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Expression of Fos-related antigens in the nucleus accumbens and associated regions following exposure to a cocaine-paired environment

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Abstract

This study examined whether conditioned hyperactivity measured in a cocaine-paired environment was associated with increased expression of Fos-related antigens (FRA) within the nucleus accumbens (NAc) and associated forebrain regions of rats. Three groups of rats were given repeated injections of either cocaine in the test environment and saline in the colony room (group Paired), saline in the test environment and cocaine in the colony room (group Unpaired), or saline in both environments (group Control). All rats were subsequently given a drug-free test for conditioned hyperactivity in the test environment, and their brains were removed so that FRA immunohistochemistry could be conducted. Rats in the Paired group showed conditioned hyperactivity during the conditioning test, and this behavioural response was associated with increased FRA expression within the caudal NAc, the medial prefrontal cortex and the lateral septum relative to the Unpaired and Control groups. Paired rats also showed increased FRA expression within the orbital prefrontal cortex, the claustrum, the caudal amygdala (basolateral and central regions), the paraventricular thalamic nucleus, the subiculum of the hippocampus, and the lateral habenula relative to the Control group. However, the FRA levels in these latter sites were not significantly increased relative to those of Unpaired rats, indicating that genomic responses in these regions were not entirely context dependent. The correspondence between conditioned hyperactivity and enhanced FRA expression within the caudal NAc, the medial prefrontal cortex and lateral septum suggests that these regions may participate in the expression of conditioned responses to cocaine-related stimuli.

Introduction

Environmental stimuli previously associated with cocaine administration can elicit a variety of conditioned physiological and subjective responses that may contribute to drug-seeking and relapse in human cocaine addicts (Childress *et al*., 1988; Ehrman *et al*., 1992; Newlin, 1992). Conceivably, pharmacological treatments that attenuate these conditioned responses may be useful as pharmacotherapies for cocaine abuse. However, the development of such treatments requires a greater understanding of the neurobiological mechanisms underlying the conditioning of cocaine's effects to environmental stimuli.

Efforts to identify central nervous system (CNS) sites that mediate conditioned behavioural responses to cocaine-related stimuli have focused primarily on the nucleus accumbens (NAc) and regions anatomically connected to the NAc. The NAc mediates a substantial component of the unconditioned behavioural activation produced by psychostimulant drugs (Pijnenburg *et al*., 1975; Kelly & Iverson, 1976; Carr & White, 1987; Clarke *et al*., 1988; Delfs *et al*., 1990), and several studies have suggested that this region may likewise participate in the expression of conditioned responses to psychostimulant-related cues (Gold *et al*., 1988; Hiroi & White, 1991; Layer *et al*., 1993; Gratton & Wise, 1994; Bespalov & Zvartau, 1996; Kiyatkin & Stein, 1996; Di Ciano *et al*., 1998a, b; although see Brown & Fibiger, 1992; Neisewander *et al*., 1996). Previous studies also have implicated forebrain regions that project to the NAc in the expression of conditioned responses to cocaine-related cues (Brown & Fibiger, 1993; Hitchcott & Phillips, 1997, 1998; Franklin & Druhan, 1999). In one such study, Fos immunohistochemistry was used to determine whether conditioned locomotor responses to an environment previously associated with cocaine were accompanied by increased Fos-related antigen (FRA) expression within specific forebrain regions (Brown & Fibiger, 1993). Changes in neuronal activity or intracellular signalling often stimulate the synthesis of intracellular constituents, e.g. FRAs, which can be detected immunohistochemically to identify CNS regions influenced by specific pharmacological or behavioural manipulations (Sagar *et al*., 1988; Morgan & Curran, 1991; Cullinan *et al*., 1996; Hatakeyama *et al*., 1996). Using this technique, Brown *et al*. (1992) observed conditioning-related increases in FRA expression within several forebrain regions that project to the NAc. Surprisingly, they found no evidence of increased FRA expression within the NAc itself, suggesting that conditioned hyperactivity was not related to increased neuronal activity within the NAc. However, FRA expression was quantified in only a limited area of accumbens tissue in that study. There is substantial anatomical and functional heterogeneity within the NAc (Zahm & Brog, 1992; Pennartz *et al*., 1994; Heimer *et al*.,

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1997), and conditioning-related increases in FRA expression could have occurred in subregions of the NAc that were not examined. To test this possibility, we quantified FRA expression within different subdivisions of the rat NAc (i.e. the shell, core and rostral pole) following tests for conditioned hyperactivity in a cocaine-associated environment. Detailed regional analyses of FRA expression in forebrain areas projecting to the NAc were also performed in this study to provide a more thorough topographical analysis of conditioning-related changes in neuronal function than has previously been attempted.

Materials and methods

Subjects

The subjects were 27 male Wistar rats (Charles River, Wilmington, MA, USA), weighing 250–300 g at the start of the experiment. The animals were housed in clear plastic cages (two rats per cage) in an AAALAC-approved animal-care facility maintained at 21 °C with a 12 h light/dark cycle (lights on at 07.00 h). Food and water were available *ad libitum* throughout the experiment. All rats were handled and weighed daily the week before the experiment. The experiment was conducted at the same time each day, during the light phase of the light/dark cycle. All experimental procedures were approved by an Institutional Animal Care and Use Committee in accordance with NIH guidelines.

Apparatus

Preliminary screening sessions were conducted in eight activity chambers ($30 \times 30 \times 30$ cm) constructed of black PVC walls, semi-transparent Plexiglas ceilings, and stainless-steel rod or aluminium mesh floors. Each chamber had seven photobeam emitters mounted 5 cm above the floor and 8–10 cm apart along two adjacent walls (three on one wall and four on the other). The associated photobeam detectors were mounted on the remaining walls, directly opposite the emitters. All photobeam detectors were connected via Med Associates controllers (ENV-256C) and interface ports (DIG-712) to an IBM 486 computer with Med-PC software (Med Associates, Georgia, VT, USA) which recorded interruptions of the photocell beams. The chambers were located within a small dimly lit testing room that was not used for any other purpose.

The pairing and conditioning test sessions were conducted in eight chambers ($40 \times 40 \times 40$ cm; San Diego Instruments, San Diego, CA, USA) that were distinct from the chambers used for preliminary screening. Each chamber had black and white striped walls, a smooth Plexiglas floor, and eight pairs of photocell emitters and detectors stationed 3.5 cm above the floor and 3.5 cm apart around the perimeter. The photocell emitters and detectors were connected via an electrical interface to an IBM 486 computer with PAS software (San Diego Instruments) that recorded interruptions of the photocell beams. The chambers were contained within a sound-attenuated, dimly lit room with white noise (70 dB) presented continuously to mask extraneous noise. Lemon-scented tissue paper was placed in the testing room to provide a distinctive smell to the environment.

