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Cocaine Differentially Regulates Activator Protein-1 mRNA Levels and DNA-Binding Complexes in the Rat Striatum and Cerebellum

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SUMMARY

Cocaine is a psychomotor stimulant that exerts many of its behavioral and physiological effects through alteration of catecholamine reuptake systems. One early cellular response to cocaine administration is a brain region-specific alteration in the transcriptional pattern of immediate early genes belonging to the Fos/Jun family of nucleotide sequence-specific [activator protein-1 (AP-1)] DNA-binding proteins. The work described here compares cocaine-induced transcriptional regulation of immediate early gene mRNA levels, as well as AP-1 DNA-binding activity, within the striatum and cerebellum. In the striatum, acute cocaine administration increases cellular levels of c-*fos* and *jun*-B mRNA, whereas transcriptional effects in the cerebellum are limited to c-*fos* mRNA. After chronic cocaine treatment a desensitization of c-*fos* mRNA induction is observed in the striatum, with sensitization of the same transcriptional effect occurring in the cerebellum. Pharmacological studies further reveal that the dopamine D1, dopamine D2, γ-aminobutyric acid type B, and *N*-methyl-D-aspartate receptor systems mediate the effects of cocaine on cerebellar neurons, whereas striatal effects are modulated through D1 and *N*-methyl-D-aspartate receptors. Gel retention analysis using antibodies to the various Fos and Jun proteins was used to characterize cocaine-dependent alterations in the composition of striatal and cerebellar AP-1 DNA-binding complexes. In striatum, cocaine increases the relative levels of c-Fos, Fos-B, Jun-B, and Jun-D proteins that bind the AP-1 DNA sequence element, whereas in the cerebellum only c-Fos and Jun-D binding activities are increased. These data suggest two possible neuroanatomical sites where tolerance and sensitization to cocaine can be examined at the genomic level.

Transforming short-lived chemical signals into long term cellular changes is paramount in fundamental biological processes such as cellular differentiation and neuronal plasticity. The need for novel protein synthesis associated with the establishment and maintenance of some forms of long term cellular changes has suggested that alterations in specific patterns of gene transcription are necessary. Research over the last several decades has begun to demonstrate how short-lived chemical signals elicit altered patterns of gene expression. Thus, it is becoming apparent that some long-lasting forms of neuronal plasticity involve regulation of specific patterns of gene expression (1, 2).

The IEG protein products are critical components of the neuronal signal-transcription coupling machinery. Many of these proteins bind to DNA in a nucleotide sequence-specific fashion, subsequently modulating expression of specific target genes. The Fos and Jun family members represent the best studied examples of such IEG proteins (3). These proteins are able to dimerize via the formation of leucine zippers, bind to a specific nucleotide sequence element (TGAGTCA, referred to as the AP-1 binding site) near the promoter region of target genes, and subsequently regulate transcription of those genes. After numerous types of neuronal stimulation, transcriptional activation of specific Fos and Jun family member genes is rapid (within minutes), transient (most *fos* and *jun* mRNA levels return to basal values within a few hours after induction), and independent of *de novo* protein synthesis (4).

Tolerance, sensitization, and dependence resulting from repeated use of addictive drugs involve plastic and long-lasting changes within specific neural networks. Indeed, recent studies have implicated specific IEGs as factors that may regulate some of the genomic events associated with this type of neuronal plasticity (5). Numerous studies in this area have focused on

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ABBREVIATIONS: IEG, immediate early gene; AP-1, activator protein-1; GABA, γ-aminobutyric acid; NMDA, *N*-methyl-D-aspartate; DTT, dithiothreitol; bp, base pair(s); EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; CRE, cAMP response element; CTF/NF-1, cellular transcription factor/nuclear factor-1; SP-1, SV40 promoter factor 1; PLSD, protected least significant difference.

cocaine administration, because the neuronal pathways and neurotransmitter receptor/transporter systems through which cocaine acts are well characterized (6, 7). For example, it is now firmly established that acute administration of cocaine induces expression of c-*fos* and *jun*-B mRNA in the rat striatum (8–12). This induction is blocked by the dopamine D1 receptor antagonist SCH-23390, reflecting a receptor-mediated effect of cocaine, but could well be mediated by both D1 and D2 receptors (13). It is noteworthy that such changes occur in the striatum, because this area is a crucial component of the neuronal circuitry underlying reward and motor behavior (6, 7). IEG transcriptional changes after acute cocaine administration have also been reported in the nucleus accumbens for mRNAs encoding c-*fos*, *jun*-B, and *zif*-268 (14, 15), as well as *fos*-B and c-*jun* (10). This effect is not observed after chronic cocaine administration, however, suggesting desensitization of the ability of cocaine to regulate these specific IEGs. Once again, it is noteworthy that the nucleus accumbens exhibits cocaine-induced transcriptional changes, in that this brain region plays a key role in the reinforcing properties of drugs of abuse such as cocaine, morphine, nicotine, and alcohol (6, 16). Demonstrating induction of IEG transcripts in the striatum and nucleus accumbens by acute cocaine treatment is a first step towards elucidating the role of gene expression in the development of tolerance and dependence.

In addition to cocaine-dependent alterations in IEG mRNA levels, changes in the levels of specific IEG proteins are also observed. In the striatum, acute cocaine administration significantly increases the level of at least four immunoreactive proteins belonging to the Fos family, for up to 24 hr (12, 17). Within the nucleus accumbens, AP-1 binding activity (reflecting the presence of Fos and/or Jun family members) is elevated for at least 3 days after the last dose of cocaine administered twice daily for 2 weeks (10). Thus, both short and long term alterations in the level and composition of AP-1 binding complexes may have profound effects on target genes whose protein products play a role in processes involved in cocaine addiction.

Whereas the effects of cocaine within the striatum are well documented, the stimulatory properties of cocaine in other brain areas have received considerably less attention. The cerebellum, whose principle physiological function is the modulation of motor activity, represents one such target site for cocaine. The cerebellum receives a dense noradrenergic projection from the locus coeruleus, with minor dopaminergic input from the ventral tegmental area, and is rich in cocaine binding sites (18). Cocaine can modulate cerebellar neurotransmission by enhancing the inhibitory effect of GABA on Purkinje cell spontaneous activity (19). At the genomic level, a recent study using *in situ* histochemistry has described an increase in c-*fos* mRNA in the granule cell layer of the cerebellum 15 min after cocaine-induced seizures (20). This induction is apparently not dependent on the onset of seizure activity, because cocaine administered at a subseizure dose still induced c-*fos* expression.

