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Fos after single and repeated self-administration of cocaine and saline in the rat: emphasis on the basal forebrain and recalibration of expression

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Abstract

The effects of addictive psychostimulant drugs on the brain change over repeated administrations. We evaluated a large sample of brain structures, particularly ones comprising basal forebrain macrosystems, and determined in which the immediate-early gene product, Fos, is expressed following a single and repeated self-administrations of cocaine. The caudate-putamen and accumbens, comprising the basal ganglia input structures, and the hypothalamic supraoptic and paraventricular nuclei, lateral and medial habenula, mesopontine rostromedial tegmental nucleus and anterior cingulate cortex exhibited Fos expression enhanced by acute self-administration of cocaine, but desensitized after repeated administrations. Fos expression was mainly enhanced by acutely self-administered cocaine in basal ganglia output and intrinsic structures and the intermediate nucleus of lateral septum, medial division of the central amygdaloid nucleus and zona incerta, but, in contrast, was sensitized in these structures after repeated administrations. Acute and repeated self-administration of cocaine left Fos expression unaffected or marginally enhanced in most extended amygdala structures, of which nearly all, however, exhibited robustly increased Fos expression after repeated saline self-administration, occasionally to levels exceeding those elicited by cocaine. Thus, self-administered cocaine mainly elicits Fos expression, which persists or increases with repeated administrations in some structures, but declines in others. In addition, Fos expression is sensitized in most extended amygdala structures merely by the act of repeated self-administering. Similar spatiotemporal patterns of cocaine- or saline-elicited Fos expression characterize functionally-related clusters of structures, such as, e.g., basal ganglia input

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structures, basal ganglia output structures, extended amygdala and structures in the brainstem to which forebrain macrosystems project.

Keywords

drug abuse; addiction; relapse; psychostimulant; dopamine

Cocaine, a tenaciously addictive drug (de Wit and Stewart, 1981; Childress et al., 1988; O'Brien et al., 1990; Ehrman et al., 1992; Bauer and Kranzler, 1994; Bossert et al., 2005; O'Brien, 2005; Shaham and Hope, 2005), acts mainly by inhibiting the dopamine transporter (Koe, 1976; Ritz et al., 1987; Woolverton, 1987; Einhorn et al., 1988; Balster, 1990; Vanover, 1992; Howell and Byrd, 1995; Roberts et al., 1999; Wee and Woolverton, 2004), resulting in an accumulation of extracellular dopamine and consequent hyperstimulation of postsynaptic dopamine receptors. This, in turn, increases Fos expression preferentially in structures within the trajectory of the mesotelencephalic dopaminergic pathway (Graybiel et al., 1990; Young et al., 1991). Fos, encoded by the immediate-early gene, c-*fos*, contributes to transcriptional regulation of numerous macromolecules and is oft exploited to map neuronal activation (Curran and Morgan, 1985; Morgan and Curran, 1991; Sharp et al., 1993; Farvivar et al., 2004).

Recently, Zhang et al. (2006) showed that a conditional knockout of c-*fos*, preferentially targeted to postsynaptic D1 receptor-expressing neurons in the ventral and dorsal striatum, attenuates some of the behavioral and neurochemical effects of cocaine. Indeed, a number of other measures of neural activation, including glucose metabolism in the rat (London et al, 1986; Porrino et al., 1988; Sharkey et al., 1991; Porrino, 1993; Macey et al., 2004), monkey (Graham and Porrino, 1995; Lyons et al., 1996; Porrino et al., 2002), and human (London et al., 1990), as well as cerebral blood flow in the monkey (Howell et al., 2002), and BOLD (blood oxygen level detection) signaling in the human (Breiter et al., 1997) indicate that the mesotelencephalic dopaminergic axis is engaged during acute cocaine administration, such that agents that attenuate neural activation within this axis should be expected to modulate the behavioral effects of cocaine. Accordingly, numerous investigations of cocaine-elicited neuroadaptations that linger long after the cessation of drug use have focused on structures that receive dense dopaminergic input, such as the dorsal and ventral striatum (e.g., Nestler, 2001a; b; 2005; 2008; LaRowe et al., 2007; Zavala et al., 2007; Conrad et al., 2008; Thomas et al., 2008; Moussawi et al., 2009). Similarly, most reports describing the effects of cocaine on Fos expression also contain observations limited to striatopallidum (e.g., Graybiel et al., 1990; Hope et al., 1992; Cenci et al., 1992; Moratalla et al., 1993; 1996; Uslaner et al., 2001a; Crombag et al., 2002; Willuhn et al., 2003; 2006; Samaha et al., 2004).

However, the basal forebrain contains, in addition to striatopallidum, the extended amygdala and septal-preoptic projection system (Heimer, 2003; Heimer and Van Hoesen, 2006; Zahm, 2006; Heimer et al., 2008), which share fundamental neuroanatomical similarities with striatopallidum (Alheid and Heimer, 1988; Swanson, 2000; Zahm, 2006) and also receive substantial dopaminergic innervation (Fallon and Moore, 1978a; b; Fallon et al., 1978). Each is a potential substrate for neuroadaptation if it expresses cocaine-elicited Fos, particularly if

in a manner that changes during a course of repeated cocaine administrations. Cocaine-elicited Fos expression has been reported in the extended amygdala (Day et al., 2001), as well as some other forebrain and brainstem structures, such as the subthalamic nucleus (Uslaner et al., 2001b; 2003) and rostromedial tegmental nucleus (Geisler et al., 2008; Jhou et al., 2009), that, interestingly, do not receive dense dopaminergic terminations. Furthermore, systemically and locally applied cocaine acutely modulates responses of cortical somatosensory neurons (Jiménez-Rivera and Waterhouse, 1991; Bekavak and Waterhouse, 1995; Rutter et al., 1998; Drouin and Waterhouse, 2004) and cerebellar Purkinje cells (Jimenez-Rivera et al, 2000), both clearly beyond the reach of dopaminergic projections. Together, these considerations encourage casting the net beyond the striatum if the range of possible cocaine-induced neuroadaptations is to be adequately appreciated. One way to approach this is to better characterize the breadth of the collection of structures in which Fos expression is elicited by cocaine administration. Accordingly, we undertook to determine if Fos is expressed following acute and repeated self-administration of cocaine in a select sample of brain structures, mostly representing different parts of the several basal forebrain macrosystems, and whether levels at which Fos is expressed after a single self-administration session are recalibrated following repeated administrations.

Material and Methods

Forty-five male Sprague-Dawley rats (Harlan, Indianapolis, IN, USA) weighing 225-250g entered the study, which was carried out in accordance with guidelines mandated in the National Institutes of Health Guide for the Care and Use of Laboratory Animals. Twenty-five rats had been used in a previous study in which the retrograde tracer FluoroGold (FG) was injected unilaterally into the ventral tegmental area at the time that rats were also instrumented with intra-jugular catheters for intravenous (i.v.) administration of cocaine or saline (see Geisler et al., 2008, for details). A second cohort of twenty rats that did not receive FG injections was instrumented with catheters at a subsequent time. All of the rats were singly housed on a 12 hr 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

The rats were deeply anaesthetized by intraperitoneal injections of a cocktail consisting of 45% ketamine (100 mg/ml), 35% xylazine (20mg/ml) and 20% saline at a dose of 0.16 ml/100g of body weight. A silastic catheter (20 μl 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. As noted above, some of the rats also received iontophoretic injections of FG into one side of the mesencephalic ventral tegmental area prior to awakening (see Geisler et al., 2008, for details). The rats were kept warm until they awakened and, thereafter, the catheters were flushed daily with sterile saline to prevent clogging.

Self- and investigator-administration of cocaine and vehicle

Seven to ten days after surgery, the animals were placed in self-administration chambers (Med. Associates, St. Albans, VT) and allowed to self-administer cocaine (500 μg/kg per 30 μl infusion) or saline for 2h/day, during the dark part of the dark/light cycle, as previously

described (Marinelli et al., 2003). Nose-poking in an active hole delivered an infusion (cocaine or saline) and illuminated the hole with an LED light for 30 sec, whereas nose poking in an inactive hole had no programmed consequences. Self-administration was regarded as established when nose pokes were significantly more numerous in the active than inactive hole. During the self-administration period, a separate group of rats was placed in separate chambers located in the same environment as the self-administration chambers and given infusions of cocaine or saline, also by infusion pump, while the rats were held gently in the hand of the investigator. These investigator-administered infusions were given in numbers and at time-intervals reflecting the averages delivered on the same day to the rats in the cocaine self-administering group. Patency of the catheters was confirmed with an infusion of 200 μl of ketamine/xylazine mixture through the catheters on the last day of the experiment. All rats succumbed within 3-5 s following this treatment, indicating that all of the lines remained open throughout the study.

Experimental groups

Groups, shown diagrammatically in Figure 1, comprised the following: a single session of saline (SAS 1d; n=6) or cocaine (SAC 1d; n=6) self-administration; self-administration of saline (SAS 6d; n=8) or cocaine (SAC 6d; n=9) or investigator-administration of cocaine (IAC 6d; n=8) in six consecutive daily sessions; and self-administration of cocaine (n=4) or saline (n=4) in five consecutive daily sessions with no infusion given on the sixth day (SAC 5d and SAS 5d, respectively). Rats were perfused 2h following the start of the final drug or vehicle administration session, except for rats in the SAC 5d and SAS 5d (basal Fos) groups, which were anesthetized on the sixth day in the home cage and perfused immediately thereafter. Only rats in the 6d cocaine (SAC 6d and IAC 6d) and vehicle (SAS 6d) administration groups received VTA FG injections, which have been described separately (Geisler et al., 2008).