Behavioural testing procedures

The behavioural component of the study consisted of three phases: an initial screening session; a pairing phase; and a final test for conditioned hyperactivity. The initial screening session was conducted 3 days prior to the start of the pairing phase in chambers that were dissimilar to the activity boxes used in the subsequent pairing and test phases of the experiment (see above). Each rat was placed into a separate activity chamber for 30 min and baseline activity levels were recorded in the absence of any drug treatment. The activity scores from this test were then used to assign rats to treatment groups (seven to nine rats/group) that were equated with respect to baseline activity levels (range of means and SEMs for the 30 min screening session was 1404 ± 121 to 1464 ± 173).

The pairing phase was conducted over a 12-day period, with test environment exposures occurring on odd-numbered days and colony room treatments given on even-numbered days. Rats in one group (the 'Paired' group) were given an injection of cocaine (10 mg/kg, i.p.) prior to each test environment exposure, and an injection of saline on colony room treatment days. Rats in a second group (the 'Unpaired' group) received saline prior to test environment exposures and cocaine (10 mg/kg, i.p.) on colony room treatment days. A third group of rats (the 'Control' group) received saline injections on both test environment and colony room treatment days. Activity was monitored for 60 min following each injection in the test environment, and animals were returned immediately to their home cages following colony room treatments.

On the conditioning test day, all rats received saline injections prior to placement into the test environment and locomotor responses were measured in the absence of any drug treatments for 60 min.

Cocaine (NIDA Drug Supply, Research Triangle Park, NC, USA) was dissolved in 0.9% saline. All drug and vehicle solutions were injected in a volume of 1 mL/kg.

Immunohistochemistry

The rats were anaesthetized with sodium pentobarbital (50 mg/kg, i.p.) immediately after being removed from the test cage and perfused transcardially with 100 mL of saline followed by 800 mL of fixative (4% paraformaldehyde in 0.1 M phosphate buffer). The brains were removed from the cranial cavity, placed in fixative for 90 min at 4 °C, then transferred to a 20% sucrose solution and stored at 4 °C. These brains were later sliced into 40-μm coronal slices and collected in wells containing 0.1 M phosphate-buffered saline (PBS). Every fourth section was subsequently immersed for 10 min in 0.1 M PBS with 2% H_2O_2, then rinsed twice in PBS (10 min/rinse) before being stored overnight in PBS with 0.1% sodium azide (PBS–Az). The sections were then placed overnight in a solution containing 2% normal donkey serum (NDS) in PBS–Az with 0.3% Triton-X (PBS–Az–Tx), and this was followed by 48 h incubation with primary antibody (rabbit anti-Fos antiserum at 1 : 50 000 dilution, Oncogene Sciences, Cambridge, MA, USA) in PBS–Az–Tx with 2% NDS. After exposure to the primary antibody, the tissue was incubated for 90 min in biotinylated donkey anti-rabbit (1 : 500, Jackson Immunoresearch Laboratories, West Grove, PA, USA) in PBS–Tx at room temperature, and then transferred to avidin–biotin complex (1 : 500, Jackson Immunoresearch Laboratories) in PBS–Tx for 30 min at room temperature. FRA-positive cells were visualized by placing the tissue in 3,3′-diaminobenzidine (DAB, 0.02%, Sigma, St. Louis, MO, USA) with 0.0002% peroxide and 0.6% nickel ammonium sulphate in 0.01 M Tris buffer for 3.5 min. This reaction was arrested by immediate transfer into 0.05 M Tris buffer (pH 7.6).

The tissue was rinsed three times (10 min/rinse) in PBS–Tx between each incubation phase, and was given an additional rinse in 0.05 M Tris buffer (pH 7.6) prior to the DAB reaction. The sections were given two final rinses in PBS–Az following arrest of the DAB reaction and then mounted on gelatin-coated slides or stored at 4 °C for later mounting.

Quantification and statistical analyses

Activity scores measured during the pairing phase were subjected to a one-way within, one-way between-groups analysis of variance

(ANOVA) with pairing session as the within-groups factor and conditioning group as the between-groups factor. The conditioning test activity scores were analysed using one-way between-groups ANOVAs. Significant main effects were subjected to further *post hoc* analysis using Fisher's LSD test with significance levels set at $P < 0.05$.

Forebrain areas chosen for FRA quantification included the major subdivisions of the NAc and numerous regions that project to the NAc. Thus, FRA expression was examined in the shell, core and rostral pole subdivisions of the NAc, in the infralimbic, prelimbic, cingulate and secondary motor areas of the medial frontal cortex, in the claustrum and orbital regions of the lateral frontal cortex, in the rostral and caudal levels of the basolateral amygdala, in the dorsal and ventral hippocampus, in the paraventricular nucleus of the thalamus, in the lateral septum and in the lateral habenula. FRA expression was also examined at rostral and caudal levels of the central nucleus of the amygdala (which does not project to the NAc), as this region is known to be important for the control of classically conditioned responses (Everrit *et al.*, 1999) and initial visual inspection of this region revealed substantial FRA expression in Paired rats.

FRA quantification was accomplished by first identifying a coronal section that could serve as a representative cross-section for each region of interest (Fig. 1). Coronal brain slices closely matching these representative sections were then selected for each rat, and camera lucida drawings of each brain slice were constructed using light microscopy at 10 × magnification. Boundaries between structures and related subregions were determined by comparing observable landmarks and boundaries within each brain slice with those

