.4	10.2 \pm 2.1

* $p < 0.001$ compared to SAS (one-way ANOVA, Fishers LSD *post hoc* test).

** $p < 0.05$ compared to SAS (Kruskal–Wallis test, Student–Newman–Keuls *post hoc* test).

$p < 0.02$ compared to subcommissural VP (one-way ANOVA, Fishers LSD *post hoc* test).

(1) the brainstem of SAS groups and (2) the forebrain of SAS, SAC, and YAC groups (Table 1). Maximal double-labeling in the brainstem coincided with the position of a spot-like aggregation of densely packed Fos-ir neurons observed only in the brains of rats from the SAC and YAC groups (Figure 5). This dense cluster of Fos-ir neurons was present bilaterally and many neurons within it on both sides of the midline were double labeled, albeit more so ipsilateral to the injections site (Figures 5a–e and 6a–d). The rostral-most part of the cluster was embedded in the caudal subnucleus paranigralis of the VTA (Supplementary Figure 1A and B) just above the medial extremity of the medial lemniscus, although no evidence was found for colocalized Fos-ir and TH-ir (a marker of VTA dopaminergic neurons) in immunoperoxidase (Supplementary Figure 1) or immunofluorescence (data not shown) material. The mesopontine cluster of Fos-ir neurons was incrementally larger in successively more caudal sections (Figures 5a–c, e and