Fixation of brains and immunocytochemistry

Upon induction of deep anesthesia by an infusion of ketamine/xylazine mixture through the catheters, the rats were perfused transaortically with a solution of 0.1 M Sorensen's phosphate buffer (SPB) containing 4% freshly depolymerized paraformaldehyde (pH 7.4) and 2.5 % sucrose. The brains were removed, post-fixed in fresh fixative solution for four hours, rinsed in SPB, sunk in a 25% sucrose solution, and sectioned frozen on a sliding microtome. Five adjacent series of 50 μm thick sections were collected, each representing an entire brain from frontal pole to caudal medulla. 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. In order to promote uniform immunoreactions and minimize processing-related variability across the different cases, series of sections from rats in each of the various experimental groups were run "*in tandem*", i.e., immersed for identical times in aliquots of the immunohistochemical processing solutions. However, *in tandem*, i.e., batch, processing of the 6d SAS, SAC and IAC cohorts was done separately from that of the 1d SAS, 1d SAC and basal Fos cohorts, because the self-administration sessions for the two groups were run at different times.

Immunohistochemical processing steps were carried out at room temperature unless otherwise indicated, and under gentle agitation on a horizontal rotator (Lab-Line, Fisher, Pittsburgh, PA). As mentioned, for each step, several entire series of free floating sections were processed in aliquots from batches of the relevant solutions. First, a rinse in 0.1 M Sorenson's phosphate buffer (SPB, pH 7.4) was followed by immersion in 1 % sodium borohydride for 15 minutes and subsequent thorough rinsing in 0.1 M SPB. A brief pretreatment with 0.1M SPB containing 0.2 % Triton X-100 immediately followed, after which the sections were transferred into a solution of 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 various series of sections were immersed in aliquots of SPB-t containing a biotinylated antibody against rabbit IgGs made in donkey and used at a dilution of 1:200 (Jackson ImmunoResearch Laboratories Inc., West Grove, PA) for an hour. Afterward, the sections were rinsed in SPB-t 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 then were rinsed and 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 was absent when the primary or secondary antibodies or ABC reagents were omitted and following preabsorption with the cognate peptide (Calbiochem) used at a dilution of 50 ng/μl.

Immunofluorescence

Series of sections from SAC 1d brains were processed as described in the preceding subsection, but using the following sequence of immunoreagents: [1] rabbit anti-c-Fos (Calbiochem) used at a dilution of 1:5000 overnight, [2] biotinylated anti-rabbit from donkey (Jackson) used at 1:200 for 1 hour, [3] ExtrAvidin conjugated to fluorescein isothiocyanate (FITC, Sigma) used at 1:200 for 1 hour, mouse (monoclonal) anti-neuronal nuclear antigen (Chemicon, Temecula, CA), a marker of nearly all types of neurons in the vertebrate CNS (Mullen et al., 1992), used at 1:30,000 overnight, and anti-mouse conjugated to Texas Red (Jackson) used at a dilution of 1:100 for 1 hour. The sections were mounted on subbed glass slides and coverslipped with ProLong Gold (Invitrogen, Eugene, OR). Sections were viewed and photographed with an Olympus BX51 light microscope in epifluorescence mode using a dual band filter block (Semrock, Rochester, NY — catalogue designation: FITC/TxRed-A, excitation centers [bandwidths] in nm: 479[38] and 585[27], emission centers[bandwidths] in nm: 534[29] and 628[33]). The relevant required excitations and emissions values (provided by Semrock) are, respectively, 492 and 520 (FITC) and 595 and 617 (Texas Red). Images were captured with a DVC-2000C digital camera and DVC View software (both DVC Company, Austin, TX). Red and green images in a single focal plane were acquired sequentially by tuning out non-relevant wavelength sensitivities with the software, which avoided shifts in the image and/or focal plane that accompany switching out

filter blocks. For the purpose of illustration, magenta was substituted for red with Photoshop CS2 software (Adobe), after which merges were done with ImageJ software (NIH).

Analysis of Fos-ir neuron density

Appropriate to the goal of establishing differences between groups in sectioned material identical in all respects other than numbers of Fos-expressing neurons, we chose to use density measurements (Saper, 1996) rather than stereological estimates (Coggeshall and Lekan, 1996) as the independent variable. This decision was in large part dictated by the large number of cases and structures requiring evaluation, which was done using a semi-automated approach described as follows. Sections were initially surveyed under brightfield optics with the aid of a Nikon Optiphot light microscope in brightfield mode at low magnification. Subjective estimates were made of the densities Fos-immunoreactive (ir) neurons in numerous structures in the brains of the saline self-administering controls (basal Fos and SAS 1d and 6d groups, see Table 1). Quantitative measurements of the density of Fos immunoreactive neurons in the corresponding SAS, SAC and IAC groups were then done in a subset of these structures (bracketed in Table 1). The quantitation was done by first identifying regions of interest (ROIs) at low magnification (Fig. 2). The ROIs were then refocused with the 20X objective and digital images were captured using a Sony DXC-970MD video camera interfaced by means of an A-D converter to a dedicated software package (Northern Eclipse, Missisauga, ON) with the aid of which the digitized images were converted to gray scale and densitometric thresholding was utilized to select optically dense Fos-ir nuclei. Thresholding was further refined by digitally rejecting detected objects too large or small to be Fos-ir nuclei. Selected Fos-ir nuclei also were pseudo-colorized, which allowed the evaluator to visually inspect the sample for undetected Fos-ir nuclei and objects detected by the software that should not have been. Either condition necessitated re-thresholding to optimize detection of Fos-ir nuclei. When this was achieved, the ROI was digitally circumscribed and the indicated area and number of detected objects within it were exported to a spreadsheet for calculation of object density and organization of the data for statistical analysis. In cases in which the ROIs did not approximate the entire field of view, the sizes and shapes of circumscribed ROIs were standardized across the six experimental groups. Measurements were done in quadruplicate, i.e., in two sequential sections on both sides of the brain. Rarely, a missing section or section defect precluded acquisition of four measurements and the average was calculated instead with two or three. Group means were calculated and, using GB-STAT software (Dynamic Microsystems, Silver Spring, MD), statistically tested with a one-way ANOVA (density) followed as indicated by the LSD (least significant difference) post hoc test.

A note on the ROIs

The ROIs shown in Figure 2 were selected both to represent a relatively broad sample of brain structures, with an emphasis on forebrain macrosystems, and test whether Fos expression elicited by self-administration of saline and cocaine differs in different subdivisions of the same structures, such as, e.g., accumbens core, shell and rostral pole (Fig. 2A and B), central and ventrolateral parts of the caudate-putamen (Fig. 2D), dorsolateral, dorsomedial and ventral divisions of the bed nucleus of stria terminalis (Fig. 2H), medial and lateral divisions of the central amygdaloid nucleus (Fig. 2I), intermediate

and ventral divisions of the lateral septal nucleus (Fig. 2J), medial and lateral divisions of the lateral habenular nucleus (Fig. 2K), and rostral and caudal parts of the rostromedial tegmental nucleus (Fig. 2M and N).

Certain of the ROIs require a sentence or two in further explanation. The rostromedial tegmental nucleus is a recently described structure in the ventral mesencephalon comprising a rostral part (RMTg r; Fig. 2M), which is embedded among the dopaminergic processes of the caudomedial VTA, and caudal part (RMTg c, Fig. 2N), which occupies the medial tegmentum caudal to the VTA. Both parts of the RMTg exhibit strong Fos expression in the cocaine exposed brains (Geisler et al., 2008; Jhou et al., 2009). The ROI for the suprachiasmatic nucleus (SCN) comprises not the SCN itself, but rather a Fos-dense cap region suprajacent to it (Fig. 2Q). The ROI for the piriform cortex (Fig. 2S) includes only the dense cell layer. ROIs for the S1 cortex (Fig. 2T) correspond to the Fos-dense lamina 3.

Results

Behavior

Rats in the SAC 1d, SAC 6d, and IAC 6d groups received about the same number of cocaine infusions in all sessions (Fig. 3A), indicating that the rats in each of these groups on average had similar amounts of cocaine on-board on day of sacrifice and that the self-administration history for the SAC 6d and IAC 6d rats was on average similar. However, as indicated in Figures 3B and C, responding (numbers of nose pokes) and locomotor activity, respectively, declined over several daily sessions in the SAC 6d group to values significantly below the mean of the SAC 1d group, likely reflecting the tendency of SAC 6d rats to make fewer ineffective approaches and nose pokes as they accumulate experience in self-administering. SAS rats were about half as active and delivered about 35% of the infusions recorded for SAC rats. However, responding and locomotor activity in the SAS 6d group were comparable to that of SAS 1d rats throughout the course of six consecutive self-administration sessions.

Some rats in the group that self-administered cocaine for 5 days and was anesthetized in the home cage on day 6 (SAC 5d “basal Fos” group) delivered lesser amounts of cocaine than did rats in the SAC 1 and 6 day groups (Fig. 3A). Two such rats actually stopped self-administering on days 4 and 5. Despite these drug intake anomalies, uniformly low Fos expression was detected in all of the “basal Fos” rats, whether SAS or SAC and whether they discontinued self-administering or persisted through the full 5 days (see below).

Fos immunoreactivity

Immunoreaction product generated with the Fos antibody occupied exclusively cell nuclei (Figs. 4 and 5). The impression that such immunolabeled nuclei represented a range of sizes consistent with mainly neuronal labeling was confirmed with immunofluorescence double-labeling with the neuron-specific marker NeuN (Mullen et al., 1992) in sections from SAC 1d brains, which revealed in several structures, including the accumbens, caudate-putamen (Fig. 4) and piriform cortex, that Fos-ir nuclei were confined almost exclusively to NeuN

immunoreactive profiles. Fos labeling was present in many brain structures in densities that varied depending on the treatment group (Table 1; Figs. 6, 7, 8 and 9)

Saline controls

SAS 1d (Fig. 5A and B) and 6d (Fig. 5C and D) brains exhibited abundant and widespread Fos-ir nuclei in numerous brain structures, whereas fewer and more lightly immunostained Fos-ir nuclei were present in the brains of rats that self-administered cocaine (SAC 5d) or saline (SAS 5d) on 5 consecutive days and then were anesthetized in the home cage on day 6 and killed immediately thereafter (Fig. 5E and F, Table 1). The estimated relative densities of Fos-ir cells in the saline and basal Fos groups are shown in Table 1. Estimates given in Table 1 were validated by objective quantitation in 32 structures [bracketed in Table 1] that were selected as regions of interest (ROIs) for quantitative analysis. The ROIs (Fig. 2) are described in the Materials and Methods.