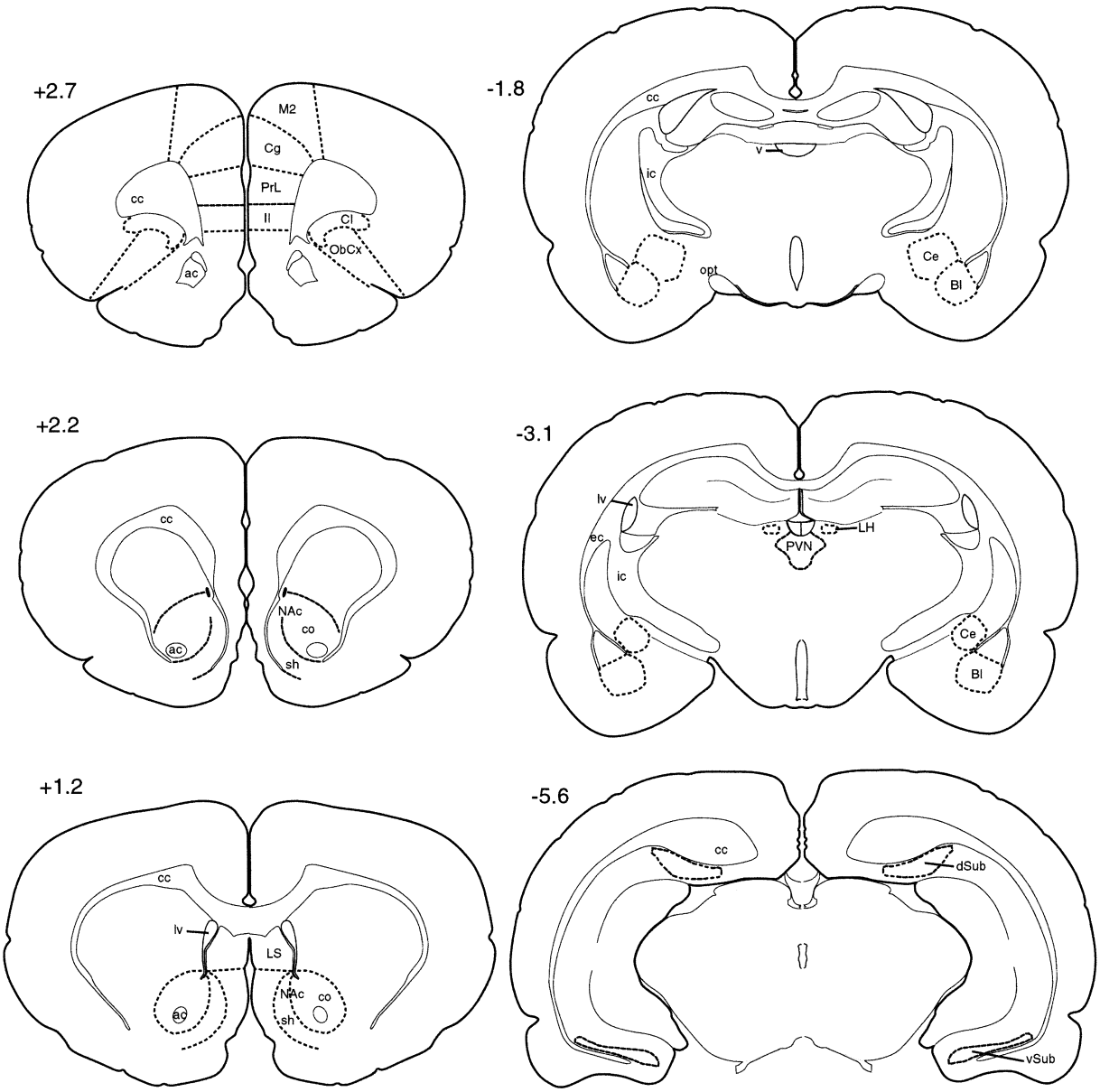

FIG. 1. Schematic representations of sections used for quantification of FRA-positive nuclei. The boundaries of each area quantified are indicated by dashed lines within the sections. Coordinates are listed to the left of each representative section and are in accordance with the atlas of Paxinos & Watson (1998). Abbreviations: ac, anterior commissure; Bl, basolateral amygdaloid nucleus; cc, corpus callosum; Ce, central amygdaloid nucleus; Cg, cingulate cortex; Cl, claustrum; co, core of the accumbens; dSub, dorsal subiculum of the hippocampus; ec, external capsule; ic, internal capsule; Il, infralimbic cortex; LH, lateral habenula; LS, lateral septum; lv, lateral ventricle; M2, secondary motor cortex; NAc, nucleus accumbens; ObCx, orbital cortex; opt, optic tract; PrL, prelimbic cortex; PVN, paraventricular nucleus of the thalamus; sh, shell of the accumbens; v, third ventricle; vSub, ventral subiculum of the hippocampus.

displayed in corresponding sections from the rat brain atlas of Paxinos & Watson (1998). FRA-positive cells were identified by the presence of dense immunohistochemical staining within cell nuclei (Fig. 2) and were transcribed onto the camera lucida drawings. All visibly labelled nuclei within a tissue section were counted from the camera lucida drawings, and the mean number of cells per subregion (averaged across both hemispheres) was calculated. The construction of the camera lucida drawings and quantification of FRA-positive nuclei were performed blindly to eliminate experimenter bias. The numbers of FRA-positive nuclei counted in each region or subregion of a representative section were transformed to their logarithmic values to equate the variances among means, and these values were then analysed using one-way between, one-way within-groups analyses of variance (ANOVAs). The conditioning group served as the between-groups factor, and the subregions of a structure within each tissue section served as the within-groups factor. Significant main effects were analysed further using Fisher's LSD test with significance levels set at $P < 0.05$.

Results

Behavioural conditioning

Analyses of activity scores measured during the pairing phase indicated that Paired rats that received cocaine prior to each pairing session were significantly more active than Unpaired and Control animals across all pairing sessions ($F_{2,24} = 45.50$; $P < 0.0001$; data not shown). Thus, the dose of cocaine employed for this study produced robust locomotor activating effects during pairing sessions. Significant group differences in activity were also observed during the test for conditioning (Fig. 3; $F_{2,24} = 13.49$; $P < 0.0001$). These group differences were particularly prominent during the first 20 min of the conditioning test (Fig. 3; $F_{2,24} = 16.12$; $P < 0.0001$), with *post hoc* tests revealing that Paired rats were significantly more active than Unpaired and Control animals during this period ($P < 0.0001$ and $P < 0.001$, respectively). Given the considerable lag-time required for synthesis and expression of FRAs to occur (see Dragunow & Robertson, 1987; Dragunow & Faull, 1989), it is likely that much of the FRA expression measured in this study was induced during this initial period when conditioned hyperactivity was evident in the Paired group. Significant group differences were also observed during the second 20 min of the conditioning test (Fig. 3; $F_{2,24} = 15.80$; $P < 0.0001$), although these differences were due to lower activity in the Unpaired group relative to Paired and Control rats ($P < 0.0001$ for both comparisons) rather than conditioned hyperactivity in the Paired group. Analysis of the activity scores from the third 20-min period did not reveal any significant group differences. This decline in conditioned hyperactivity during the latter phases of testing is consistent with findings obtained previously with this paradigm (e.g. Brown & Fibiger, 1992; Di Ciano *et al*., 1998a).