The studies described here seek to better characterize the effect of cocaine administration on the cerebellar AP-1 system and to compare any observed changes with those appearing in the striatum. Alterations in *fos* and *jun* mRNA levels in the aforementioned brain regions were assessed by Northern blot analysis, whereas gel shift analysis coupled with the use of antibodies to specific Fos and Jun proteins served to characterize the nature of AP-1 DNA interactions. The observed effects of cocaine on the cerebellar AP-1 system may thus be relevant to the short and long term effect of cocaine on cerebellar activity.

Materials and Methods

Animals, Tissue Preparation, and Pharmacological Protocols

Adult (90-day-old) male Sprague-Dawley rats were housed under a 12-hr light/12-hr dark cycle and given food and water *ad libitum*. For either acute or chronic studies, a single dose of cocaine (20 mg/kg) dissolved in saline was given intraperitoneally. Chronic treatment studies involved daily administration of cocaine for 12 days. For *in vivo* pharmacology studies, all drugs were dissolved in saline and administered intraperitoneally 30 min before injection of cocaine; the animals were then sacrificed 1 hr after the cocaine injection. Pharmacological compounds were obtained from Research Biochemicals (Cambridge, MA), with the exception of cocaine (Sigma Chemical Co., St. Louis, MO). After the specific drug administration paradigm, animals were anesthetized with halothane to unconsciousness (<1 min) and decapitated. The whole brain was removed and rinsed in ice-cold phosphate-buffered saline for 1 min before dissection. Cerebellum and striatum were dissected and immediately stored on dry ice. All tissue samples were stored at −70°.

RNA Extraction and Northern Blot Analysis

Total RNA was extracted from cerebellum and striatum, and the RNA concentration was determined spectrophotometrically. For Northern blot analysis, total RNA (7–10 μg) was separated by electrophoresis on 6% formaldehyde/1.2% agarose gels. The RNA was transferred to Magna NT nylon membranes (Micron Separations, Westboro, MA) by capillary action, in 5× (0.75 M sodium chloride, 0.075 M sodium citrate) standard saline citrate, followed by UV cross-linking (Stratalinker; Stratagene, La Jolla, CA). The membranes were then rinsed in water and allowed to dry until use. Membranes were prehybridized for 3–24 hr at 60° in hybridization buffer [5% sodium dodecyl sulfate, 400 mM sodium phosphate, pH 7.0, 1 mM EDTA, 1 mg/ml bovine serum albumin (fraction V), 50% formamide]. The prehybridization buffer was discarded and replaced with fresh hybridization buffer including a specific cRNA hybridization probe. Hybridization proceeded for 16–24 hr at 60°. Membranes were subsequently washed with 1% sodium dodecyl sulfate, 0.05× standard saline citrate, 1 mM EDTA, at 70–75° for 1–4 hr. After washing, membranes were exposed multiple times to Kodak XAR-5 film, to obtain a range of hybridization signal intensities for semiquantitative densitometric analysis. For each RNA sample, autoradiographic signals within the linear range of film sensitivity were digitized using a tabletop laser scanner (model MSF300ZS; X-Ray Scanner Corp.) and Adobe Photoshop XSF software. Relative intensities were quantified using Image software. IEG mRNA signal intensities were standardized to cyclophilin mRNA, 28 S rRNA, or 18 S rRNA signal intensities for each sample.

Gel Shift Assays

Whole-cell protein extracts. Two groups of five adult male Sprague-Dawley rats were each administered saline or 20 mg/kg cocaine-HCl intraperitoneally and sacrificed 2 hr later. Cerebellum and striatum were dissected and homogenized (with a Dounce homogenizer) in buffer A (0.25 M sucrose, 15 mM NaCl, 5 mM EDTA, 1 mM EGTA, 150 μM spermine, 500 μM spermidine, 1 mM DTT, 0.1 mM phenylmethylsulfonyl fluoride, 2 μg/ml leupeptin, 5 μg/ml aprotinin, 1 μg/ml pepstatin, 0.1 mM *p*-aminobenzamide) on ice. Extracts were centrifuged at 2000 × *g* for 10 min. The resulting pellet was resuspended in buffer B (0.5 M HEPES, pH 7.9, 0.75 mM $MgCl_2$, 0.5 mM EDTA, 0.5 M KCl, with protease inhibitors) by gentle mixing for 30 min, followed by centrifugation at 14,000 × *g* for 30 min. The resulting supernatant was dialyzed for 2 hr against 1 liter of 10 mM Tris·HCl, pH 7.9, 1 mM EDTA, 5 mM $MgCl_2$, 10 mM KCl, 10% glycerol, with protease inhibitors,

with a change of dialysis buffer after 1 hr. All of the aforementioned manipulations were performed at 4°. Samples were divided into aliquots, flash frozen in a dry ice/95% ethanol bath, and stored at −70°. Protein concentrations were determined by the Bio-Rad protein assay.

Gel retention analysis. Various amounts of whole-cell protein extracts were mixed with binding buffer (20 mM HEPES, pH 7.9, 50 mM KCl, 0.5 mM EDTA, 1% glycerol, 0.5 mM DTT, added fresh each time), 1 μg of poly(dI·dC), and 1×10^4 cpm of a double-stranded, ^{32}P-labeled, AP-1 oligonucleotide (CGCTTGATGAGTCAGCCGGAA; Promega, Madison, WI), in a final volume of 20 μl. For supershift assays, the mixture also contained rabbit polyclonal IgG (Santa Cruz Biotechnology, Santa Cruz, CA) recognizing specific Fos (c-Fos or Fos-B) or Jun (c-Jun, Jun-B, or Jun-D) proteins. The reactions were incubated at 37° for 15 min, mixed with gel loading buffer, and subjected to electrophoresis on a 4% acrylamide gel. Gels were then dried and exposed to Kodak XAR-5 film for multiple autoradiographic exposures. Semiquantitative densitometric analysis was performed as described.

Antiserum specificity. Antiserum specificity was analyzed by various means. The antisera employed in these studies were raised against synthetic 13–17-residue peptides that represent unique sequence elements of the known Fos and Jun proteins. Thus, antisera directed against any specified Fos or Jun protein epitope would not be expected to cross-react with other members of the family. One characterization study used epitope-specific and nonspecific peptides in AP-1 DNA-binding reactions (data not shown). For every antiserum used, only the epitope-specific peptide was able to successfully compete with the formation of a supershifted complex, whereas nonrelated epitope peptides had no effect. Other immunochemical forms of analysis further support the notion of specificity.[1] Nuclear and cytoplasmic extracts have been prepared from a wide variety of mammalian cell lines (e.g., KNRK, Hela, A431, and NIH/3T3 cells) and subjected to both immunoprecipitation and Western blot analysis. Results from both types of analysis further confirm that antisera directed against a given Fos or Jun protein are unable to recognize other members of the family. However, this analysis does not rule out the possibility that other cellular proteins unrelated to Fos or Jun proteins are capable of cross-reactivity.