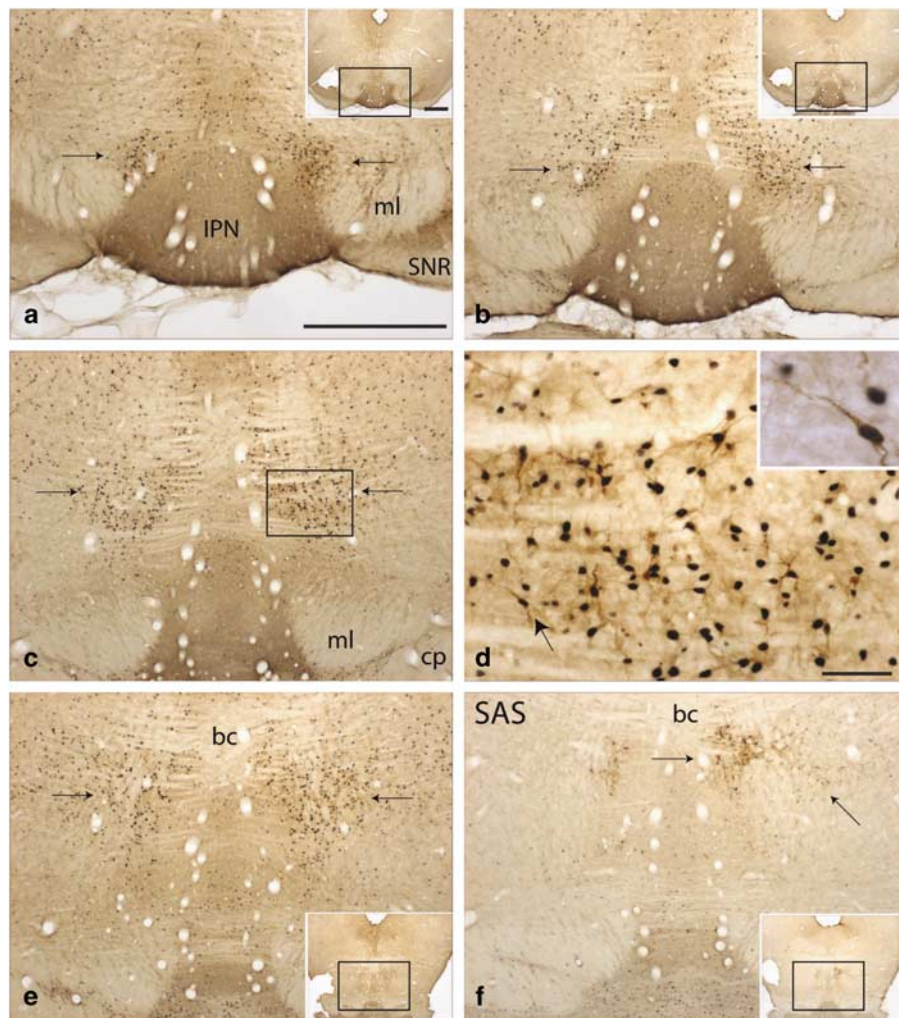


Figure 5 Micrographs illustrating a formation of densely packed Fos-immunoreactive (-ir) neurons (arrows in a–e) that occupy the ventromedial mesopontine tegmentum following the administration of cocaine. The micrographs are enlargements of the boxed areas in the insets. These micrographs also show Fluoro-Gold-ir neurons, which are inconspicuous except in (d), an enlargement, and (f), where they are unobscured by dense Fos-ir. The inset in (d), an enlargement of the box in (c), shows a double-labeled neuron at higher magnification. (f) A section from a rat that self-administered saline (SAS) at approximately the same rostrocaudal level as (e). Brainstem sections from SAS brains lack the formation of densely packed Fos-ir neurons, but FG-ir neurons (arrows) can be observed and moderate numbers of Fos-ir nuclei can be seen in other parts of the brainstem, although they are inconspicuous, because Fos stains lightly in the control brains. bc, brachium conjunctivum (superior cerebellar peduncle); cp, cerebral peduncle; IPN, interpeduncular nucleus; ml, medial lemniscus; SNR, substantia nigra pars reticulata. Scale bars: (a–c, e, f)–1 mm; (d)–100 μ m; insets–1 mm.