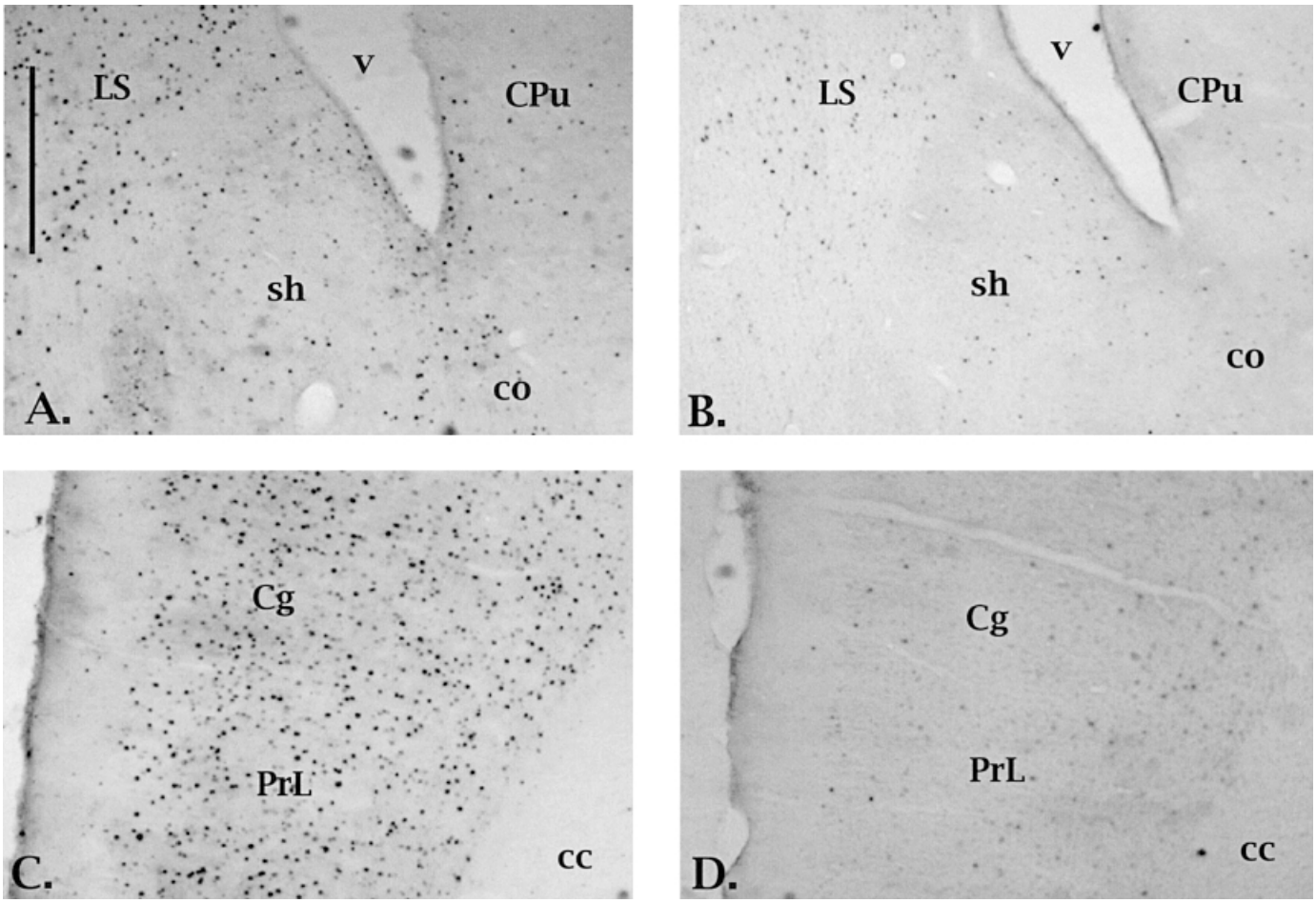

FIG. 2. Photomicrographs showing FRA-positive nuclei within representative brain regions. (A and B) FRA expression within the caudal NAc (+1.2 relative to Bregma) of representative Paired and Control rats, respectively. The photographs show the dorsomedial region of the NAc where FRA expression was the densest. FRA expression within the ventrolateral septum can also be seen in these photographs. (C and D) FRA expression in the cingulate region of the medial prefrontal cortex in the same representative Paired and Control rats. Calibration bar, 400 µm. Abbreviations: CPu, caudate putamen; LS, lateral septum; V, lateral ventricle.

Immunohistochemical analysis of FRA expression

The mean numbers of FRA-positive nuclei counted in separate brain regions of Paired, Unpaired and Control rats are shown in Figs 4–8. Figure 4A shows that the number of FRA-positive nuclei within the caudal NAc was increased in both shell and core subregions of Paired rats relative to that measured in Unpaired and Control animals. Statistical analyses of the log-transformed FRA data confirmed the significance of this result, with a between/within ANOVA indicating a significant overall effect of conditioning group ($F_{2,24} = 3.97$; $P < 0.03$), and *post hoc* analyses revealing significantly greater numbers of FRA-positive cells in Paired rats than in Unpaired or Control animals. Although there were visible trends toward increased FRA expression in the rostral NAc of Paired rats (Fig. 4B), statistical analyses indicated that these trends were not significant regardless of whether shell and core subregions were analysed together in a between/within ANOVA ($F_{2,22} = 1.57$; $P = 0.23$), or independently in separate between-groups ANOVAs ($F_{2,22} = 0.73$; $P = 0.50$ for the shell, and $F_{2,22} = 2.72$; $P = 0.09$, for the core).

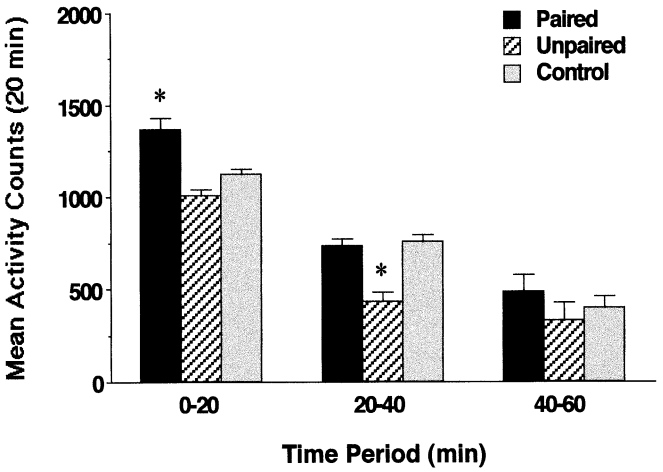

FIG. 3. Mean activity counts and SEMs recorded during successive 20-min periods of the test for conditioning. Rats in the Paired group were significantly more active than Unpaired and Control rats during the first 20 min of the conditioning test, confirming that behavioural conditioning had occurred. Rats in the Unpaired group were less active than Paired or Control rats during the second 20 min of the test session, whereas there were no group differences during the final 20 min. Significant differences ($P < 0.05$) relative to the Control group are indicated by asterisks.