AP-1 oligonucleotide radiolabeling. Double-stranded consensus AP-1 oligonucleotide was end-labeled in 50 mM Tris·HCl, pH 7.6, 10 mM $MgCl_2$, 5 mM DTT, 0.1 mM spermidine, with T4 polynucleotide kinase (BRL) and [γ-^{32}P]ATP (6000 Ci/mmol).

Plasmid Constructs and Riboprobe Synthesis

Analysis of relative *fos* and *jun* mRNA levels was carried out with cRNA probes after subcloning of specific DNA fragments into the plasmid pGEM 3Z (Promega). Full-length rat cDNA clones for c-*fos* and c-*jun* were a generous gift from Dr. Tom Curran (Roche Institute of Molecular Biology, Nutley, NJ). A 1255-bp fragment (bp 1–1255, *Eco*RI/*Sac*I) of the c-*fos* clone and a 817-bp fragment (bp 1–817, *Eco*RI/*Pst*I) of the c-*jun* clone were subcloned. All other clones were of mouse origin and were obtained from the American Type Culture Collection. For *jun*-D, a 899-bp *Sph*I fragment (bp 323–1222) was subcloned. Polymerase chain reaction-amplified fragments (with *Hind*III/*Xba*I ends) of *fos*-B (bp 73–1206) and *jun*-B (bp 284–1390) were used for subcloning. A 650-bp fragment (bp 35–685, *Pst*I/*Hinc*II) of rat cyclophilin cDNA was subcloned. Radiolabeled (with [α-^{32}P]UTP) cRNA probes were synthesized *in vitro* using SP6, T3, or T7 RNA polymerase. Specific activities of cRNA probes were routinely $>3 \times 10^9$ cpm/μg of plasmid.

Statistical Analysis

Normalized values are represented as means ± standard errors unless otherwise stated. Statistical analyses were determined with a one-way analysis of variance using the Fischer PLSD test, as calculated with Statview II software.

[1] S. Horwitt, Santa Cruz Biotechnology, personal communication.

Results

Cocaine-induced alterations in striatal and cerebellar Fos/Jun mRNA levels. Riboprobe specificity was assessed by Northern blot analysis of hippocampal RNA from kainic acid-treated rats. As shown in Fig. 1, a single hybridization signal was detected after hybridization with cRNA probes for c-*fos*, *fos*-B, *jun*-B, and *jun*-D, whereas an additional minor hybridization signal (previously detected by others) was observed with the c-*jun* riboprobe. Thus, hybridization signals observed in subsequent Northern blot analyses for c-*fos*, *fos*-B, c-*jun*, *jun*-B, and *jun*-D transcripts are specific for that species of mRNA and do not represent nonspecific cross-hybridizing transcripts.

Preliminary studies and observations by others suggested that 60 min after cocaine administration represents an optimal time point for measuring IEG mRNA induction. In the following study, animals were sacrificed 60 min after intraperitoneal saline or cocaine (20 mg/kg) injection, striatum and cerebellum were isolated, and relative IEG mRNA levels were determined by Northern blot analysis (Table 1). The most dramatic changes in IEG mRNA expression were observed in the striatum. Cocaine induced c-*fos* mRNA levels by approximately 10-fold and *jun*-B mRNA levels by approximately 3-fold. Changes of >25% from control values were not observed for striatal c-*jun*, *jun*-D, or *fos*-B mRNA levels. In the cerebellum, c-*fos* was the only IEG whose mRNA level was significantly affected (approximately 6-fold induction) after acute administration of cocaine.

Northern blot analysis was next used to determine whether induction of c-*fos* mRNA levels in the striatum and cerebellum was dependent upon the dose of cocaine administered (Fig. 2). Animals were given injections of saline or cocaine at doses of 0.5, 4.0, 20.0, or 50.0 mg/kg, with striatum and cerebellum being isolated 60 min after injection. Semiquantitative densitometric analysis of the resulting autoradiographic signals indicated that in striatum maximal induction of c-*fos* mRNA levels was ob-

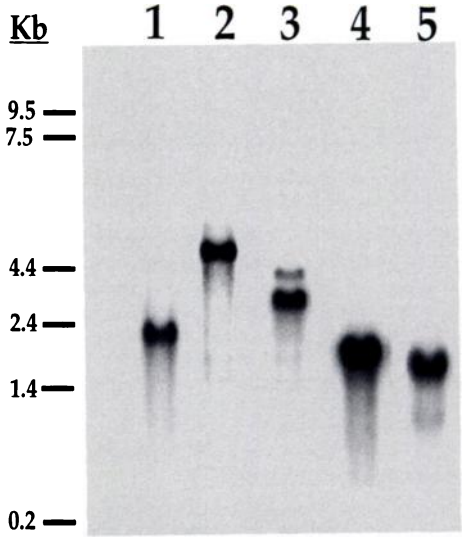

Fig. 1. Riboprobe (cRNA) hybridization conditions for Northern blot analysis of *fos* and *jun* mRNAs yield specific signals for each transcript. Total hippocampal RNA (7 μg) from kainic acid-treated rats was selected for assessing *fos* and *jun* cRNA specificity in Northern blot analyses. Specific hybridization signals are observed for the following transcripts: *lane 1*, c-*fos*; *lane 2*, *fos*-B; *lane 3*, c-*jun*; *lane 4*, *jun*-B; *lane 5*, *jun*-D. The locations of RNA molecular weight standards are noted.

TABLE 1

Effects of acute cocaine administration on striatal and cerebellar *fos* and *jun* mRNA levels

Drug-naive animals were administered an acute dose of cocaine (20 mg/kg) or saline and sacrificed 1 hr thereafter. Brain regions were dissected, and purified RNA was subjected to slot blot analysis by hybridization with individual cRNA probes. The resulting autoradiographic signals were subjected to semiquantitative densitometric analysis and normalized to cyclophilin mRNA signals. No changes (NC) greater than 25% were observed for *jun*-D, *c-jun*, or *fos*-B transcript levels after cocaine administration. The *jun*-B transcript was not detected (ND) or regulated by cocaine in the cerebellum. One-way analysis of variance was performed, and the indicated values are significantly different from saline control values at a 95% level of confidence, using the Fischer PLSD test.