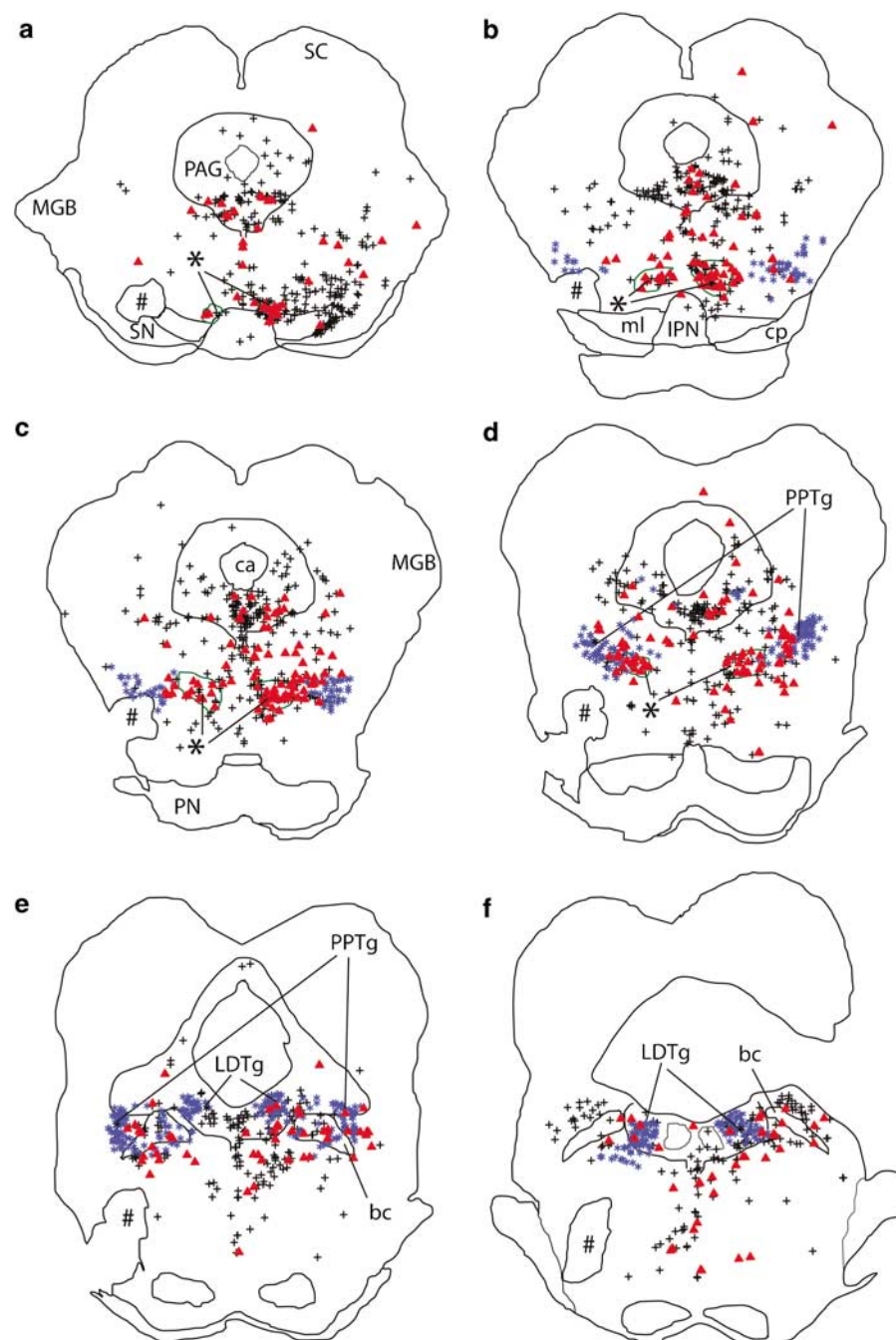


Figure 6 Plot illustrating several rostrocaudally ordered levels (a–f) of the brainstem where Fos immunoreactive VTA afferents were numerous in rats following the last of six daily cocaine administration sessions. Symbols are as in Figure 4 but include, in addition, large black asterisks in (a–d), which indicate the position of the psychostimulant-induced formation of densely packed Fos-ir neurons (see this figure and Figures 5), and blue asterisks, which indicate the position of cholinergic neurons in the PPTg nucleus and LDTg nucleus, plotted from adjacent serial sections in which these neurons were revealed with NOS immunoreactivity. Aq, cerebral aqueduct; bc, brachium conjunctivum; cp, cerebral peduncle; IPN, interpeduncular nucleus; LDTg, laterodorsal tegmentum; MGB, medial geniculate body; ml, medial lemniscus; PAG, periaqueductal gray; PPTg, pedunculo-pontine tegmentum; PN, pontine nuclei; SC, superior colliculus; SN, substantia nigra; #, tissue punch for section orientation.

6a–d), turning dorsolateralward to first lie in the ventromedial aspect of the A8 dopaminergic field, and then, leaving the DA-rich part of the ventral mesencephalon, in the medial part of the deep mesencephalic reticular formation, and, finally, in the rostromedial pontine tegmentum (Figures 5e and 6c, d and Supplementary Figure 1C). Double-labeling associated with the dense cluster of

Fos-ir neurons was densest at its center (* in Figure 7), somewhat less so in its periphery (** in Figure 7), and least in the rest of the brainstem.

The conspicuous enrichment of double-labeled neurons occupied a position medial to the PPTg nucleus, although its lateral parts did overlap somewhat with medial parts of the pars dissipata of the PPTg (Figure 6c and d). Although