Quantitative comparisons

Fos labeling, expressed as mean density of Fos-ir neuronal nuclei in ROIs after 1 session and 6 consecutive daily sessions of cocaine or saline self-administration, is shown in Figures 6-9, where saline controls (SAS) are paired with the directly comparable cocaine-receiving (SAC and IAC) groups. Each ROI (Fig. 2) is represented by a cluster of five vertical bars and a horizontal reference for “basal Fos” expression. Acute groups are represented by a pair of bars: self-administered saline (SAS 1d, n=6, white bars with hatches) and cocaine (SAC 1d, n=6, white bars). Repeated administration groups are represented by 3 bars: self-administered saline (SAS 6d, n= 8, gray bars with hatches) and cocaine (SAC 6d, n=9, gray bars) and investigator-administered cocaine (IAC 6d, n=8, black bars). Widths of the horizontal bars reflect the magnitude of the SEM above and below the mean for “basal Fos” expression. As noted above, because significant differences were not detected between the basal SAC and basal SAS groups, the data from those groups were pooled. The results of the ANOVA and LSD post hoc tests applied to the data are given in the cross-reference tables beneath the graphs (Figs. 6C - 9C) and in Table 2. Table 2 shows, in addition, the actual magnitudes of significant responses, reflected in the mean SAC and 1d values after the respective SAS and 6d means were subtracted out.

SAC vs. SAS—Cocaine, whether self-administered over 1 or 6 sessions or investigator-administered over 6 sessions, mainly increased Fos expression as compared to the relevant SAS group (Figs. 6-9; Table 2, column A). Two different kinds of response could be observed. In one, Fos expression was low in the SAS group (Fig. 10A, B, E and F) and much greater in the corresponding SAC group (Fig. 10C, D, G and H) after both 1 and 6 self-administration sessions, resulting in marked increases in both the intensity of immunostaining of Fos-ir/nucleus and density of Fos-ir neurons. Evaluated structures exhibiting this type of response included the ventral pallidum (Fig. 6A), caudate-putamen (Figs. 6B and 10), globus pallidus (Fig. 6B) subthalamic nucleus (Fig 6B), substantia nigra reticulata (Fig. 6B), intermediate division of the lateral septum (Fig. 7B), lateral division of the lateral habenula (Fig. 7B), zona incerta (Fig. 7B), ventral tegmental area (Fig. 8A) and rostromedial tegmental nucleus (Fig. 8A). The other response type, was reflected in moderate amounts of SAS-elicited Fos labeling that could be moderately greater in the

corresponding SAC groups, after either 1 or 6 self-administration session(s), but generally not both. Structures exhibiting this kind response after 1, but not 6, session(s) included the accumbens rostral pole (Fig. 6A), dorsomedial division of the bed nucleus of stria terminalis (Fig. 7A), medial division of the lateral habenula (Fig. 7B), supraoptic nucleus (Fig. 8B), and prelimbic and anterior cingulate cortex (Fig. 9). Structures that exhibited this response after 6, but not 1, sessions included the dorsolateral division of the bed nucleus of stria terminalis (Fig. 7A) and the pedunculopontine and laterodorsal tegmental nuclei (Fig. 8A).

Three (of 32) quantitatively evaluated structures - the lateral division of the central amygdaloid nucleus (Fig. 6C), ventral division of the lateral septum (Fig. 7B), and supraoptic nucleus (Fig. 8B) - exhibited a significantly lesser density of Fos-ir nuclei following cocaine administration as compared to the corresponding SAS group after 6, but not 1, self-administration sessions. Interestingly, of these, the lateral division of the central amygdaloid nucleus and supraoptic nucleus exhibited greater than control Fos expression after the first cocaine self-administration session. Note, however, that the apparent decreases in SAC Fos-expressing neurons in the lateral division of the central amygdaloid nucleus and ventral division of the lateral septum on day 6 were due not to fewer SAC-elicited Fos-expressing neurons, but rather to greatly increased SAS-elicited Fos expression (Fig. 7A and B; Table 2). In contrast, the change in density of Fos-expressing neurons between day 1 and day 6 in the supraoptic nucleus reflected a true decrease in the numbers of Fos expressing neurons (Fig. 8B and Table 2). No significant differences distinguishing SAS and SAC groups at 1 or 6 days were observed in the accumbens shell or core (Fig. 11), piriform cortex, forelimb/hindlimb somatosensory cortex or suprachiasmatic nucleus.

Day 6 vs. day 1—Table 2, column B shows that the density of Fos-ir neurons after 6 daily sessions of cocaine self-administration differed significantly in a number of brain structures from that observed in the same structures following only 1 self-administration session. The same column, in addition, provides the actual magnitudes of significant responses, reflected in the mean day 6 values after the respective day 1 means had been subtracted out. Structures in which Fos-ir neuron density was greater after 6 sessions as compared to 1 included the ventral pallidum (Fig. 6A), globus pallidus (Fig. 6B), substantia nigra pars reticulata (Fig. 6B), dorsolateral and ventral divisions of the bed nucleus of stria terminalis (Fig. 7A), lateral division of the central amygdaloid nucleus (Fig. 7A), intermediate division of the lateral septum (Fig. 7B), zona incerta (Fig. 7B) and ventral tegmental area (Fig. 8A). Structures in which Fos-ir neuronal density decreased after 6 sessions as compared to 1 included the accumbens rostral pole and shell (Fig. 6A), central and ventrolateral parts of caudate-putamen (fig. 6B), medial and lateral divisions of the lateral habenula (Fig. 7B), rostral and caudal parts of the rostromedial tegmental nucleus (Fig. 8A), hypothalamic supraoptic and paraventricular nuclei (Fig. 8B) and anterior cingulate cortex (Fig. 9). Fos-ir neuron density in the SAS group was increased in a number of structures after 6 as compared to 1 sessions. These included the dorsomedial and ventral divisions of the bed nucleus of stria terminalis (Fig. 7A), medial and lateral divisions of the central amygdaloid nucleus (Fig. 7A), ventral division of the lateral septum (Fig. 7B), zona incerta (Fig. 7B) and piriform cortex (Fig. 9). Fos-ir neuron density decreased after 6 as compared to 1 saline self-administration sessions in the central part of the caudate-nucleus (Fig. 6A), laterodorsal

tegmental nucleus (Fig. 8A), supraoptic nucleus (Fig. 8B), and the somatosensory cortical barrel field (Fig. 9).

Patterns of Fos response—One subset of evaluated structures exhibited negligible Fos expression following self-administration of saline, but robust Fos expression after both 1 session and 6 consecutive daily sessions of cocaine administration. Another group of evaluated structures exhibited moderate to robust expression of Fos following self-administration of saline and many of these showed a further, generally modest, augmentation of Fos expression, after either 1 or 6 sessions of cocaine self-administration. The different Fos responses were not randomly distributed among basal forebrain and brainstem structures, but instead clustered in patterns reflecting the identities of various components of basal forebrain macrosystems (Table 2).

Thus, evaluated basal ganglia input nuclei, which include subterritories of the accumbens and caudate-putamen, adhered to a general pattern in which cocaine mainly enhances Fos expression in a robust manner that desensitizes after repeated administrations. Fos expression in these structures was greater or statistically similar in the SAC as compared to SAS condition on days 1 and 6, while SAC-elicited Fos expression after 6 as compared to 1 sessions was mainly decreased. SAS-elicited Fos expression after 6 as compared to 1 sessions was mainly unchanged in these structures. This pattern, i.e., acute cocaine-elicited robust enhancement of Fos expression that desensitizes on repeated administrations, was also observed in the lateral habenula, rostromedial tegmental nucleus, pedunculopontine and laterodorsal tegmental nuclei, hypothalamic paraventricular and supraoptic nuclei, and anterior cingulate and barrel field cortex.

In evaluated basal ganglia output and intrinsic nuclei, including the ventral pallidum, globus pallidus, subthalamic nucleus, substantia nigra reticulata and ventral tegmental area, and also in the intermediate division of the lateral septum, lateral division of the central amygdaloid nucleus and zona incerta, the SAC as compared to SAS on days 1 and 6 was accompanied by robustly increased Fos expression. In all but the subthalamic nucleus, SAC-elicited Fos expression was further increased after 6 as compared to 1 sessions. SAS-elicited Fos expression after 6 as compared to 1 sessions was uniformly unchanged in all of these structures, except the lateral division of the central amygdala and zona incerta, where it was increased. Thus, in this pattern, cocaine robustly enhances Fos expression on day 1 *and* 6 in a manner that sensitizes with repeated administrations.

Evaluated extended amygdala structures and the ventral division of the lateral septum exhibited SAC-elicited Fos expression that was statistically similar or marginally greater than the relevant SAS control on days 1 or 6. In nearly all of these structures, however, robustly sensitized Fos responses were observed after repeated saline administrations.

SAC vs. IAC—Means for Fos-ir neuron density were significantly greater in the IAC 6d group than in the SAC 6d group in the accumbens rostral pole (Fig. 6A) and core (Fig. 6A), central part of the caudate-putamen (Fig. 6B), intermediate division of the lateral septum (Fig. 7B), medial division of the lateral habenula (Fig. 7B), zona incerta (Fig. 7B), ventral tegmental area (Fig. 8A), rostral division of the rostromedial tegmental nucleus (Fig 8A),

laterodorsal tegmental nucleus (Fig 8A), piriform cortex (Fig. 9), prelimbic cortex (Fig. 9), anterior cingulate cortex (Fig. 9) and the forelimb/hindlimb part of the S1 somatosensory area (Fig. 9). In contrast, the IAC 6d value was significantly below that of the SAC 6d group in the medial division of the central amygdaloid nucleus (Fig. 7A) and it trended toward lesser in the dorsolateral division of the bed nucleus of stria terminalis (Fig. 7A) and paraventricular nucleus of the hypothalamus (Fig. 8B). In the remaining structures the IAC 6d and SAC 6d values were not different.