	Induction	
	Striatum	Cerebellum
	fold	
c-*fos*	10.02 ± 2.23	5.69 ± 0.76
fos-B	NC	NC
jun-B	3.13 ± 0.93	ND
jun-D	NC	NC
c-jun	NC	NC

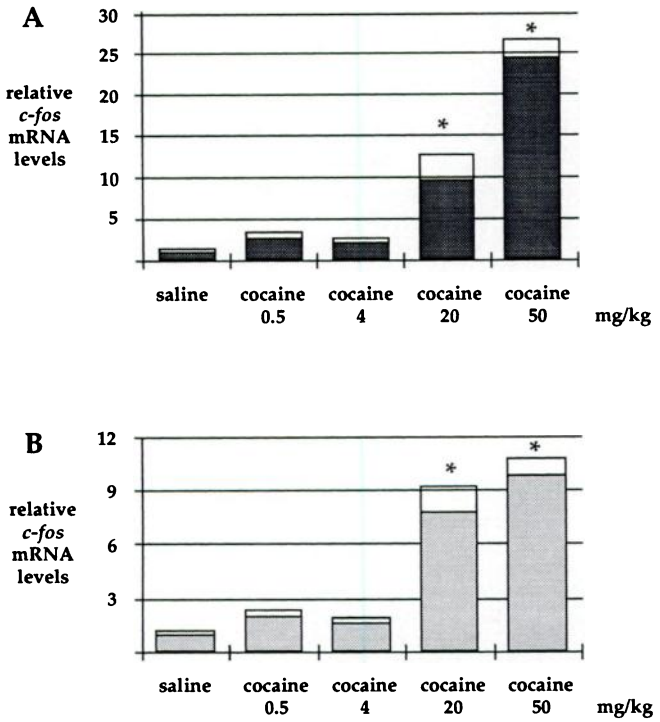

Fig. 2. Cocaine induces dose-dependent increases in striatal and cerebellar c-*fos* mRNA levels. Groups of four animals were each administered the indicated doses of cocaine or saline intraperitoneally and were sacrificed 1 hr thereafter. The data represent c-*fos* mRNA values (mean ± standard error) relative to the mean c-*fos* mRNA levels of saline-treated animals for the striatum (A) and the cerebellum (B). The c-*fos* signals were normalized to control cyclophilin mRNA signals. One-way analysis of variance was performed, and the indicated values (*) are significantly different from saline control values at a 95% level of confidence, using the Fischer PLSD test. White sections of bars represent standard error for the specific mean value presented.

served at the 50 mg/kg dose, whereas in cerebellum the 20 mg/kg dose was sufficient to produce a nearly maximal response. The steepness of the cocaine dose-response curve in the striatum has also been observed for induction of c-Fos-like immunoreactivity (12). Thus, these two brain regions differ only slightly with regard to the dose of cocaine required to elicit maximal induction of c-*fos* mRNA levels.

Another study sought to investigate the effects of chronic cocaine administration on c-*fos* mRNA levels in these two brain structures. This study was initiated as a result of previous observations describing desensitization of striatal (17) and nucleus accumbens (10) c-*fos* mRNA induction after repeated administration of cocaine. In the latter study, acute cocaine administration produced the well described robust increase in nucleus accumbens c-*fos* mRNA levels, whereas no such increase was observed after 14 days of cocaine administration. In the former study, rat caudate was isolated 30 min after acute cocaine administration or 30 min after four successive injections of cocaine at 2-hr intervals. Similar to results described above, caudate c-*fos* mRNA levels increased 6-fold after acute drug administration but were unchanged, compared with control values, after the multiple-injection paradigm.

In the study described here, rats were injected once daily with saline or 20 mg/kg cocaine. On days 1, 2, 4, 8, and 12, the animals were sacrificed 60 min after injection, with striatal and cerebellar RNA being subjected to Northern blot analysis to detect c-*fos* mRNA. The previously observed desensitization of the striatal response was also seen in this study (data not shown). Desensitization was first observed at day 4 and continued throughout the time period tested. By day 12, approximately 10% of the c-*fos* mRNA induction observed on day 1 was present. In contrast, there appeared to be a sensitization regarding induction of cerebellar c-*fos* mRNA levels, with a maximal inductive response being observed between 4 and 8 days of chronic cocaine administration (Fig. 3). Thus, these two brain regions appear to behave in opposite fashions in this chronic cocaine administration paradigm, with respect to relative c-*fos* mRNA levels; desensitization is observed in the striatum, with sensitization occurring in the cerebellum.

Putative receptor and neurotransmitter pathways mediating

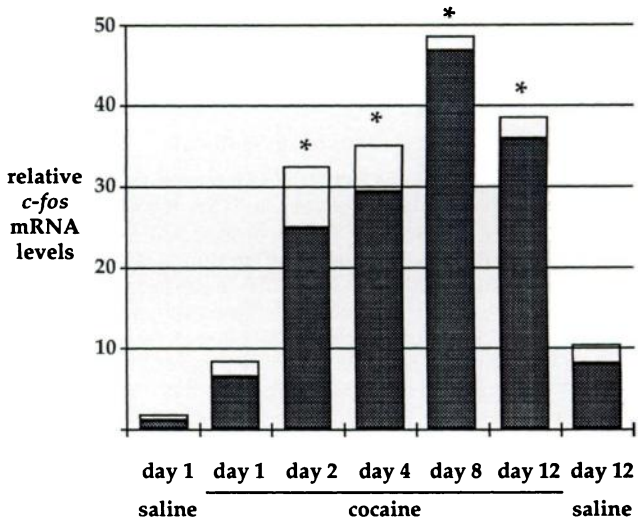

Fig. 3. Expression of c-*fos* mRNA undergoes sensitization within the cerebellum after chronic cocaine administration. Groups of four animals were each administered a daily dose of cocaine (20 mg/kg) and sacrificed 1 hr thereafter on the indicated days. Two control groups (day 1 and day 12) were instead given saline. The data represent c-*fos* mRNA values (mean ± standard error) relative to the mean c-*fos* mRNA levels of day 1 saline-treated animals. The c-*fos* mRNA values were further normalized to cyclophilin mRNA signals. One-way analysis of variance was performed and the indicated values (*) are significantly different from day 1 cocaine values at a 95% level of confidence, using the Fischer PLSD test. White sections of bars represent standard error for the specific mean value presented.

striatal and cerebellar c-*fos* transcriptional changes after acute cocaine administration were also examined. Various pharmacological agents were administered to naive rats 30 min before either saline or cocaine treatment. Striatum and cerebellum were isolated 60 min later and the corresponding RNA was subjected to Northern blot analysis to detect c-*fos* mRNA. Fig. 4 documents the results obtained after semiquantitative densitometric analysis of the resulting hybridization signals.