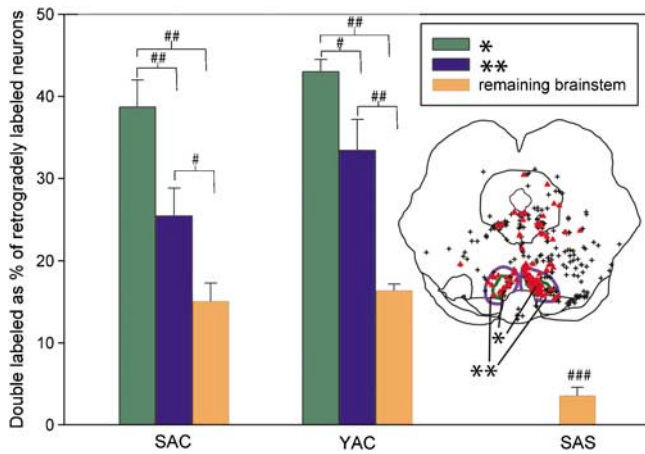


Figure 7 Graph illustrating means \pm SEM for double-labeled brainstem neurons expressed as percent of retrogradely labeled neurons for cocaine self-administering (SAC), yoked cocaine (YAC), and saline self-administering (SAS) groups. The inset is provided for reference and adheres to the description given for Figure 4. Plus signs and filled red triangles indicate retrogradely and double-labeled neurons, respectively. Maximal values for double-labeled neurons, indicated by the green bars, overlaid the aggregation of densely packed Fos-ir neurons, the position of which has been encircled by a green line (*) in the inset. As indicated by the blue bars, double-labeled neurons were also substantially enriched in a fringe adjacent to dense aggregation of Fos-ir neurons, corresponding to the territory in the diagram between the blue and green lines (**) in the inset. The numbers of double-labeled neurons in the rest of the brainstem (orange bars) also were significantly greater in the SAC and YAC groups than in the brainstem of the SAS rats, which lacked the aggregation of Fos-ir neurons, precluding the inclusion of green and blue bars within the SAS group. Statistical evaluation was made with a one-way ANOVA followed by Fisher's LSD *post hoc* test. # $p < 0.05$; ## $p < 0.005$; ### $p = 0.001$ for all comparisons except SAC and YAC remaining brainstem, where $p = 0.006$.

the extent to which double-labeling of FG-ir and cholinergic or glutamatergic PPTg neurons occurred in this zone of overlap was not determined, visibly less double-labeling of FG-ir with Fos-ir was observed there than within the cluster of Fos-ir neurons and its immediate surround (Figure 7; Table 3). In material from SAC and YAC brains processed to exhibit Fos-ir and NOS-ir, a marker of cholinergic neurons in the PPTg and LDTg nucleus (Sugaya and McKinney, 1994), only occasional double labels were observed (Supplementary Figure 2).

Behavior

All SAC and SAS rats exhibited reliable self-administration behavior. Throughout the sessions, nose poking in the active hole (paired with an infusion and light cue) was more frequent than nose poking in the inactive hole (Figure 8a; hole effect: $F_{1, 15} = 213.2$, $p < 0.001$; hole \times drug effect: $F_{1, 15} = 36.2$, $p < 0.001$). SAC and SAS rats did not differ for inactive hole responding. However, nose poking in the active hole was more than double for SAC rats compared with SAS rats (drug effect: $F_{2, 22} = 431$, $p < 0.001$; SAS vs SAC and YAC: $p < 0.001$, SAC vs YAC: NS). As a result, SAC rats took more than twice as many infusions as SAS rats (Figure 8b). These numbers were maintained by both groups during the six consecutive daily sessions (Figure 8c). YAC rats were given infusions in numbers and at time intervals nearly identical to the SAC group (Figure 8b and c;

Table 3 Means \pm SEM of Percent of Retrogradely Labeled Neurons Exhibiting Fos-ir in Named Brainstem Structures Following Self Administration of Saline (SAS) and Cocaine (YAC) or Yoked Administration of Cocaine (YAC)

	SAS	SAC	YAC
<i>Pedunculopontine tegmental nucleus</i>			
Rostral pars compacta	8.4 \pm 3.2	14.1 \pm 2.9	11.9 \pm 5.0
Caudal pars compacta	3.7 \pm 1.6	13.6 \pm 4.7	16.3 \pm 6.0
Pars dissipata	1.8 \pm 0.7	34.4 \pm 5.2*	37.4 \pm 5.0*
Laterodorsal tegmental nucleus	4.3 \pm 1.3	13.2 \pm 2.3*	18.9 \pm 2.6*
Dorsal raphe	6.2 \pm 2.2	14.1 \pm 6.1	19.0 \pm 1.3
Cuneiform nucleus	0.6 \pm 0.6	4.2 \pm 3.1	13.2 \pm 2.8
Average	4.2 \pm 1.2	15.6 \pm 4.1**	17.8 \pm 4.2**

* $p < 0.002$ compared to SAS (one-way ANOVA, Fishers LSD *post hoc* test).

** $p < 0.05$ compared to SAS (Kruskal–Wallis test, Student–Newman–Keuls *post hoc* test).

Note that the averages are essentially equivalent to those for the corresponding groups in the 'remaining brainstem' category in Figure 7.

drug effect: $F_{2, 22} = 40.4$, $p < 0.001$; SAS vs SAC and YAC: $p < 0.001$, SAC vs YAC, NS). The average intake of cocaine over 2 h was 21.5 ± 1.7 and 21.9 ± 0.4 mg/kg in the SAC and YAC groups, respectively. Horizontal locomotion was more than twice greater for SAC as compared to SAS rats (Figure 8d; drug effect: $F_{1, 15} = 7.0$, $p < 0.02$) and, whereas the locomotion of YAC rats was not measured objectively, it appeared upon visual inspection to be substantially greater than that of SAS rats and comparable to that of SAC rats.