Discussion

Data described herein suggest that the expression and recalibration of Fos reflect roles that various functional-anatomical subdivisions of the forebrain have in mediating behavioral activation associated with early phases of cocaine and saline self-administration.

Methodological considerations

While Fos expression unambiguously marks cells as opposed to axons, is not necessarily an indication of “activation”, but rather can be triggered by changes in intracellular signaling or neuronal activity, or both, depending on the stimulus and brain structure evaluated (Sharp et al., 1993; Farvivar et al., 2004). It is also important to emphasize that a pattern of brain Fos-labeling observed at any given point during a course of cocaine self-administration is not representative of other points (White and Kalivas, 1998; Nestler, 2001a; b). We selected 1d and 6d groups to represent, respectively, acute responses and the responses when rats have habituated to self-administering, but do not exhibit overt signs of withdrawal between sessions.

SAS rats were slightly less active in the self-administration chambers than SAC rats, particularly on days 3-6 (Fig 3B and C), but the slight differences in locomotion seem unlikely to explain the robustly greater Fos expression observed in the SAC rats. Actually, the expression of Fos in the SAS rats was equivalent to that in SAC brains in about one third of the evaluated structures. In contrast, rats that were anesthetized in the home cage (basal Fos group) exhibited little Fos expression. Presumably, much Fos expression observed in both SAS and SAC brains was related to arousing stimuli associated with transferring the rats from home cage to the self-administration environment, akin to the enhanced effects on Fos expression that Robinson and colleagues have associated with self-administration in a novel environment (Day et al., 2001).

Similar amounts of cocaine and saline were delivered per session from the first through the sixth day, such that the 6d and 1d groups were exposed to the same number of infusions. While less effort to achieve this result was exerted on successive days (Fig. 3B and C), it is unlikely that the greater activity of SAC 1d rats explains the differences in Fos expression because so many structures in the less active SAC 6d rats exhibited significantly greater Fos expression (Table 2). In an earlier relevant study (Neisewander et al., 2000), Fos expression associated with locomotion occurred only in the accumbens core, where no differences distinguished the SAC 1d and 6d groups in the present study.

Observed differences in the corresponding 1d and 6d SAS and SAC groups could stem from systematic differences attached to the presence of tracer injections in 6d rats or the independent self-administration sessions and immunohistochemical batches to which the 1d and 6d groups were subjected, or a combination of these, rather than actual differences in the respective Fos responses. Arguing against this notion, some SAS 6d (n=2) and SAC 6d (n=2) rats batch-processed together with the corresponding 1d groups produced amounts of Fos-expression comparable to the previously run 6d brains. Second, had either the 1d or 6d batch been subjected to suboptimal self-administration or immunohistochemistry, sections from that batch should have had uniformly less Fos-labeling, but this was not observed.

IAC vs. SAC

Fos-ir neuron densities in the group that received investigator-administered cocaine (IAC 6d) exceeded those in the SAC 6d group in several structures, possibly because IAC 6d rats, while receiving the same amount of cocaine as SAC 6d rats, got fewer, larger injections. The argument against this idea is that structures showing enhanced Fos expression in IAC 6d rats as compared to SAC 6d rats were outnumbered by those with equivalent or lesser Fos densities. Another possibility is that volitional control of repeated drug administration, i.e., agency, may act to suppress cocaine-elicited Fos expression in some structures. Consistent with this notion, structures with less Fos expression in the SAC 6d rats, which learned a task, occupied mainly the cortex and basal ganglia, including the caudate-putamen and accumbens, of which all contribute to planning and initiating learned voluntary actions (Pasupathy and Miller, 2005) and become less active as task acquisition proceeds (Kleim et al., 1996; Nordquist et al., 2003).

Basal ganglia input, output and intrinsic structures

Robust increases in Fos expression in the striatum and structures to which it projects were among the most striking effects of acute cocaine self-administration observed in the present study. Whereas Fos expression was further potentiated after 6 consecutive days of cocaine self-administration in structures to which striatum projects, including the globus pallidus, ventral pallidum, substantia nigra reticulata and ventral tegmental area (Heimer et al., 1995; Lu et al., 1998; Zahm, 2006), it was desensitized at 6 days in the caudate-putamen and accumbens. These observations can be explained on the basis of connectivity, transmitter phenotypes and empirical observations. To wit, neurons that give rise to striatal outputs are GABAergic (Ribak et al., 1979; Heimer et al., 1995) and mainly inhibited *in vivo* by cocaine via dopamine D-1 and D-2 receptors in anesthetized preparations (Henry et al., 1989, Henry and White, 1991; White et al., 1993) and unanesthetized preparations during cocaine self-administration sessions (Carelli and Deadwyler, 1994; 1997; Peoples et al., 1998). Inhibition of striatal neurons by dopamine following acute self-administration of cocaine should disinhibit, i.e., “activate”, the output and intrinsic structures to which GABAergic striatal neurons project, consistent with expression of Fos in those structures. Repeated administrations of cocaine further enhance striatal dopamine release (Kalivas and Duffy, 1990), produce dopamine D-1 receptor supersensitivity (Henry et al., 1989; Henry and White, 1991; 1995) and transiently up-regulate accumbens dopamine D-2 receptor density (Kleven et al., 1990; Peris et al., 1990), which together further inhibit striatal neurons (Henry and White, 1995) and presumably potentiate Fos expression in striatal output and

intrinsic structures, as observed on day 6 in the present study. Because behavioral activation has long been recognized to be inversely correlated with the activity of striatal neurons, but directly correlated with that of striatal output structures, such as the ventral pallidum/substantia innominata (Mogenson and Yang, 1991; Mogenson et al., 1993), we can postulate that the pattern of cocaine-elicited basal ganglia Fos expression observed in the present study identifies pathways by which behaviorally activating effects of cocaine, which are exerted directly mainly on striatal neurons, are disseminated widely through the brain via basal ganglia output structures and, in turn, the reticular formation, to which basal ganglia output structures project (Zahm, 2006). Consistent with this idea, Fos expression was potentiated in the zona incerta and pedunculopontine and laterodorsal tegmental nuclei at 6 days in the present study.

While nearly all striatal medium spiny neurons are electrophysiologically inhibited following the administration of cocaine (White et al., 1993), expression of Fos in response to acute administration of cocaine occurs in only about half, i.e., those in which D-1 is the dominant dopamine receptor subtype (Young et al., 1991). Conditional knockout of Fos expression in these neurons disrupts the expression of several proteins normally induced by cocaine and prevents the development of cocaine-elicited behavioral sensitization (Zhang et al., 2006). In addition, a gradual accumulation of other Fos family transcription factors, including FosB and ΔFosB, a long-lived splice variant of FosB (Chen et al., 1995), occurs in striatal D-1 expressing neurons during repeated cocaine administration (Kelz et al., 1999). A build-up of ΔFosB in the accumbens, which is attenuated in the conditional knockout of Fos expression described above (Zhang et al., 2006), correlates with the development of augmented locomotor responses to cocaine, enhanced sensitivity to the rewarding properties of cocaine and enhanced self-administration of lower doses of cocaine (Nestler, 2008). ΔFosB also disrupts normal transcriptional regulation of proteins induced by cocaine (Kelz et al., 1999), including Fos (Renthal et al., 2008) and some reported to be perturbed by Fos (Zhang et al., 2006). Expression of the alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptor subunit 2 (GluR2) is regulated by Fos (Zhang et al., 2006) and increased by ΔFosB (Nestler, 2008) with a resulting decrease in AMPA channel conductance and accompanying sensitivity to glutamate. This, together with the development of decreased sodium and calcium voltage-activated currents (Zhang et al., 1998; 2002), contributes to the decreased activity and increased inhibition of medium spiny neurons by dopamine that accompany repeated cocaine. Further, it indicates that Fos and ΔFosB expression not only signal, but are necessary for the enhanced inhibition. Inhibition of Fos expression by ΔFosB is the likely basis for the desensitized striatal Fos response observed at 6 as compared to 1 day of cocaine self-administration in the present study (see also Moratalla et al., 1996).

Extended amygdala and ventral lateral septum

A majority of extended amygdala structures evaluated in this study exhibited Fos expression equivalent to or greater than the controls following acute self-administration of cocaine. We included the ventral division of the lateral septum with these structures because it lies adjacent to the extended amygdala (i.e., the bed nucleus of stria terminalis) and shares with it similar connections and, presumably, functions (Risold and Swanson, 1997). After 6 days

of cocaine self-administration, responses in these structures were mixed, with some showing greater, others equivalent, and others less Fos expression than their counterparts in SAS brains. Interestingly, most extended amygdala subdivisions of rats that self-administered *saline* exhibited robustly increased Fos expression on day 6 as compared to day 1, such that the tendency for SAC as compared to SAS Fos expression in extended amygdala to be greater on day 1 was reversed on day 6. For example, the ventral division of the lateral septum and medial division of the central amygdaloid nucleus went from exhibiting significantly greater Fos expression in the SAC as compared to SAS groups on day 1 to exhibiting significantly less on day 6. In view of this effect of repeated saline self-administration, increased Fos expression observed in SAC rats in most extended amygdala structures on day 6 as compared to day 1 might be due as much to the act of self-administering (as in saline self-administration) as to the effects of cocaine. Insofar as extended amygdala and lateral septum are implicated not only in neural responses to fear and anxiety (LeDoux et al., 1988; LeDoux, 1992; Duncan et al., 1996; Petrovic and Swanson, 1997; Davis and Shi, 1999; Sheehan et al., 2004; Phelps and LeDoux, 2005; Zahm and Trimble, 2008), but also arousal (Asmus and Newman 1994; Sheehan et al., 2004; Deurveilher et al., 2006; Valdés et al., 2006; Schlitz et al., 2007), increased Fos expression in the extended amygdala on day 6 may reflect arousal or anxiety that accompanies repeated self-administration.