Figure 5 shows FRA expression within subregions of the medial and lateral prefrontal cortex. As with the caudal NAc, the numbers of FRA-positive nuclei in each subregion of the medial prefrontal cortex were higher in Paired rats than in Unpaired or Control animals (Fig. 5A). A between/within ANOVA of the log-transformed FRA data in these medial regions confirmed a significant effect of conditioning group ($F_{2,23} = 6.17$; $P < 0.01$), and *post hoc* analyses indicated that this effect was due to overall increases in FRA expression within the medial regions of Paired rats relative to both Unpaired and Control rats. This ANOVA also revealed a significant effect of region ($F_{3,23} = 33.45$; $P < 0.0001$) due to the overall lower numbers of FRA-positive cells in the infralimbic cortex relative to the secondary motor, cingulate and prelimbic cortices, but there was no significant interaction between group and region factors. Analysis of the FRA expression in lateral regions of the prefrontal cortex also revealed significant effects of conditioning group ($F_{2,22} = 4.80$; $P < 0.05$) and region ($F_{1,22} = 59.76$; $P < 0.0001$), but no interaction between these variables (Fig. 5B). However, *post hoc* analyses of the group effect indicated that, while FRA counts in these lateral regions were significantly increased in Paired rats relative to Control animals, there were no significant differences between Paired and Unpaired groups.

FRA expression in the basolateral and central amygdala is shown in Fig. 6. In caudal sections of these nuclei, the numbers of FRA-positive cells were elevated in both Paired and Unpaired groups relative to Controls (Fig. 6A). This effect was confirmed by a significant group effect in a between/within ANOVA of the log-transformed FRA counts ($F_{2,22} = 5.29$; $P < 0.01$), and subsequent *post hoc* tests. In contrast, there were no group differences observed in these nuclei when FRA expression was quantified from sections of the rostral amygdala (Fig. 6B).

The results obtained from the ventral and dorsal subiculum of the hippocampus were similar to those obtained from the lateral prefrontal cortex (Fig. 7). Although a significant group effect was observed in a between/within ANOVA of the log-transformed values ($F_{2,16} = 3.72$; $P < 0.05$), *post hoc* tests revealed that FRA expression in Paired rats was increased relative to Control rats but not Unpaired animals. Significant group differences were also observed for FRA

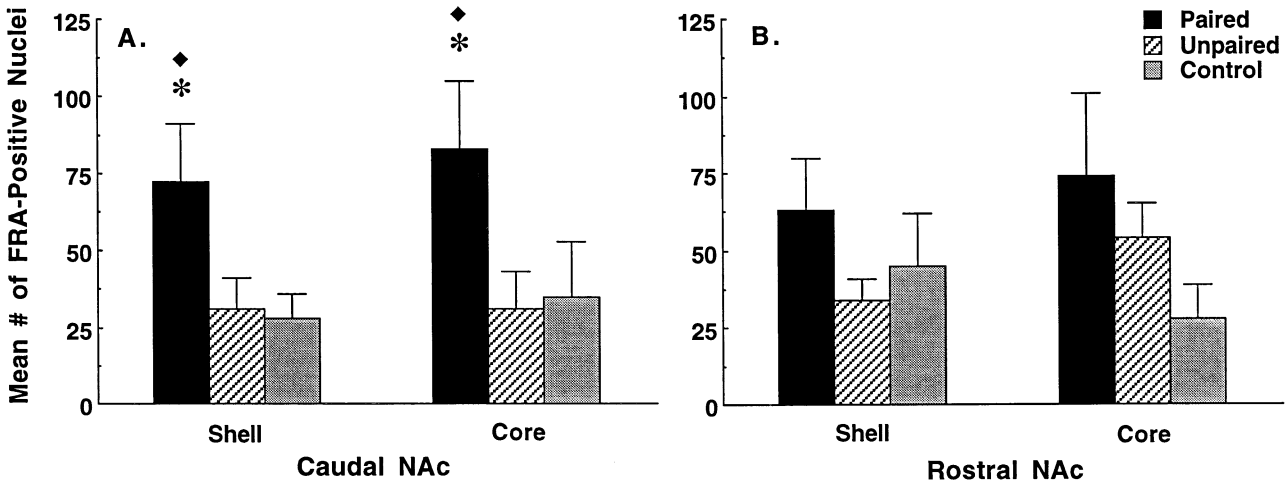

FIG. 4. Mean numbers (± SEMs) of FRA-positive nuclei in the shell and core of the caudal (A) and rostral (B) NAc measured after the test for conditioning. Analyses of the log-transformed data revealed significantly higher numbers of FRA-positive nuclei in the caudal shell and core subregions of Paired rats relative to those seen in Unpaired and Control animals ($P < 0.05$). In contrast, group differences in FRA expression were not observed for either subregion of the rostral NAc. Significant differences ($P < 0.05$) relative to the Unpaired group are indicated by diamonds, whereas differences relative to the Control group are indicated by asterisks.