To establish base-line values, animals were injected with saline 30 min before a second injection of either saline or cocaine. Cocaine treatment, as observed previously, elicited an approximately 10-fold increase in striatal c-*fos* RNA levels and a 5-fold increase in cerebellar c-*fos* RNA levels. Pretreatment with SCH-23390, a D1 receptor-preferring antagonist, completely blocked cocaine-induced changes in both striatal and cerebellar c-*fos* mRNA levels. (−)-Sulpiride, a selective D2 receptor antagonist, by itself was able to elevate striatal c-*fos* mRNA levels by approximately 5-fold but did not significantly alter the effect of cocaine; that is, it failed to produce an additive effect with cocaine. In contrast, (−)-sulpiride by itself had no statistically significant effect on cerebellar c-*fos* mRNA levels but was capable of partially blocking (by approximately 50%) cerebellar c-*fos* mRNA increases produced by cocaine. (±)-Baclofen, a $GABA_B$ receptor agonist, had no statistically significant effect on basal striatal c-*fos* mRNA expression or that observed after administration of cocaine. However, (±)-baclofen was able to suppress the cocaine-induced increase in cerebellar c-*fos* mRNA levels by 80%. Lastly, (+)-MK-801, a noncompetitive NMDA receptor antagonist, had no effect on basal striatal or cerebellar c-*fos* mRNA levels but blocked the ability of cocaine to stimulate c-*fos* mRNA levels by 70% in the striatum and by nearly 100% in the cerebellum. Thus, dopamine D1 and NMDA receptor antagonists are both able to nearly completely block the effects of cocaine in induction of c-*fos* mRNA levels in both striatum and cerebellum, whereas modulation of D2 and $GABA_B$ receptors affects the ability of cocaine to alter c-*fos* mRNA levels in the cerebellum only.

Cocaine-induced alterations in striatal and cerebellar AP-1 DNA-binding complexes. Changes in *fos* and *jun* mRNA levels are indicative of a corresponding alteration in the intracellular levels of the encoded Fos and Jun proteins. Such a change in Fos and Jun protein levels would then predictably result in alterations of AP-1 DNA-binding activity, a semifunctional assay for active Fos and Jun dimer complexes.

Gel shift analysis using a radiolabeled, consensus AP-1 (TGAGTCA), double-stranded oligonucleotide was first used

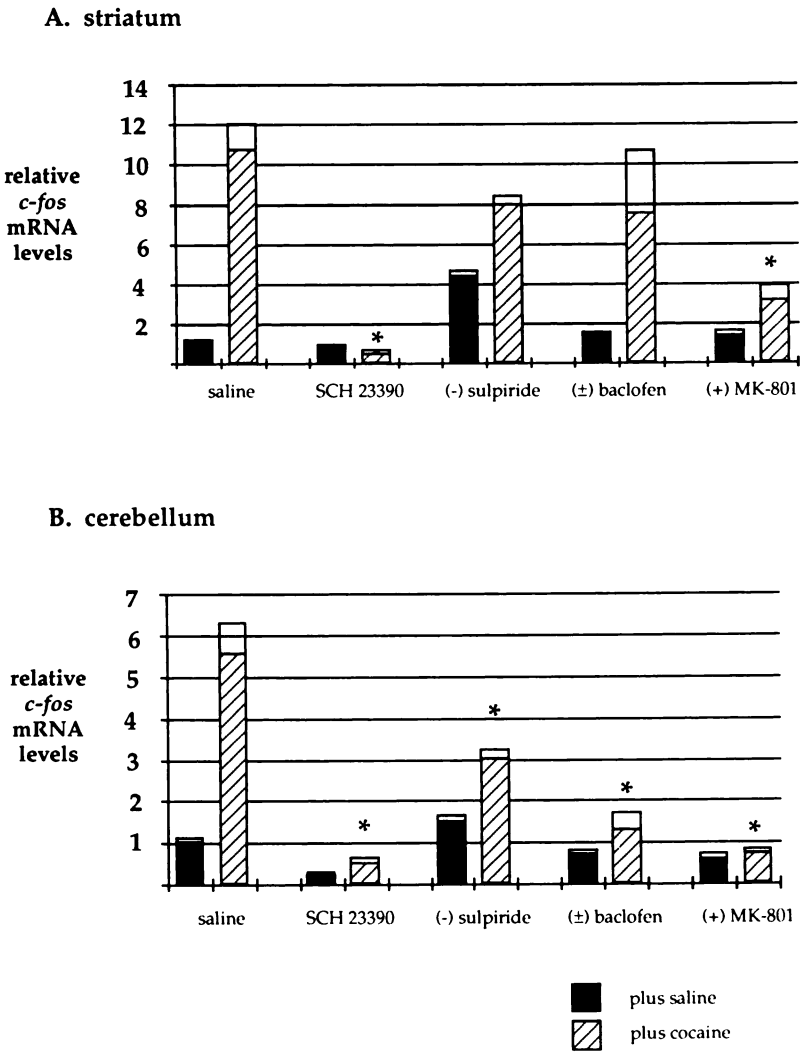

Fig. 4. Specific pharmacological compounds alter the effect of cocaine on striatal and cerebellar c-*fos* mRNA levels. Groups of animals (n = 5) were injected with the following agents 30 min before administration of saline (■) or 20 mg/kg cocaine (▨): saline, SCH-23390 (0.5 mg/kg), (−)-sulpiride (40 mg/kg), (±)-baclofen (1 mg/kg), and (+)-MK-801 (1 mg/kg). Animals were sacrificed 1 hr after the second injection. The c-*fos* mRNA values were normalized to cyclophilin mRNA signals. The data represent relative c-*fos* mRNA levels, compared with control animals that received only saline (i.e., saline plus saline). One-way analysis of variance was performed and the indicated values (*) are significantly different from drug plus cocaine values at a 95% level of confidence, using the Fischer PLSD test. White sections of bars represent standard error for the specific mean value presented.