DISCUSSION

Self-administration and investigator-administered infusions of cocaine increased the numbers of VTA-projecting brainstem neurons co-expressing Fos, the protein product of the immediate early gene *c-fos*. The peak density of such neurons occupied a neuronal aggregation in the ventromedial mesopontine tegmentum that in single sections appears spot-like, but it can be inferred from the information in a rostrocaudally ordered series of sections to represent a more or less cone-shaped volume with an apex embedded in the caudal part of the VTA/retrorubral field (RRF) and base situated in the rostromedial pontine tegmentum. Insofar as (1) no evidence was found for colocalized Fos-ir and TH-ir and (2) as many or more of the Fos-ir and double-labeled neurons lay outside of the VTA/RRF as in it, it was concluded that the Fos-ir and double-labeled neurons comprise a functional (Fos labeling)-anatomical (FG labeling) entity distinct from the VTA/RRF. With hindsight, a distinct condensation of VTA afferents coinciding with the position of this aggregation of Fos-ir neurons is clearly visible in previous descriptions (Figures 8d, 9c, and 11b in Geisler and Zahm, 2005), but, lacking the Fos-ir neurons as a reference in the earlier paper, the implications of these afferents for psychostimulant effects could not have been surmised. In further support, we have unpublished

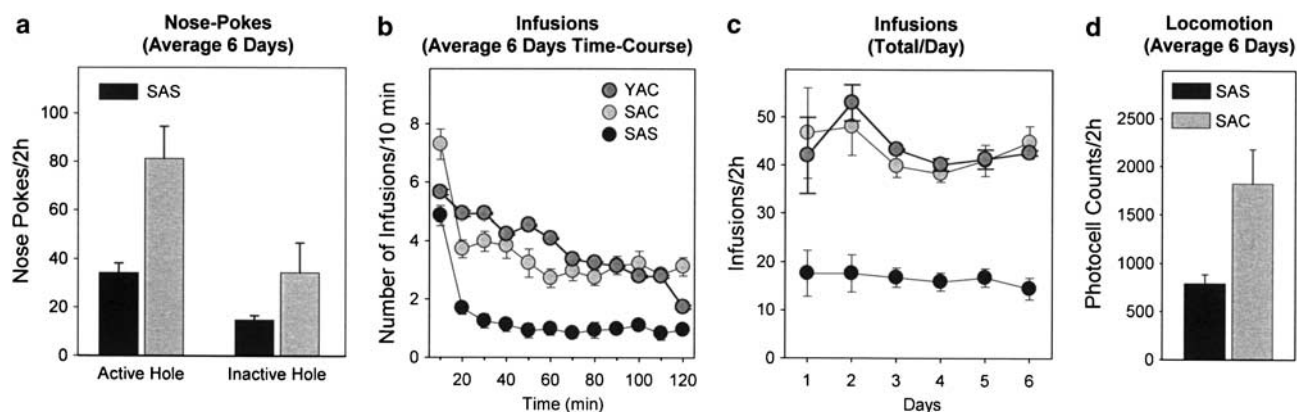


Figure 8 (a) Rats that self-administered cocaine (SAC) and saline (SAS) acquired self-administration and nose poked more in the active than in the inactive hole (hole effect: $p < 0.001$; hole \times drug effect: $p < 0.001$). SAC rats responded in the active hole more than twice as much as SAS rats (drug effect: $p < 0.01$), while responding in the inactive hole did not differ. (b) During the course of each 2 h session, SAC rats took more than twice as many infusions as SAS rats and rats in the yoked administration of the cocaine group (YAC) received infusions in numbers and at intervals equivalent to those self-administered by the SAC rats (SAS vs SAC and YAC: $p < 0.001$, SAC vs YAC: NS). (c) During the course of the six sessions, SAS showed low intake of saline; SAC rats showed high drug intake and maintained similar daily levels of cocaine self-administration that were matched in the YAC group (SAS vs SAC and YAC: $p < 0.001$, SAC vs YAC, NS). (d) SAC rats exhibited significantly greater locomotion during the daily 2 h self-administration sessions ($p < 0.02$).

anterograde tracing data indicating that neurons in this cluster give rise to a robust axonal projection that terminates in the VTA.