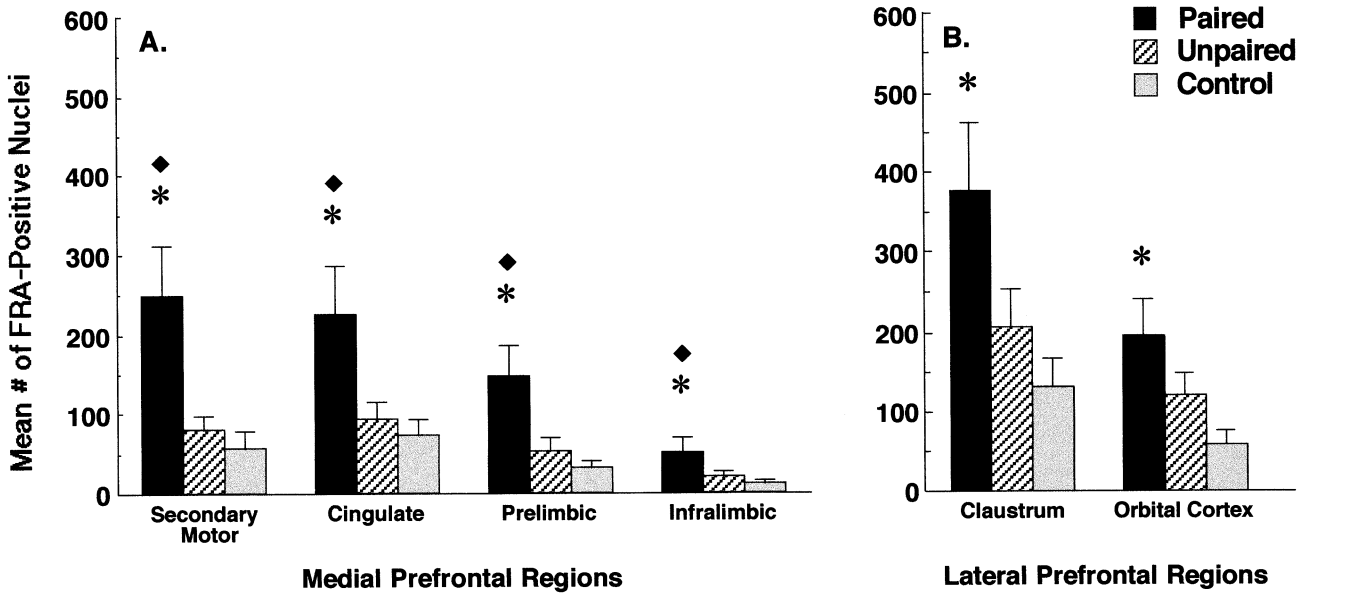

FIG. 5. Mean numbers of FRA-positive nuclei (± SEMs) in the medial (A) and lateral (B) prefrontal cortex. Analyses of the log-transformed data revealed that FRA expression in Paired groups was significantly higher than in Unpaired and Control groups throughout the medial prefrontal cortex. In lateral prefrontal regions, FRA expression in Paired rats was significantly elevated relative to Controls, but not Unpaired rats. Significant differences ($P<0.05$) relative to the Unpaired group are indicated by diamonds, whereas differences relative to the Control group are indicated by asterisks.

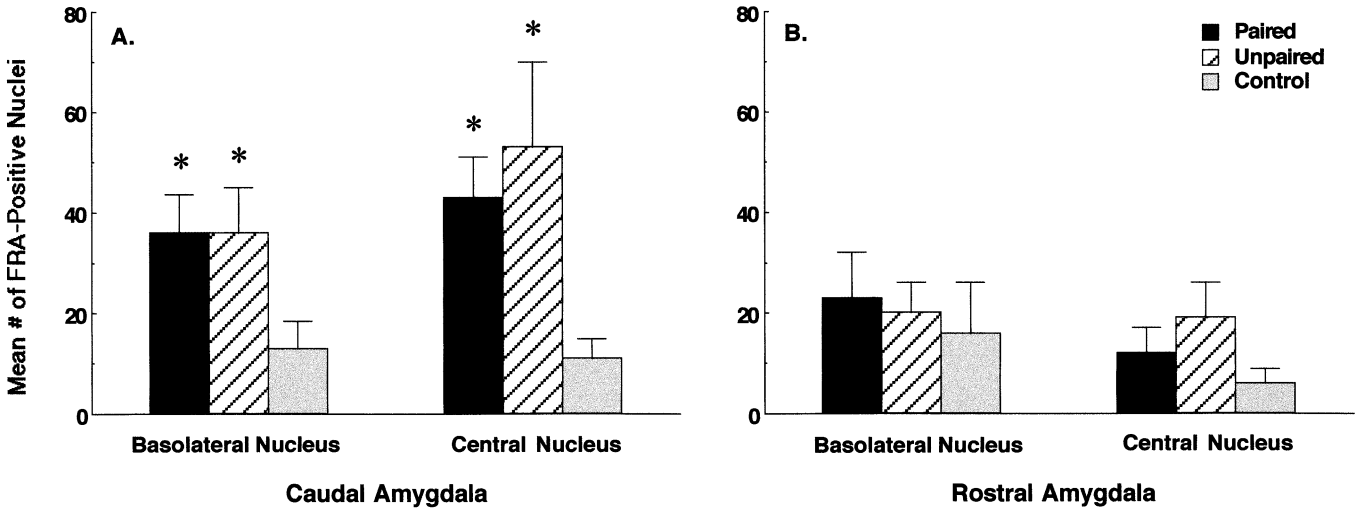

FIG. 6. Mean numbers of FRA-positive nuclei (± SEMs) in the caudal (A) and rostral (B) amygdala. Analyses of the log-transformed data revealed that FRA counts were elevated in the basolateral and central nuclei of the caudal amygdala in both Paired and Unpaired groups relative to the Control group (asterisks indicate $P<0.05$). In contrast, there were no group differences in FRA expression within either nucleus of the rostral amygdala.

expression in the paraventricular nucleus of the thalamus ($F_{2,23}=4.88$; $P<0.02$), which were due to increased numbers of FRA-positive cells in both Paired and Unpaired rats relative to controls (Fig. 8A). For the lateral habenula, significant group differences in FRA expression ($F_{2,23}=3.46$; $P<0.05$) were related to increased numbers of FRA-positive nuclei in Paired rats relative to Controls, but FRA expression in Paired animals was not greater than in Unpaired rats (Fig. 8B). The lateral septum was the only region examined other than the NAc and medial frontal cortex where FRA expression appeared to be increased selectively in Paired rats relative to both Unpaired and Control animals (Fig. 8C). Although the ANOVA for this region narrowly missed significance ($F_{2,24}=3.21$; $P=0.058$), the pattern of group differences showed clear trends towards increased FRA expression in Paired rats relative to Unpaired and Control animals.

Discussion

Consistent with previous studies (Barr *et al.*, 1983; Beninger & Herz, 1986; Brown *et al.*, 1992; Martin-Iverson & Reimer, 1994; Damianopoulos & Carey, 1995; Cervo & Samanin, 1996), Paired rats that received cocaine repeatedly in the test environment during the pairing phase were hyperactive in that environment during the test for conditioning relative to Unpaired and Control rats. Immunohistochemical analyses of the brains from these rats revealed increased numbers of FRA-positive nuclei within the caudal NAc (both core and shell subregions), the medial prefrontal cortex (including infralimbic, prelimbic, cingulate and secondary motor areas), and the lateral septum of Paired animals relative to Unpaired and Control rats. This correspondence between locomotor and genomic responses during the test for conditioning indicates that

conditioned behavioural responses to cocaine-related stimuli are associated with functional changes in the caudal NAc, medial prefrontal cortex and lateral septum.