to determine whether cocaine treatment altered the levels of AP-1 DNA-binding activity within the striatum and cerebellum. Fig. 5 reveals that both striatal and cerebellar protein extracts from cocaine-treated rats contained significantly higher levels of AP-1 DNA-binding activity, compared with extracts prepared from saline-injected control animals. Semiquantitative densitometric analysis of the resulting autoradiographic signals indicated an approximately 6-fold increase in striatal AP-1 DNA-binding activity and a 3-fold increase in cerebellar AP-1 DNA-binding activity after cocaine treatment. The nucleotide sequence specificity of AP-1 complex formation was also evaluated by competition analysis using nonradiolabeled oligonucleotides containing consensus AP-1, CRE (TGACGTCA), CTF/NF-1 (TGGCATGCTGCCA), and SP-1 (GGGCGG) DNA binding sites (Fig. 6). For both striatal and cerebellar protein extracts, the unlabeled AP-1 oligonucleotide effectively competed with complex formation, as expected. Significant competition (>80%) was observed at 10-fold molar excess, with nearly total competition being observed at 50-fold molar excess. A CRE-containing oligonucleotide also competed, although less effectively. This observation was expected, because the consensus AP-1 and CRE sequence elements differ by only a single base. Synthetic oligonucleotides containing nonrelated CTF/NF-1 and SP-1 DNA binding sites were unable to compete with AP-1 complex formation at a molar excess as high as 250-fold. Thus, the retained complex formed with a radiolabeled AP-1 oligonucleotide and proteins present within striatal and cerebellar extracts is specific for recognition of the consensus AP-1 nucleotide sequence.

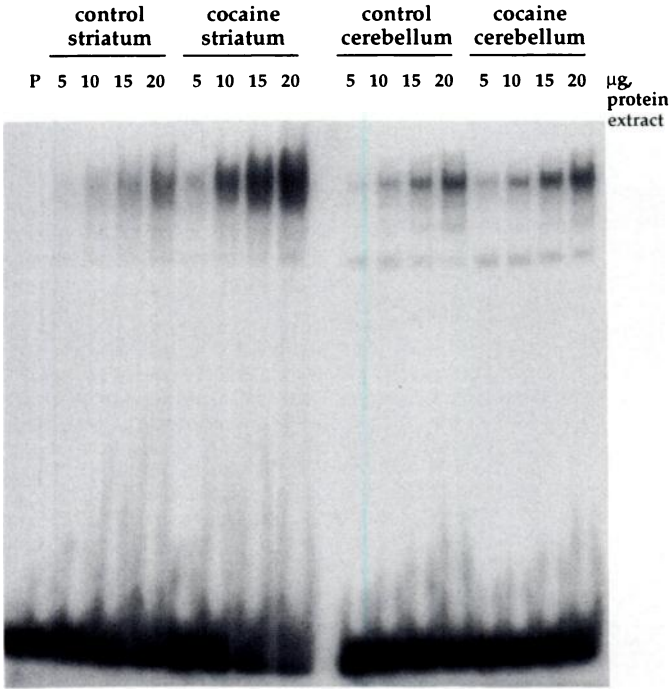

Fig. 5. Acute cocaine administration differentially regulates AP-1 DNA-binding activity in striatal and cerebellar extracts. Gel retention analysis of AP-1 DNA-protein complexes was performed with a ^{32}P-end-labeled consensus AP-1 oligonucleotide and increasing protein concentrations (5, 10, 15, and 20 μg) of whole-cell extracts from tissues isolated 2 hr after administration of cocaine (20 mg/kg) or saline (control). AP-1 DNA-protein complexes are seen in the top of the autoradiograph, whereas the faster migrating unbound oligonucleotide is at the bottom. *P*, AP-1 oligonucleotide probe alone.

Lastly, a series of antibody supershift studies were performed to begin to identify the specific Fos and Jun protein factors that constitute the retained AP-1 complex formed from striatal and cerebellar protein extracts. This type of analysis makes use of serum IgG antibodies that are directed against synthetic peptides representing unique sequences within the various characterized Fos and Jun proteins (see Materials and Methods for a more detailed description of antiserum characterization and specificity). The antiserum is added to the binding reaction before gel electrophoresis and, if a peptide epitope to which the antiserum is directed is present within the retained AP-1-DNA complex, then the antibody binds to the complex and a supershifted band results after autoradiography. Presumably, the peptide epitope represents a unique segment of the targeted Fos or Jun protein, although cross-reactivity with a nonrelated AP-1 DNA-binding protein cannot be formally excluded.

In the study presented in Fig. 7, antisera directed against specific epitopes of rat c-Fos, Fos-B, c-Jun, Jun-B, and Jun-D were individually added to AP-1 DNA-binding reactions containing striatal and cerebellar protein extracts from both saline- and cocaine-treated animals. In striatal protein extracts, cocaine treatment served to increase the apparent relative levels of c-Fos, Fos-B, Jun-B, and Jun-D present in AP-1 complexes. The relative degree of induction of these AP-1 DNA-binding proteins also varied dramatically within the striatal extracts. For example, saline-treated animals appeared to contain moderate levels of Fos-B and Jun-D in striatal extracts, with cocaine treatment increasing the relative levels of these factors by approximately 2–3-fold. In contrast, no detectable levels of c-Fos or Jun-B were observed in striatal extracts from saline-treated animals. Thus, for these latter factors, cocaine treatment appears to result in the formation of new classes of AP-1-DNA complexes that are not detectable in the striatum under basal conditions.

Results observed with cerebellar protein extracts were significantly different, in that cocaine treatment appeared to produce relatively small but significant increases in relative levels of c-Fos and Jun-D only. Both factors appeared to be present in cerebellar extracts from saline-treated controls, with cocaine treatment increasing the relative levels of these factors by 2–4-fold. A low level of c-Jun was also present in cerebellar AP-1 complexes; however, cocaine treatment had no observable effect on relative levels. Thus, in the cerebellum, cocaine treatment appears to increase the level of two pre-existing Fos and Jun proteins and does not result in the apparent formation of new AP-1 complexes that are undetectable in extracts from control animals.