In contrast to the brainstem, the numbers of forebrain VTA-projecting neurons that co-expressed Fos-ir were no greater in cocaine self-administering rats and rats that received investigator-administered infusions of cocaine than in those that self-administered saline, except in the pallidum, including the globus pallidus and ventral pallidum, but these activated pallidal neurons are a minor part of the overall descending input to the VTA. Consequently, the present data suggest that afferents ascending from the mesopontine tegmentum provide the predominant activated input to the VTA during cocaine exposure. What, specifically, activates these brainstem VTA afferents remains to be determined.

Methodological Considerations

All of the SAC and SAS rats exhibited consistent and stable self-administration behavior through six daily sessions. They were killed immediately after the sixth session in order to assess the possibility that activated VTA afferents contribute to the actions of cocaine during active operant responding to acquire it. Insofar as the 6 day time point is but one window into a continuously developing drug response (White and Kalivas, 1998; Nestler, 2001), the Fos-labeling of VTA afferents, as described here, cannot be presumed representative of other time points and may turn out to be an evolving neuroadaptation to cocaine. Cocaine given i.p. at 40 mg/kg produces sporadic seizures in some animals during the second week of daily injections (Kilbey *et al*, 1979). Insofar as the rats in the present investigation self-administered on average 21.7 mg/kg/day, and i.v. administration is thought to produce 5–10 times the brain concentrations of i.p. administered cocaine, the possibility must be considered that proconvulsant levels of cocaine were achieved in the brains of some rats in the present investigation, particularly those that received

the investigator-administered drug (Dworkin *et al*, 1995). However, no seizures were observed in the present investigation, in which self-administration lasted for only 6 days and involved numerous small infusions of drug distributed over the course of 2 h. All of the self-administering rats rapidly established similar self-titrated, controlled patterns of self-administration (Yokel, 1987; Pettit and Justice, 1989, 1991; Wise *et al*, 1995; Ahmed and Koob, 1998), which were maintained during 6 days. Patterns of neuronal Fos-ir labeling throughout the brains were very consistent across animals in the various treatment groups (Zahm, unpublished data). Thus, it is concluded that the cocaine-elicited activations of brain reached in the present investigation were fairly consistent across the rats in the SAC and YAC groups and reliably below seizure thresholds.

Rats receiving cocaine infusions were compared with a control group that self-administered saline. The saline-administering rats took about half as many infusions as those nose poking for cocaine, suggesting that i.v. saline paired with a light cue provides at least a moderate level of reinforcement and associated locomotion, as shown previously (Marinelli *et al*, 2003). Although not a perfect control, rats self-infusing saline are regarded as superior to naive controls or rats receiving vehicle injections, due not only to the presumed mild motivation and associated motor activation, noted above, but also to the fact that both they and the cocaine-infused rats, including those that received investigator-administered cocaine, were exposed during testing to similar stimuli accompanying daily removal from the home cage to a separate test chamber. As regards the potential impact of stress, all of the rats used in the present study were subjected daily throughout the course of the study to gentle human handling in conjunction with the flushing of the catheters, in an effort to minimize as well as equalize across groups the effects of handling as a stress variable. In view of this precaution, it is suggested that the consistent trend observed in the present study toward greater numbers of Fos-ir VTA afferents in rats receiving

investigator- as compared to self-administered cocaine is not due to the handling associated with investigator administration of drug (Barrot *et al*, 1999), but it may instead reflect the superimposition of passive drug administration stress on the drug response (Mutschler and Miczek, 1998; Marinelli, 2007). There also may be some concern that cues associated with investigator administration of cocaine render it a signaled rather than truly noncontingent delivery of drug and thus possibly possessing some rewarding quality (Browman *et al*, 1998). Consequently, the observed Fos-labeling, which did not differ between the investigator- and self-administered treatment groups, seems most likely to have been due strictly to the pharmacological actions of cocaine, but an effect of the cognitive-motivational state of the rats cannot be entirely ruled out.