The correlative nature of immunohistochemical data prevents us from inferring a causal relationship between increased FRA expression within in the NAc, medial prefrontal cortex, and lateral septum and conditioned locomotor activity. The increased FRA response in these regions could have been a secondary consequence, rather than a cause, of the heightened behavioural activity in Paired rats. Nevertheless, the present findings concur with a growing body of evidence implicating roles for the NAc and medial prefrontal cortex in the control of conditioned motivational responses. Thus, the finding of increased FRA expression within the NAc of Paired rats is consistent with evidence that the accumbens plays an important role in conditioned responding to stimuli associated with both natural and psychostimulant reinforcers (Kelly & Iverson, 1976; Gold *et al.*, 1988; Everitt *et al.*, 1991; Hemby *et al.*, 1992; Bespalov & Zvartau, 1996; Di Ciano *et al.*, 1998a, b). Likewise, the conditioning-related increases in FRA expression within medial regions of the prefrontal cortex are consistent with findings from behavioural, anatomical, electrophysiological, neurochemical and neuroimaging studies implicating the medial prefrontal cortex in Pavlovian conditioning processes (Peterson, 1986; Isaac *et al.*, 1989; Brown *et al.*, 1992; Carey & Damianopoulos, 1994; Maxwell *et al.*, 1994; Powell *et al.*, 1994, 1996; Yoshioka *et al.*, 1995, 1996; Wedzony *et al.*, 1996; Bussey *et al.*, 1997; Maas *et al.*, 1998; Tzschentke & Schmidt, 1998; Childress *et al.*, 1999; McLaughlin & Powell, 1999). Interestingly, previous disconnection experiments have found that approach responses elicited by an appetitive conditioned stimulus can be blocked by combined unilateral lesions of the cingulate cortex and the NAc in opposite hemispheres (Everitt *et al.*, 1999). Disconnection experiments are based on the reasoning that lesions of a common serial circuit at different sites within opposite hemispheres should disconnect the circuitry bilaterally and disrupt behaviours mediated by that circuit. The fact that combined unilateral lesions of the NAc and cingulate cortex were able to disrupt conditioned approach responses suggests that these regions are part of a common serial circuit involved in the control of Pavlovian conditioned behaviours. The present evidence for increased FRA expression within these regions of rats exposed to a cocaine-related environment indicates that this circuit could also be important for regulating responses to cocaine-associated stimuli.

It is noteworthy that increased FRA expression within the NAc was regionally specific, with significant increases observed in the caudal shell and core subregions but not in the rostral accumbens. This regional specificity could explain why conditioned increases in FRA expression were not observed within the NAc of rats exposed to a cocaine-paired environment in a previous study by Brown *et al.* (1992). In their study, Brown *et al.* (1992) examined FRA expression in only a small section of tissue from the rostral portion of the NAc. The present findings confirm that conditioned FRA expression is not detected at rostral levels of the NAc, but they repudiate the conclusions reached by Brown *et al.* (1992) by providing evidence that conditioning-related increases in FRA expression can occur

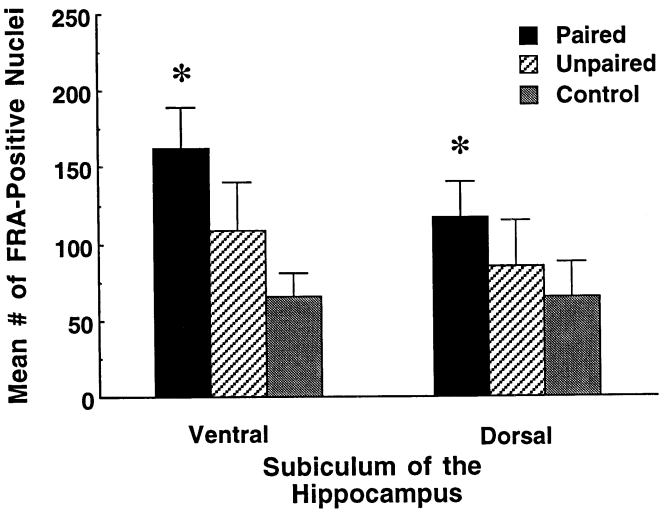

FIG. 7. Mean numbers of FRA-positive nuclei (± SEMs) in the dorsal and ventral subiculum. Analyses of the log-transformed data revealed that FRA expression in the Paired group was elevated relative to the Control group (asterisks indicate $P < 0.05$), but not the Unpaired group, in both the dorsal and ventral regions of the subiculum.

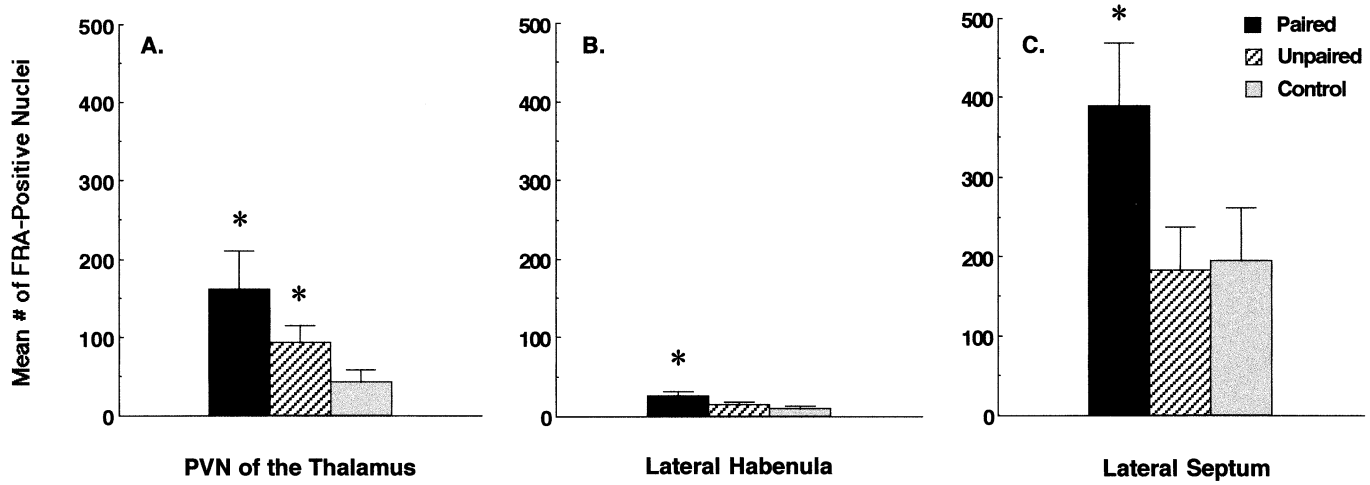

FIG. 8. Mean numbers of FRA-positive nuclei (± SEMs) in the paraventricular thalamus, lateral habenula and lateral septum. Analyses of the log-transformed data revealed that FRA expression in the paraventricular thalamus (A) was elevated in the Paired and Unpaired groups relative to the Control group ($P < 0.05$). FRA expression in the lateral habenula (B) was elevated in Paired animals relative to the Control group ($P < 0.05$), but not the Unpaired group. FRA expression in the lateral septum (C) was increased in Paired rats relative to Unpaired and Control groups, although only the increase relative to Control animals reached statistical significance ($P < 0.05$). The asterisks in each panel indicate significant differences relative to the Control group.