Discussion

Cocaine is a powerful central nervous system stimulant with high abuse liability. It acts as an indirect agonist at dopaminergic, noradrenergic, and serotonergic synapses by inhibiting the synaptosomal reuptake of these neurotransmitters. Cocaine is similarly effective in attenuating the uptake properties of cloned rat dopamine, noradrenaline, and serotonin transporters for their respective amines (21–23). Its rewarding, motivational, and locomotor stimulatory properties are mediated in part by its effects on mesolimbic and mesostriatal dopaminergic neurons (6, 7, 24). However, increased arousal, alertness, and mental ability reported in humans (25) and enhanced sensitivity to audiogenic and visual sensory stimuli in animals (19) are

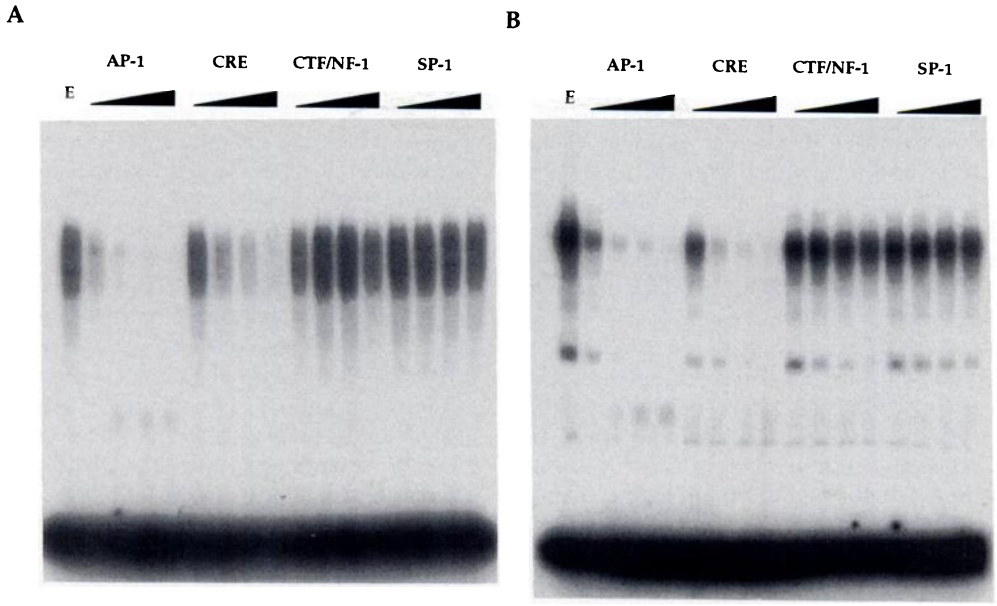

Fig. 6. AP-1 sequence-specific DNA/protein complexes are present within striatal and cerebellar whole-cell extracts. Protein extracts (10 μg) from the striatum (A) and cerebellum (B) of animals treated with 20 mg/kg cocaine for 2 hr were preincubated with a 10-, 50-, 100-, or 250-fold molar excess of nonradiolabeled AP-1, CRE, CTF/NF-1, or SP-1 oligonucleotides before incubation with the ^{32}P-end-labeled consensus AP-1 oligonucleotide. *Broadening of arrowhead*, increasing concentrations of competing nonradiolabeled oligonucleotides. *E*, oligonucleotide probe plus extract alone.

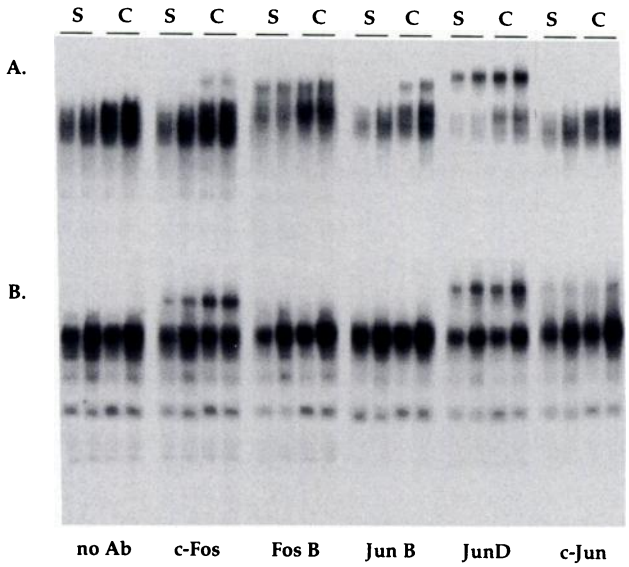

Fig. 7. Acute cocaine administration differentially regulates apparent relative levels of Fos and Jun proteins, within striatal and cerebellar extracts, capable of forming AP-1 DNA-protein complexes. Polyclonal antisera against unique peptide epitopes within c-Fos, Fos-B, Jun-B, Jun-D, or c-Jun were added to preformed radiolabeled AP-1 DNA-protein complexes before electrophoresis. Ten or 15 μg of striatal (A) or 5 or 10 μg of cerebellar (B) extracts were taken from animals treated with either saline (*S*) or 20 mg/kg cocaine (*C*) for 2 hr. The presence of Fos and Jun proteins within AP-1 DNA-protein complexes results in a more slowly migrating (or supershifted) complex. Antibody (*Ab*) specificity was assessed as described in Materials and Methods.

not easily explained by the direct action of cocaine on this dopaminergic circuit. Furthermore, increased sensitivity to sensory input may be linked to the reinforcing properties of cocaine as a drug of abuse. Nevertheless, other target sites within the brain that could mediate these stimulatory events associated with cocaine administration have not been rigorously investigated. The cerebellum receives a large noradrenergic input from the locus coeruleus and exhibits a high density of cocaine binding sites (18). The cerebellum is also the primary biofeedback center of motor sensory inputs and thus plays a critical role in modulating motor behavior. However, little is known about the effects of cocaine on cerebellar neurons or the role of the cerebellum in cocaine-stimulated behavior. The data presented in this study represent an attempt to determine the effects of cocaine on AP-1 mRNA levels and DNA-binding activity in the cerebellum and to compare any observed changes with those occurring in the striatum.

The first set of studies focused on cocaine-induced transcriptional regulation of *fos* and *jun* genes. In the striatum, acute cocaine treatment elevated steady state levels of c-*fos* and *jun*-B transcripts, whereas in the cerebellum the only transcript to respond was that for c-*fos*. These observations in the striatum are similar to those described previously (8, 11, 15, 17) and confirm that cocaine treatment affects transcription of a limited subset of *fos* and *jun* genes in a brain region-specific fashion. The transcriptional response of the cerebellum was more restricted, in part because of more limited basal expression of *fos* and *jun* transcription factors; *jun*-B mRNA was undetectable within the cerebellum. These observations suggest that the transcriptional responses induced by cocaine exhibit specificity at both the genomic and neuroanatomical levels.