Concluding comments

Whereas current thought emphasizes a putative shift of processing from ventral to dorsal striatum as drug-seeking become habitual (Everett and Robbins, 2005; Belin and Everitt, 2008), the present data reveal that both dorsal and ventral striatopallidum are robustly engaged by cocaine from the very earliest stages of a course of self-administration, which is consistent with previous studies (Graybiel et al., 1990; Young et al., 1991), and recalibrate Fos expression similarly, at least during the first 6 days. Fos expression accompanies cocaine-elicited inhibition of striatum, but, because Fos often signals “activation,” it is reasonable to speculate that the potentiation of Fos expression in basal ganglia output structures reflects activation and accompanying dissemination of behaviorally activating effects of cocaine widely through the brain. Fos expression in the extended amygdala, zona incerta and ventral division of the lateral septum, while either unaffected or mildly increased following acute self-administration of cocaine, was robustly increased following repeated self-administration of cocaine, although whether due to cocaine or arousing aspects of repeated responding is uncertain. Nonetheless, if in these instances Fos does reflect neuronal activation, it would appear that striatopallidum, extended amygdala and related structures contribute together to the crescendo of behavioral activation and accompanying reinforcement that occurs early during a course of cocaine self-administration, impelling the descent toward addiction.

Fos expression in *inhibited* medium spiny neurons seeds the expression of longer-lived Fos family transcription factors (Chen et al., 1995; Kelz et al., 1999; Zhang et al., 2006) that, in turn, modulate the transcription of numerous proteins, including Fos, in such a way as to disrupt neuronal homeostasis and facilitate addictive behaviors (Nestler et al., 2008). Several other structures evaluated in this study, including the lateral and medial habenula,

rostromedial tegmental nucleus, supraoptic and paraventricular nuclei, and anterior cingulate cortex, exhibited an absolute decline in cocaine-elicited Fos expression upon repeated exposures. In addition, as described above, a decline in repeated cocaine-elicited Fos expression relative to repeated saline-elicited Fos expression occurs in the central nucleus of the amygdala and ventral division of the lateral septum, suggesting that a reduction of Fos expression in response to repeated cocaine also involves the extended amygdala. Whether a ΔFosB-related neuroadaptation accounts for the attenuation of cocaine-elicited Fos expression over repeated administrations in all of these structures is unknown, but early results suggest that this is so in the prelimbic cortex (Winstanley et al., 2008) and rostromedial tegmental nucleus (Kaufling et al., 2009). To the extent that such neuroadaptations may ultimately to contribute to profound dysregulations of neuronal function, the present data indicate that cocaine-elicited pathology may be more widespread than previously thought.

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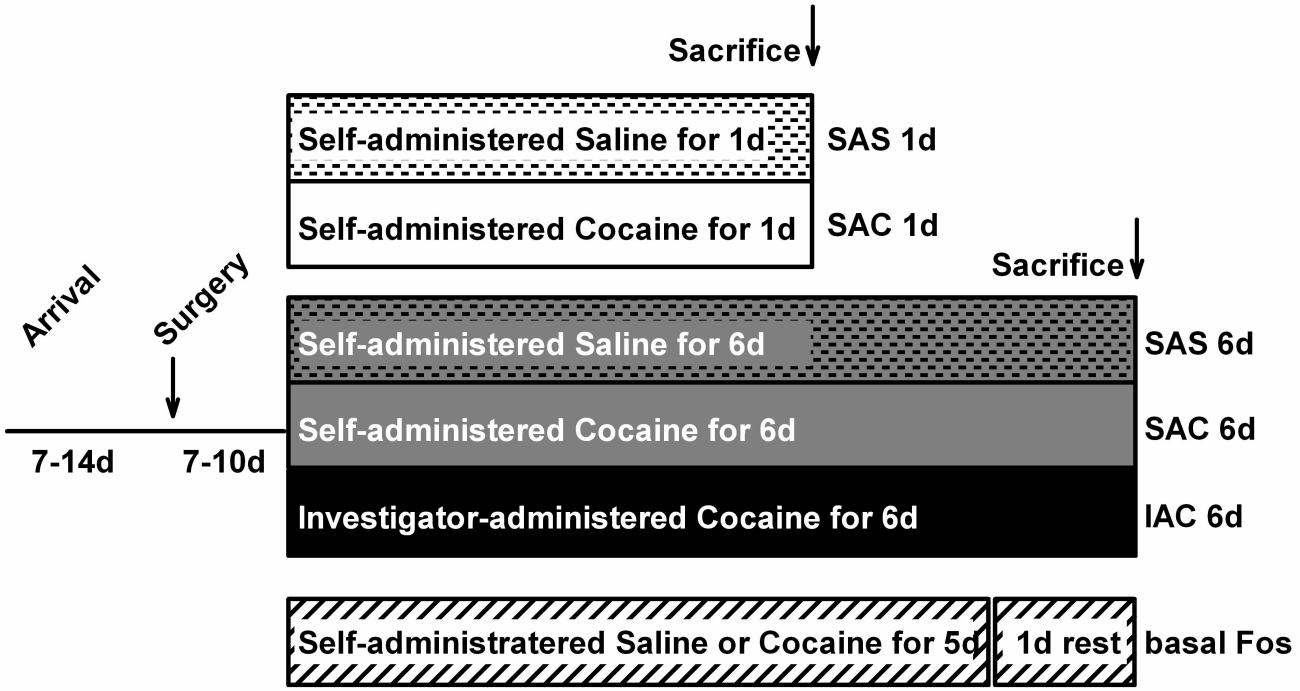
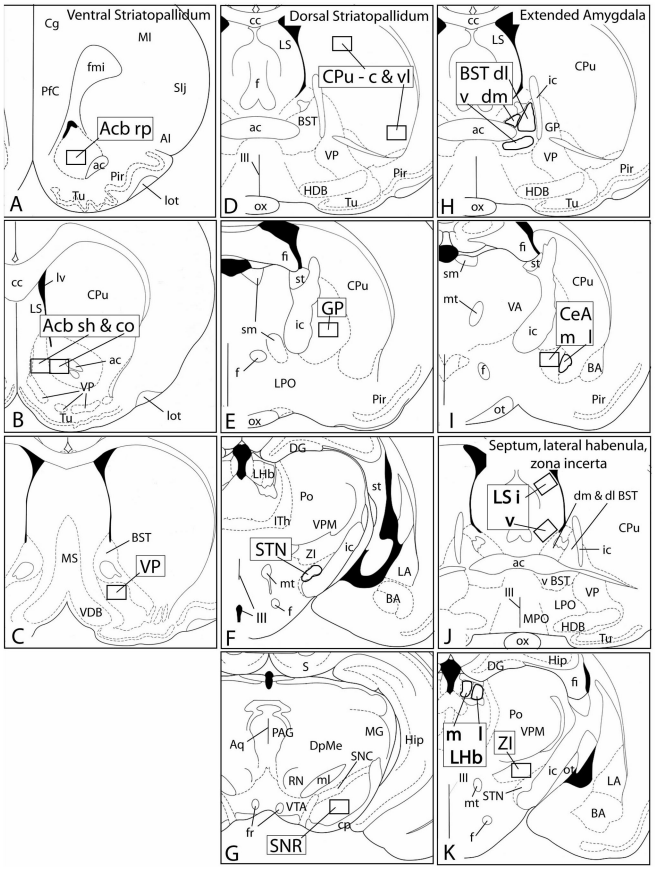


Figure 1.

Diagram illustrating the experimental protocol and resulting groups. Abbreviations: d — days; h — hours; IAC — investigator-administered cocaine; SAC — self-administered cocaine; SAS — self-administered saline.



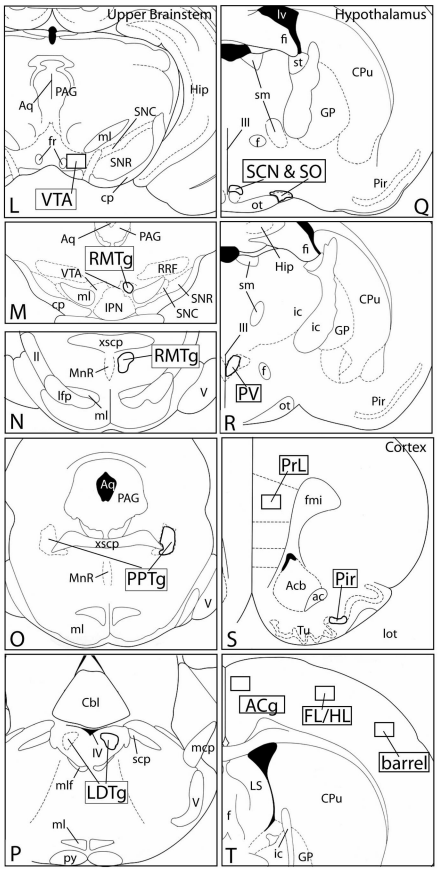


Figure 2.