In another recent study (Colussi-Mas *et al*, 2007), D-amphetamine was administered acutely by subcutaneous injection to drug-naïve rats and produced a generalized increase in Fos-labeling in VTA afferents, including within numerous forebrain structures. Although no mention was made in that report of a dense spot-like aggregation of Fos-ir VTA afferents located in the ventromedial mesopontine tegmentum, as was observed in the present study, we have observed such a pattern of Fos-ir neurons in rats given subcutaneous injections of methamphetamine at 10 mg/kg (unpublished results), suggesting not only that this Fos expression pattern generalizes across different psychostimulant drugs but also that it is pharmacologically rather than motivationally driven. Apart from the issue of this brainstem cluster of Fos-ir neurons, the stress of a first psychostimulant drug exposure, distinct pharmacological action of cocaine as compared to D-amphetamine, different pharmacokinetics of catheter as compared to s.c. delivered drug, titration of cocaine dose by the self-administering rats themselves, and perhaps other as yet unidentified, factors may account for the differences in Fos-labeling of forebrain afferents of the VTA following administration of D-amphetamine (Colussi-Mas *et al*, 2007) and cocaine (present study).

In order to uniformly sample from VTA-projecting neurons, FG injections were administered in all rats using, to the extent possible, identical pipette tips, current parameters, and stereotaxic coordinates, which led to a potential shortcoming of the study, that is, the coordinates employed produced FG injections mainly in the lateral part of the VTA. However, our unpublished anterograde tracing study revealed dense axonal projections from the Fos-ir, VTA-labeled neuron cluster to lateral and medial parts of the VTA, suggesting that the medial VTA is also related by connections to the aggregation of Fos-ir neurons observed following cocaine administration.

Insofar as retrograde labeling by FG is complete a day or two after FG is injected (Zahm *et al*, 2001), FG-ir should have 'pre-labeled' similar numbers of VTA afferents in the three treatment groups. That this was achieved is confirmed by Table 1, which shows statistically indistinguishable numbers of retrogradely labeled neurons in the forebrain and brainstem in the four evaluated cases from each of the three treatment groups. This being so, it can be argued that rather than calculating what percent of retrogradely labeled neurons they comprised, the

double-labeled neurons could simply have been counted. Indeed, simple counts of double-labeled neurons, despite exhibiting more data scatter than the percentages, did reveal statistically valid increases in the brainstem and pallidum, but not in the rest of the forebrain, following cocaine administration (Table 1).

Distinct Patterns of Cocaine-Elicited Fos Labeling of VTA Afferents in the Forebrain and Brainstem

The present data reveal that the administration of cocaine is accompanied by activation of VTA afferents residing mainly in the mesopontine tegmentum and, to a lesser extent, pallidum. In interpreting this finding, the following points need to be kept in mind. (1) Failure to detect Fos-ir in a neuron does not necessarily mean that it has not been activated. (2) Increased activity of DA neurons following psychostimulant administration may be due to the removal of tonic inhibitory influences, which would not be revealed by the detection of Fos-ir. (3) Increased Fos expression could signal increased activity in inhibitory afferents of the VTA. Thus, it is apparent that the extent to which the observed activation of afferents causes an excitation of DAergic neurons was not addressed in the present study nor necessarily predictable from the data.

With these caveats in mind, a number of points can be made, nonetheless, regarding potential roles for VTA afferents in the control of DA neuron activity following cocaine administration. Ventral pallidal afferents of the VTA, which are known to contact DAergic neurons (Groenewegen *et al*, 1994), were classically regarded as GABAergic (Kalivas *et al*, 1993) and thus, upon activation, might be expected to inhibit DAergic neurons. Indeed, Floresco *et al* (2003) have reported that inhibition of pallidal afferents by infusion of a muscimol/baclofen cocktail selectively increases the population activity of DA neurons in the VTA. However, it is likely that VP neurons also target GABAergic neurons residing in the VTA (eg Carr and Sesack, 2000) or occupying other structures that project to the VTA (Geisler and Zahm, 2005), either of which might then disinhibit DA neurons. Also noteworthy in this regard is the recent observation that 35% of the ventral pallidal neurons projecting to the VTA is glutamatergic (Geisler *et al*, 2007). Consistent with the cautionary considerations listed above, such potential disinhibitory and excitatory actions may have important physiological roles that would not necessarily be revealed within a milieu of neurons profoundly silenced by muscimol/baclofen infused into the VP.