within caudal sections of the NAc. This latter evidence for functional differentiation of rostral and caudal subregions of the NAc is consistent with behavioural results showing that locomotor activity and self-stimulation of the ventral tegmental area are enhanced by infusions of d-amphetamine or dopamine receptor agonists into the caudal, but not rostral NAc (Essman *et al*., 1993; Ranaldi & Beninger, 1994). On the other hand, the lack of differential FRA expression between core and shell subregions is surprising in the light of substantial evidence for anatomical and functional differences between these sites (Zahm & Brog, 1992; Maldonado-Irizarry & Kelley, 1994; Maldonado-Irizarry *et al*., 1995; Everitt *et al*., 1999). It is possible that these subregions are similarly activated during conditioned responding, but that they influence different aspects of behaviour.

Although cells within the lateral septum are known to project to the NAc (Brog *et al*., 1993), the role of this region in regulating conditioned or unconditioned responses to drug-related stimuli has rarely been examined. Electrophysiological studies have shown learning-related neuronal activity within the lateral septum during aversive and appetitive conditioning (Berger & Thompson, 1978; Yadin & Thomas, 1981; Yadin *et al*., 1992), and immunohistochemical mapping studies have demonstrated increased FRA expression in this region after presentations of fear- or cocaine-related conditioned stimuli (Brown *et al*., 1992; Yadin *et al*., 1992; Campeau *et al*., 1997). Moreover, infusions of the muscarinic receptor antagonist scopolamine into the lateral septum blocked conditioned heart rate changes produced during aversive conditioning procedures (Powell *et al*., 1985). One line of evidence suggests that the lateral septum may be important for inhibiting conditioned anxiety responses, and for negative reinforcement produced by the alleviation of anxiety states (Yadin & Thomas, 1981; Yadin *et al*., 1992). If this hypothesis is correct, then the increased FRA expression observed within the lateral septum of Paired rats in this study could reflect a conditioned compensatory response that might normally serve to offset the anxiogenic properties of cocaine (Ettenberg & Geist, 1991, 1993).

Several of the regions examined showed increases in FRA expression that could not be attributed to excitatory conditioning. In particular, FRA expression in the orbital frontal cortex, the claustrum, the caudal basolateral and central nuclei of the amygdala, the lateral habenula, the paraventricular thalamic nucleus, and the dorsal and ventral subiculum of the hippocampus of Paired rats was significantly higher than that measured in Control animals, but it was not higher than that observed in Unpaired rats. For most of these regions, FRA levels in Unpaired rats were midway between those observed in Paired and Control groups. In the paraventricular thalamus, the caudal basolateral amygdala and the caudal central amygdala, FRA expression was significantly elevated in both Paired and Unpaired animals relative to Controls. Such results are most readily attributable to non-specific increases in neuronal activity within these regions as a consequence of repeated cocaine treatment. On the other hand, non-specific adaptations to cocaine are not the only explanation for this pattern of results. It is conceivable that cells in these regions may have responded both to the excitatory conditioned stimulus properties of the cocaine-associated environment in Paired rats, and to inhibitory conditioned stimulus attributes of this environment (resulting from its association with the omission of cocaine) in Unpaired rats. This explanation would be consistent with evidence implicating the basolateral and central amygdala, the paraventricular thalamus, and subiculum of the hippocampus in both excitatory and inhibitory conditioning (Winocur *et al*., 1987; Everitt *et al*., 1991; Seldon *et al*., 1991; Ono *et al*., 1995; Robledo *et al*., 1996; LaBar & LeDoux, 1996; Hitchcott & Phillips, 1997; Muller *et al*., 1997; Young & Deutch, 1998; Maren, 1999). It is also noteworthy that Unpaired rats were hypoactive relative to Paired and Control animals during the second 20-min period of the conditioning test, suggesting that a moderate degree of conditioned inhibition may have been present in the Unpaired group. However, the present findings do not provide definitive evidence linking the increased FRA expression in Unpaired rats to conditioned inhibition, and the changes in FRA expression observed within the aforementioned regions cannot be attributed to either excitatory or inhibitory conditioning at the present time.

It is of interest that the pattern of changes in FRA expression observed in the present study concurs with the results of behavioural studies indicating that prefrontal–accumbens pathways may be more important for Pavlovian conditioned responding than the basolateral amygdala and ventral subiculum. Studies of Pavlovian conditioned behaviours measured using an autoshaping task revealed that the development of simple approach responses to a food-related stimulus could be blocked by 6-hydroxydopamine (6-OHDA) lesions of the NAc and by excitotoxic lesions of the anterior cingulate cortex, but not by excitotoxic lesions of the basolateral amygdala or ventral subiculum (Bussey *et al*., 1997; Everitt *et al*., 1999). Likewise, we have recently found that the expression of conditioned hyperactivity in a cocaine-paired environment can be blocked when the NAc or sites within the prelimbic/cingulate cortex are inhibited by local infusions of a γ-aminobutyric acid (GABA) receptor agonist, but not when the basolateral amygdala or ventral subiculum are inhibited in this manner (Franklin & Druhan, 1999). Lesions of the basolateral amygdala also failed to block expression of conditioned hyperactivity in two previous studies (Brown & Fibiger, 1993; Ahmed *et al*., 1995). Although one must always interpret negative FRA data with caution, behavioural findings such as these raise the possibility that the lack of context-specific FRA expression in the basolateral amygdala, ventral subiculum and other areas reflects a more limited involvement of each site in the regulation of Pavlovian conditioned hyperactivity responses to cocaine-associated stimuli. Coordinated neuronal activity within these regions may only be necessary during the performance of more complex behaviours involving discriminated operant responses for conditioned reinforcement (Everitt *et al*., 1999).

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Abbreviations

6-OHDA, 6-hydroxydopamine; CNS, central nervous system; DAB, 3,3′-diaminobenzidine; FRA, Fos-related antigen; GABA, γ-aminobutyric acid; NAc, nucleus accumbens; NDS, normal donkey serum; PBS, phosphate-buffered saline.

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