Diagrams illustrating the 32 regions of interest (ROIs) subjected to computer-assisted counting in the present study. ROIs are shown as boldly outlined rectangles or irregular shaped areas, labeled with large, bold font, that conform to the size and shapes of the areas actually evaluated. Abbreviations: ac — anterior commissure; Acb - accumbens; AI — agranular insular cortex; aq — cerebral acqueduct; BST — bed nucleus of stria terminalis; cc — corpus callosum; c — central division; BA — basal amygdaloid nucleus; Cbl — cerebellum; CeA — central nucleus of the amygdala; Cg — anterior cingulate cortex; co — core; cp — cerebral peduncle; CPu — caudate-putamen; DG — dentate gyrus; dl — dorsolateral division; dm — dorsomedial division; DpMe — deep mesencephalic nucleus; f — fornix; fi —fimbria fornix; FL/HL — primary somatosensory cortex, forelimb/hindlimb area; fr — fasciculus retroflexus; GP — globus pallidus; Hip — hippocampus; HDB — horizontal limb of the diagonal band; I — intermediate division; ic — internal capsule; IPN — interpeduncular nucleus; ITh — intralaminar nuclei of the thalamus; l — lateral division; LA — lateral nucleus of the amygdala; lfp — longitudinal fasciculus of the pons; LHb — lateral habenula; LS — lateral septum; lot — lateral olfactory tract; LPO — lateral preoptic area; lv — lateral ventricle; m — medial division; mcp — medial cerebellar peduncle; MG — medial geniculate body; MI — first primary motor cortex; ml —medial lemniscus; mlf — medial longitundinal fasciculus; MnR — median raphe; MPO — medial preoptic area; MS — medial septum; mt — mammillothalamic tract; ot — optic tract; ox — optic chiasm;

PAG — periacqeductal gray; PfC — prefrontal cortex; Pir — piriform cortex; Po — posterior nucleus of the thalamus; PPTg — pedunculopontine tegmental nucleus; PrL — prelimbic cortex; py — medullary pyramid; RMTg — rostromedial tegmental nucleus; RN — red nucleus; rp — rostral pole; RRF — retrorubral field; sh — shell; SIj — primary somatosensory cortex, jaw area; sm — stria medullaris; SNC — substantia nigra compacta; SNR — substantia nigra reticulata; spc — superior cerebellar peduncle; st — stria terminalis; STN — subthalamic nucleus; Tu — olfactory tubercle; v — ventral division; V — trigeminal nerve; VA — ventral anterior nucleus of the thalamus; VDB — vertical limb of the diagonal band; vl — ventrolateral division; VP — ventral pallidum; VPM — ventral posteromedial thalamic nucleus; VTA — ventral tegmental area; xscp — crossing of the superior cerebellar peduncle; ZI — zona incerta; II — optic tract; III — third ventricle; IV — fourth ventricle.

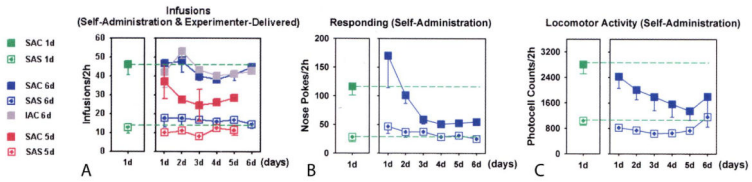


Figure 3.
Diagrams illustrating the behavior of the rats used in the study. Means and SEMs are shown.

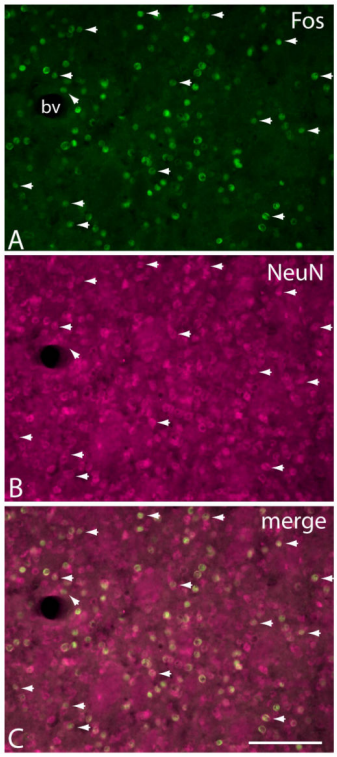


Figure 4.

Fluorescence micrographs of the central caudate-putamen (see Fig. 2D) of a SAC 1d rat illustrating localization of Fos-immunoreactive nuclei (A) within cells identified in panel B as neurons on the basis of immunreactivity against the neuronal nuclear antigen (NeuN). An obliquely oriented arrowhead near a blood vessel (bv) marks the only Fos immunoreactive structure in the field not co-localized with a NeuN immunoreactive structure. Horizontally oriented arrowheads mark examples illustrating that NeuN immunoreactive structures, because NeuN often stains both the cell body and nucleus, are usually larger than the co-localized Fos-labeling. The disparity in the size and shape of co-localized Fos- and NeuN-immunoreactive structures, also apparent in the merged image (C) rules out the possibility that the near complete assocation of Fos-ir nuclei with NeuN structures is due to bleed-through of the FITC signal. Scale bar: 100 μm.

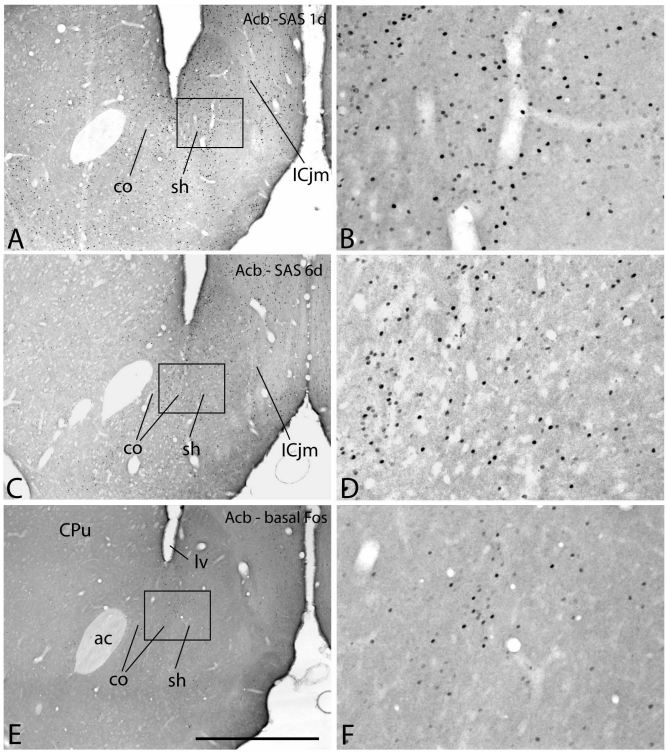


Figure 5.

Photomicrographs illustrating Fos immunoreactive neurons in the accumbens (Acb) in the 1 (1d) and 6 (6d) day saline-self administration (SAS) groups and in the basal Fos expression group, which comprised rats that self-administered saline for 5 days and were killed on the sixth day with no further opportunity to self administer. B, D and E are enlargements of the boxed areas in A, C and E, respectively. Additional abbreviations: co — Acb core; sh — Acb shell; ICjm — Island of Calleja magnus; lv — lateral ventricle. Scale bar: 1 mm in A, C and E; 200 um in B, D and F.

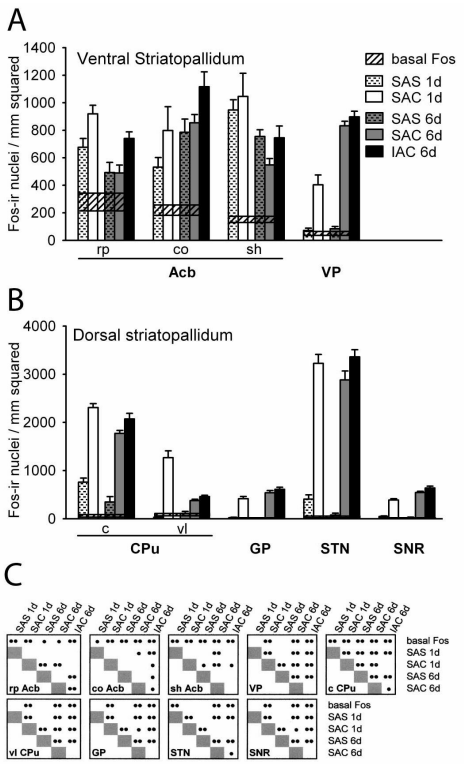


Figure 6.

Graphs illustrating the density of Fos-immunoreactive nuclei in the ventral striatopallidum (A), including the accumbens (Acb) and ventral pallidum (VP), and dorsal striatopallidum (B), including the caudate-putamen (CPu), globus pallidus (GP), subthalamic nucleus (STN), and substantia nigra reticulata (SNR), for the experimental groups as described in the text. Means and SEMs are shown. Widths of the horizontal bars reflect the magnitude of the SEM above and below the mean for “basal Fos” expression. Additional abbreviations: c — central division of CPu; co — Acb core; IAC — investigator-administered cocaine; rp — Acb rostral pole; SAC - self-administered cocaine; SAS — self-administered saline; sh — Acb shell; vl — ventrolateral division of CPu.

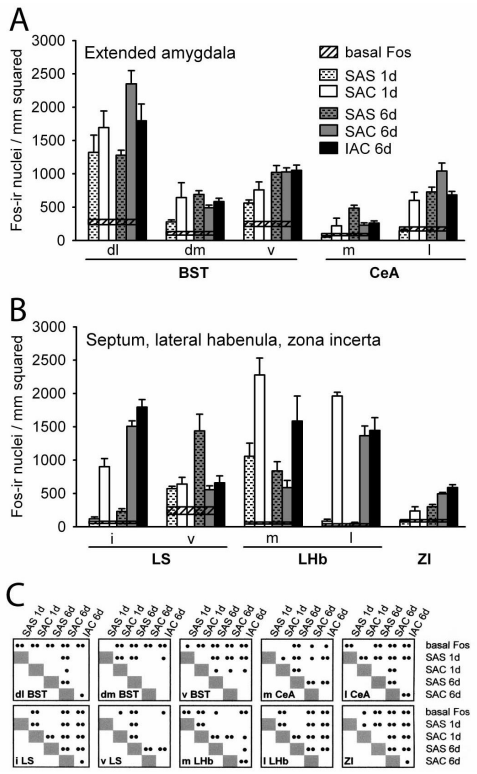


Figure 7.