In view of the robust cocaine-elicited Fos expression observed in numerous brainstem afferents of the VTA located in the caudal ventromedial mesencephalon and rostromedial pons, it is interesting to recollect that a 'midbrain locomotor region' (MLR), that is, a district occupied by sites where electrical stimulations elicit coordinated locomotor movements in anesthetized cats (Shik *et al*, 1966) and rats (Coles *et al*, 1983), is present at the same brainstem levels. This coincidence calls to mind the possibility that the Fos-activated neurons detected in this study might belong to the MLR and so exert the acute locomotor activating effects of cocaine directly. In considering this possibility, it is pertinent that the MLR

was first reported to coincide with the cuneiform nucleus (Shik *et al*, 1966) and then the PPTg nucleus (Garcia-Rill, 1986), both of which lie well lateral to the striking condensation of VTA afferents exhibiting cocaine-elicited Fos-expression described herein. However, in an incisive review of the literature, Inglis and Winn (1995) later maintained that neither of these structures represents an accurate neuroanatomical correlate of the MLR. On the contrary, as studies continued to promulgate, more diverse and complex movements were observed following stimulations in an increasingly broad expanse of mesopontine sites. Consequently, although the possibility cannot be excluded at present that Fos-activated VTA afferents contribute directly to the acute locomotor activating effects of cocaine via local mechanisms exerted within the MLR or an expanded conceptualization of it, the connections with the VTA seem more likely to be critical in this regard.

Any suggestions regarding the phenotype(s) or synaptic relationships of the densely packed cocaine-activated brainstem afferents of the VTA must also be regarded as speculative. We do know that these neurons are not DAergic (present results) and neither is their prominently ventromedial position consistent with much overlap with the cholinergic/glutamatergic PPTg-LDTg nuclear complex, although such overlap clearly occurs to a limited extent in the ventromedial, so-called dissipated parts, of the PPTg and less so in its other parts (Table 3). The LDTg, reported to provide the major cholinergic projection to the VTA (Oakman *et al*, 1995), contained numerous retrogradely labeled neurons, but only moderate double-labeling (Table 3). Accordingly, we also saw little double-labeling of Fos-ir in NOS-ir (cholinergic) neurons in the PPTg or LDTg (Supplementary Figure S2). It is unclear how the present functional-anatomical data will ultimately reconcile with the results of functional studies identifying the LDTg specifically as critical to the capacity of VTA DA neurons to burst fire (Lodge and Grace, 2006) and the compact part of the PPTg as critical to stimulating such burst firing (Floresco *et al*, 2003).

The glutamatergic phenotype also seems to be not strongly represented in the VTA-projecting neurons occupying the ventromedial mesopontine tegmentum (Figure 6j in Geisler *et al*, 2007), which leaves GABA as an obvious compound of interest, but with essentially no empirical evidence for or against. Thus, pending further investigation, it may be imprudent to speculate further about whether the Fos-activation of VTA afferents might indicate facilitation or opposition to previously described mechanisms (eg Cameron and Williams, 1994; Marinelli *et al*, 2003; Shi *et al*, 2004; Liu *et al*, 2005) thought to contribute to maintaining the activity of VTA DA neurons against autoreceptor-mediated inhibition in the presence of cocaine. Nonetheless, to the extent that repeated *c-fos* expression has been associated with the development of compensatory neuroadaptations to cocaine in other brain structures (eg Hope *et al*, 1992; Kelz *et al*, 1999), it seems quite reasonable to entertain the possibility that cocaine-activated Fos-ir afferents of the VTA may be a substrate for additional neuroadaptations that could directly influence the responses of VTA DA neurons during cocaine-seeking, withdrawal, and relapse.

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DISCLOSURE/CONFLICT OF INTEREST

The authors declare that, except for income received from their primary employer, no financial support or compensation has been received from any individual or corporate entity over the past 3 years for research or professional service and there are no personal financial holdings that could be perceived as constituting a potential conflict of interest.

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