Graphs illustrating the density of Fos-immunoreactive nuclei in the extended amygdala (A), including the bed nucleus of stria terminalis (BST) and central nucleus of the amygdala (CeA), and in (B) the lateral septum (LS), lateral habenula (LHb) and zona incerta (ZI), for the experimental groups as described in the text. Means and SEMs are shown. Widths of the horizontal bars reflect the magnitude of the SEM above and below the mean for “basal Fos” expression. Additional abbreviations: dl — dorsolateral division; dm — dorsomedial division; i— intermediate division; IAC — investigator-administered cocaine; l — lateral division; m — medial division; SAC - self-administered cocaine; SAS — self-administered saline; v — ventral division.

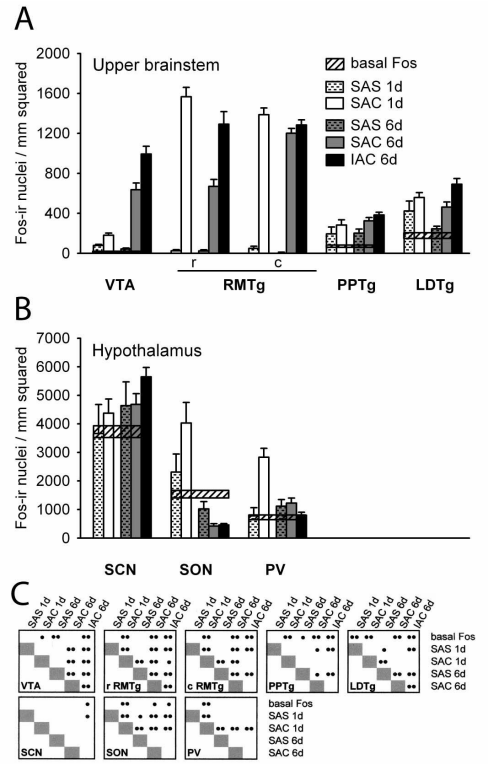


Figure 8.

Graphs illustrating the density of Fos-immunoreactive nuclei in some structures in the upper brainstem (A), including the ventral tegmental area (VTA), rostromedial tegmental nucleus (RMTg), and pedunculopontine (PPTg) and laterodorsal (LDTg) tegmental nuclei, and in the hypothalamus (B), including the suprchiasmatic nucleus (SCN), supraoptic nucleus (SON) and paraventricular nucleus (PV), for the experimental groups as described in the text. Means and SEMs are shown. Widths of the horizontal bars reflect the magnitude of the SEM above and below the mean for “basal Fos” expression. Additional abbreviations: c — caudal division; IAC — investigator-administered cocaine; r — rostral division; SAC - self-administered cocaine; SAS — self-administered saline.

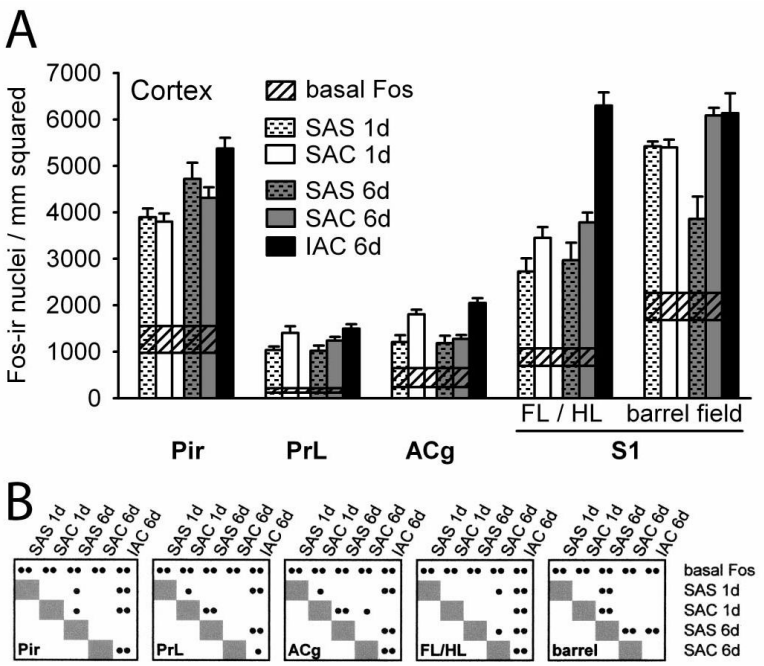


Figure 9.

Graphs illustrating the density of Fos-immunoreactive nuclei in some cortical structures, including the piriform (Pir), prelimbic (PrL), anterior cingulate (ACg), and primary somatosensory (S1), for the experimental groups as described in the text. Means and SEMs are shown. Widths of the horizontal bars reflect the magnitude of the SEM above and below the mean for "basal Fos" expression. Additional abbreviations: FL / HL — forelimb / hindlimb area; IAC — investigator-administered cocaine; SAC - self-administered cocaine; SAS — self-administered saline.

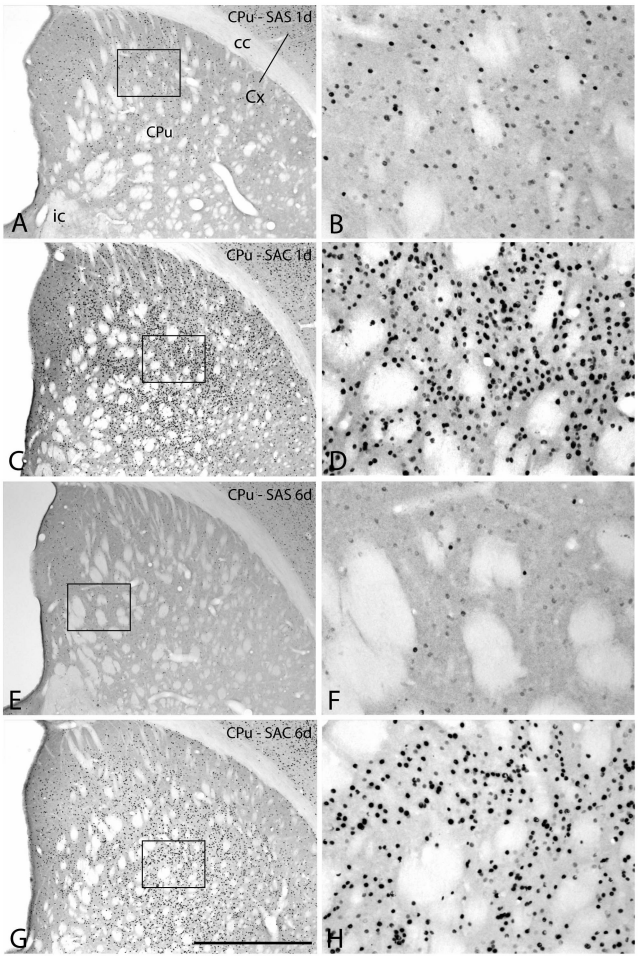


Figure 10.

Photomicrographs illustrating Fos immunoreactive neurons in the caudate-putamen (CPu) in the 1 (1d) and 6 (6d) day saline-self administration (SAS, panels A, B, E and F) and cocaine self-administration (SAC, panels C, D, G and H) groups. B, D, F and H are enlargements of the boxed areas in A, C, E and G, respectively. Additional abbreviations: cc — corpus callosum; Cx — cortex; ic — internal capsule. Scale bar: 1mm in A, C, E and G; 200 um in B, D, F and H.

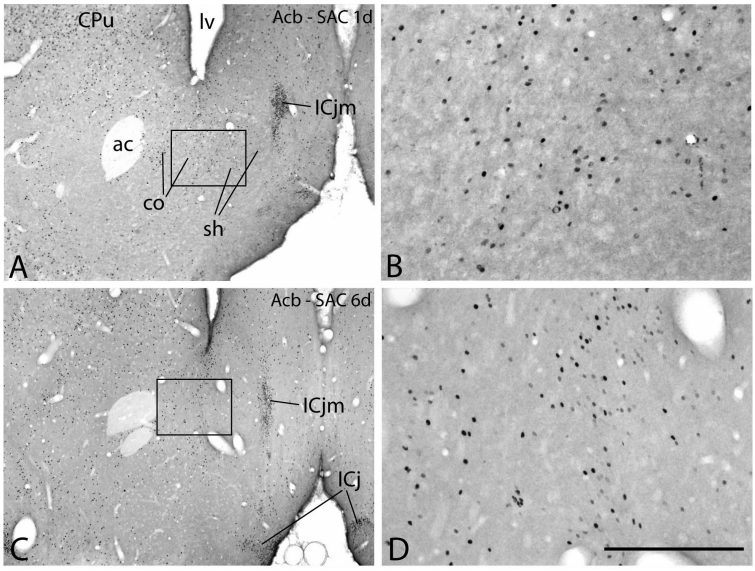


Figure 11.

Photomicrographs illustrating Fos immunoreactive neurons in the accumbens (Acb) in the 1 (1d) and 6 (6d) day cocaine-self administration (SAC) groups. B and D are enlargements of the boxed areas in A and C, respectively. Additional abbreviations: ac — anterior commissure; co — Acb core; sh — Acb shell; CPu — caudate-putamen; ICj — island of Calleja; ICjm - island of Calleja magnus; lv — lateral ventricle. Scale bar: 1mm in A, C and E; 200 um in B, D and F.

Table 1

Estimated densities of Fos-ir neurons in the saline control groups

	Fos-irdensity*				Fos-irden sity*		
	basal	**SAS 1d**	**SAS 6d**		**basal**	**SAS 1d**	**SAS 6d**
Cortex				ventromedial n	+	+	+
frontal association	+	++	+	posterior n	+	++	+
orbital	+++	+++	+++	supramammillary n	++	++	+
[piriform]	++	+++	+++	mammillary n	+	+	+
[prelimbic]	+	++	++				
primary motor	++	++	++	**Thalamus, epithalamus, subthalamus**			
granular insular	+	++	++	parataenial	+	+	+
[anterior cingulate]	+	++	+++	midline-intralaminar	++	+++	+++
[primary somatosensory]	+	+++	+++	reuniens	+	++	+
dysgranular insular	++	++	++	anterodorsal	+	++	++
dorsal hippocampus	tr	+	+	mediodorsal	tr	+	+
ventral hippocampus	nd	nd	+	reticular	0	0	0
subiculum	nd	nd	++	ventral anterior	+	+	+
granular retrosplenial	++	+++	+++	interanteromedial	++	++	++
agranular retrosplenial	++	+++	++	ventromedial	+	+	+
primary auditory	++	+++	+++	ventrolateral	+	+	+
primary visual	nd	+++	+++	ventral posterolateral	+	tr	+
perirhinal	+	++	++	ventral posteromedial	+	tr	+
amygdalopiriform	nd	+++	+	[l habenula, l]	0	tr	tr
entorhinal	nd	+++	+	[l habenula, m]	tr	++	++
				lateral geniculate	+	++	++
Basal forebrain				entopeduncular n	tr	tr	tr
anterior olfactoryn	++	+++	+++	[subthalamic n]	tr	+	tr
dorsal endopiriform n	+	++	+	[zona incerta]	++	+	+
olfactory tubercle	tr	+	+				
[accumbens rostal pole]	+	++	++	**Midbrain and pons**			
[accumbens core]	+	++	++	[ventral tegmental area]	tr	tr	tr

	Fos-irdensity*				Fos-irden sity*		
	basal	**SAS 1d**	**SAS 6d**		**basal**	**SAS 1d**	**SAS 6d**
[accumbens shell]	+	++	++	substantia nigra compacta	tr	tr	tr
Islands of Calleja	tr	+	+	[subst nigra reticulata]	tr	tr	tr
[lateral septum, i]	+	+	+	medial geniculate n	++	++	++
[lateralseptum , v]	+	++	++	[rostromed tegmental n]	0	0	0
m septum-diagonal band	+	+	++	periaqueductal gray	+	++	++
[caudate-putamen]	tr	++	+	sup colliculus, superficial	+	++	+
[ventral pallidum]	tr	+	+	sup colliculus,intermed	+	++	+
[dl bed n stria terminalis]	+	++	+	sup colliculus, deep	+	++	+
[dm bed n stria terminalis]	+	+	++	pontine n	+++	+++	+++
[v bed n stria terminal]	+	++	++	deep mesencephalic n	+	+	+
[globus pallidus]	0	tr	tr	red n	tr	+	+
[central n amygdala, l]	+	+	++	interpeducnular n	tr	+	+
[central n amygdala, m]	tr	+	++	retrorubral field	tr	+	+
basal n amygdala	+	++	++	[pedunculopont ine teg n]	tr	+	+
medial n amygdala	+	++	++	dorsal raphe n	++	+	+
				median raphe n	+	+	+
Preoptic area and lateral hypothalamus				cuneiformn	+	+	+
lateral preoptic area	+	++	++	inferior colliculus	+++	+++	+++
medial preoptic area	+	++	++	lateral lemniscus	+	+	+
[suprachiasmatic n]	+++	+++	+++	pont retic n, oral	+	+	+
[supraoptic n]	+	++	+	[laterodorsal tegmental n]	+	+	+
lateral hypothalamus	++	++	++	ventral tegmental n	++	++	++
medial hypothalamus	+	++	++	dorsal tegmental n	++	++	++
[paraventricular n]	+	+	+	parabrachial n	+	+	++
dorsomedial n	+	++	++	trigeminal n, motor	tr	tr	+
trigeminal n, sensory	tr	+	+	lateral reticularn	tr	+	+
superior olivary n	0	+	+	n prepositus	tr	+	+
locus ceruleus	0	+	+	n tracts solitarius	tr	+	+
				n ambiguus	tr	+	+
Medulla				dorsal motor n vagus	tr	tr	+

	Fos-irdensity*				Fos-irden sity*		
	basal	**SAS 1d**	**SAS 6d**		**basal**	**SAS 1d**	**SAS 6d**
caud pontine reticular n	tr	+	+	hypoglossal n	tr	tr	+
facial n	tr	tr	tr	medullary dorsal horn	tr	+	++
abducens n	0	tr	tr				
medial vestibular n	tr	+	+	**Cerebellum**			
cochlear n	+	++	+	granule cells	+++	+++	+++
trigeminal n, sensory	tr	tr	+	deep nn	tr	tr	tr
gigantocellular retic n	tr	+	+				

*Table entries reflect the averages of three cases, which were computed by assigning observations of 0, 0-10, 11-75, 76-200 and more than 200 Fos-ir nuclei/20x field numerical values of 0, 0.5, 1, 2, and 3. Averages of 0 and in the ranges of 0.01-0.66, 0.67-1.44, 1.45-2.22, and 2.23-3 are listed in the table as 0, tr (trace), +, ++ and +++, respectively. Bracketed entries were subjected to additional objective analysis illustrated in Figures 5-8 and Table 2. Additional abbreviations: dl — dorsolateral; dm - dorsomedial; i — intermediate; ir — immunoreactive; l — lateral; m- medial; n — nucleus; nd — not determined; nn — nuclei; retic — reticularis; rostromed — rostromedial; subst — substantia; sup — superior; teg — tegmental; v - ventral

Table 2

Comparisons of means graphed in Figures 5-8 reflecting Fos densities (Fos-ir nuclei/mm^2) for (A) cocaine (SAC) vs saline (SAS) self-administration on days 1 and 6 and (B) after 6 vs 1 day of SAC or SAS. Symbols indicate that SAC (in A) and day 6 (in B) Fos expressions were greater (↑ [$p < 0.5$], ↑↑ [$p < 0.01$]), lesser (↓ [$p < 0.5$], ↓↓ [$p < 0.01$]) or not significantly different (ns) from SAS and day 1, respectively. Numbers in paretheses indicate the magnitudes of significant responses, reflecting (in A) mean SAC values after the respective SAS means had been subtracted out and (in B) mean day 6 values after the respective day 1 means had been subtracted out. Note in numerous structures, that repeated saline self-administration produces Fos responses that are significantly changed over 6 days (column B, SAS). Particularly in extended amygdala structures, septum and zona incerta, the Fos response to repeated SAS is nearly uniformly increased, such that the apparent enhancement of Fos expression in these structures after 6 as compared to 1day of SAC is accounted for in part by an increase in Fos expression due to repetition of self-administration. For similar reasons, decreased Fos expression observed in the central part of the caudate-putamen after 6 as compared to 1 day of SAC, may be accounted for in part by the decrease in Fos expression associated with repetition of self-administration.

	A. SAC vs SAS on day:		**B. Day 6 vs Day 1 for:**	
	1 (SAC-SAS)	**6 (SAC-SAS)**	**SAC (day 6— day 1)**	**SAS (day 6-day1)**
Basal ganglia - input structures (Figs. 6A and B)				
accumbens				
rostral pole	↑ (+243)	ns	↓↓ (−429)	ns
core	ns	ns	ns	ns
shell	ns	ns	↓↓ (−497)	ns
caudate putamen				
central	↑↑ (+1548)	↑↑ (+1376)	↓↓ (−534)	↓↓ (−362)
ventrolateral	↑↑ (+1236)	↑↑ (+266)	↓↓ (−889)	ns
Basal ganglia - output and intrinsic structures (Figs.6A and B)				
ventral pallidum	↑↑ (+332)	↑↑ (+748)	↑↑ (+429)	ns
globus pallidus	↑↑ (+393)	↑↑ (+539)	↑ (+124)	ns
subthalamic nucleus	↑↑ (+2816)	↑↑ (+2808)	ns	ns
substantia nigra reticulata	↑↑ (+341)	↑↑ (+523)	↑ (+156)	ns
ventral tegmental area	ns	↑↑ (+592)	↑↑ (+456)	ns
Extended amygdala, septum and zona incerta (Figs. 7A and B)				
bed nucleus of stria terminalis				
dorsolateral division	ns	↑ (+1074)	↑↑ (+657)	ns
dorsomedial division	↑↑ (+366)	ns	ns	↑↑ (+411)
ventral division	ns	ns	↑ (+271)	↑↑ (+459)
central nucleus of the amygdala				
medial division	↑ (+171)	↓↓ (−252)	ns	↑↑ (+434)
lateral division	↑↑ (+455)	↑↑ (+312)	↑↑ (+440)	↑↑ (+583)
lateral septum				
intermediate division	↑↑ (+781)	↑↑ (+1275)	↑↑ (+605)	ns
ventral division	ns	↓↓ (−883)	ns	↑↑ (+866)

	A. SAC vs SAS on day:		B. Day 6 vs Day 1 for:	
	1 (SAC-SAS)	6 (SAC-SAS)	SAC (day 6— day 1)	SAS (day 6-day1)
zona incerta	↑ (+146)	↑↑ (+193)	↑↑ (+256)	↑↑ (+208)
Habenula (Fig. 7B)				
lateral habenula				
lateral division	↑↑ (+1222)	ns	↓↓ (−1693)	ns
medial division	↑↑ (1878)	↑↑ (1319)	↓↓ (−594)	ns
Brainstem (Fig. 8A)				
rostromedial tegmental nuc				
rostral part	↑↑ (+1542)	↑↑ (646)	↓↓ (−897)	ns
caudal part	↑↑ (+1337)	↑↑ (1195)	↓↓ (−184)	ns
peduncu lopontine tegmental nuc	ns	↑ (123)	ns	ns
laterodorsal tegmental nuc	ns	↑↑ (216)	ns	↓ (−178)
Hypothalamus (Fig. 8B)				
suprachiasmatic nucleus	ns	ns	ns	ns
supraoptic nucleus	↑↑ (+1719)	↓ (−598)	↓↓ (−3608)	↓ (−1291)
paraventricular nucleus	↑↑ (+2057)	ns	↓↓ (−1611)	ns
Cortex (Fig. 9)				
piriform	ns	ns	ns	↑ (826)
prelimbic	↑ (+366)	ns	ns	ns
anterior cingulate	↑ (+597)	ns	↓ (−530)	ns
somatosensory				
forelimb/hindlimb	ns	ns	ns	ns
barrel	ns	↑↑ (+2226)	ns	↓↓ (−1565)