Repeated cocaine administration induces tolerance, sensitization, and dependence at the behavioral and cellular levels. In the studies described here, chronic cocaine administration promoted a desensitization of striatal c-*fos* responses, in accordance with previous results (10, 17), and a sensitization of cerebellar c-*fos* mRNA expression. If chronic cocaine administration induces transcriptional tolerance and sensitization in two separate neuroanatomical sites, then behavioral tolerance and sensitization may well be dissociated. Some evidence suggests that behavioral sensitization to psychomotor stimulants

is mediated by mesolimbic dopaminergic neurons (26), and one study has found sensitization of c-Fos-like immunoreactivity responses within the striatum after repeated amphetamine administration (27). However, recent studies suggest that other systems may also mediate these effects. For example, administration of competitive and noncompetitive NMDA receptor antagonists (28, 29) and nitric oxide synthesis inhibitors (30) can block amphetamine-induced behavioral sensitization. Thus, sensitization of cerebellar c-*fos* mRNA expression further expands the notion of multiple neural circuits mediating cocaine-induced behavioral sensitization.

Another goal of the studies was to generate a profile of the neurotransmitter systems mediating the effects of cocaine on c-*fos* mRNA expression within the cerebellum and striatum. These studies are preliminary in the sense that only single, but pharmacologically relevant, concentrations of various agonists and antagonists were preadministered before cocaine. The D1 receptor-preferring antagonist SCH-23390 completely blocked the inductive effect of cocaine in both the cerebellum and striatum. The effect of SCH-23390 on cerebellar c-*fos* mRNA expression is probably indirect, because the cerebellum is devoid of substantial dopamine receptors. There is, however, a minor cerebellar dopaminergic projection from the ventral tegmental area, perhaps serving to mediate a portion of the D1 response. The striatal results have been well described (8, 11, 15, 17) and are presumed to result from direct blockade of D1 receptors on striatal neurons. The D2 receptor antagonist (−)-sulpiride partially blocked the inductive effect of cocaine on cerebellar c-*fos* mRNA levels but was unable to modulate cocaine-induced effects in the striatum. Again, D2 receptor effects on cerebellar c-*fos* mRNA expression may be indirect. It is also worth noting that (−)-sulpiride by itself was able to induce basal striatal levels of c-*fos* mRNA by nearly 5-fold but did not induce cerebellar c-*fos* mRNA levels. Even highly selective D2 antagonists such as YM-90151 (8), and less selective D2 antagonists such as haloperidol (11), stimulate striatal c-*fos* expression. Blockade of striatal D2 receptors may thus serve to stimulate c-*fos* transcription through cellular disinhibition. It is well known that many behaviors mediated by the dopaminergic system, such as locomotor activity, can be mimicked only by the use of nonselective agonists or by concomitant administration of both D1 and D2 receptor agonists (31–33), which also holds true for expression of c-*fos* mRNA within the striatum (13). Precise identification of the dopaminergic receptors involved in c-*fos* transcriptional regulation within the striatum and the cerebellum will require additional pharmacological analysis (because at least five dopamine receptor subtypes have been identified), as well as more precise manipulation of dopaminergic circuits.

The two major neurotransmitters involved in cerebellar activity are GABA and glutamate. In cerebellar Purkinje cells, GABA inhibits spontaneous discharge, whereas glutamate plays an excitatory role (34). In the studies described here, pretreatment of animals with the $GABA_B$ receptor agonist baclofen or the NMDA receptor antagonist MK-801 inhibited the inductive effects of cocaine on cerebellar c-*fos* mRNA levels. These data integrate well with previous studies defining a role for these neurotransmitters in regulating cerebellar responses to cocaine. For example, coadministration of MK-801 with amphetamine or cocaine blunts the development of behavioral sensitization, i.e., the progressive enhancement of locomotor stimulatory properties (28, 29). Motor function is partially mediated through the cerebellum, and the ability of MK-801 to blunt such a response suggests the involvement of NMDA receptors in mediating cerebellar responses to cocaine. MK-801 was also capable of blunting the induction of striatal c-*fos* mRNA levels by cocaine. This observation likewise corroborates a previous study in which MK-801 was shown to reverse the inductive effects of acute methamphetamine administration on the relative levels of c-Fos-like proteins in rat caudate putamen (9). This MK-801 effect presumably involves an interaction between the striatal dopamine and glutamate systems, because MK-801 is capable of blocking D1 receptor priming in 6-hydroxydopamine-lesioned rats (35).

Additional pharmacological studies (data not shown) investigated the roles of κ-opioid receptors (by pretreatment with the κ-opioid receptor agonist U-50,488H), muscarinic receptors (by pretreatment with the M_2 receptor antagonist methoctramine or the nonselective antagonist atropine), and $GABA_A$ receptors (by pretreatment with the $GABA_A$ receptor-preferring agonist muscimol) in mediating striatal and cerebellar responses to cocaine. The aforementioned agents had little if any observable effect on the ability of cocaine to modulate either striatal or cerebellar c-*fos* or *jun*-B mRNA levels. Thus, a limited array of receptor systems appear to mediate c-*fos* transcriptional responses to cocaine.

Gel retention analysis was used to determine whether changes in striatal and cerebellar *fos* and *jun* mRNA levels after cocaine administration were functionally relevant. A substantial increase in the relative amount of AP-1 DNA-binding activity present in striatal extracts was observed 2 hr after acute cocaine administration, as shown previously (10). Similar changes in AP-1 DNA-binding activity were also noted in cerebellar extracts after cocaine treatment. Thus, cocaine appears to mediate some of its cellular effects through modulation of AP-1 DNA-binding activity in both cerebellar and striatal neurons.

Few studies to date have examined the effects of psychomotor stimulant administration on the composition and regulation of those proteins that comprise AP-1 DNA-binding complexes. The occurrence of such regulation is suggested by studies showing that cell proliferation is accompanied by a temporal change in AP-1 dimer composition (36). The use of antisera directed against regions unique to the various known Fos and Jun proteins permitted the qualitative analysis of those specific family members present in the AP-1 DNA-binding complexes described above. Peptide epitope competition studies, as well as immunoprecipitation and Western blot analyses, support the notion that the various antisera are relatively specific for the Fos or Jun family member to which they were generated. However, this type of analysis does not rule out the formal possibility that other cellular proteins, unrelated to the known Fos or Jun proteins, are capable of cross-reactivity with any given antiserum.

Acute cocaine treatment significantly increased striatal levels of c-Fos, Fos-B, Jun-B, and Jun-D, which form AP-1 DNA-binding complexes, whereas only c-Fos and Jun-D levels were increased in cerebellar extracts. Thus, cocaine appears to differentially regulate the relative levels of those specific Fos and Jun proteins that comprise AP-1 DNA-binding complexes within these two transcriptionally responsive brain structures. Some of the observed changes in AP-1 DNA binding are likely

the result of transcriptional events. For example, increases in striatal c-*fos* and *jun*-B mRNA levels after acute cocaine administration likely mediate the corresponding increases in c-Fos and Jun-B protein levels within AP-1 DNA-binding complexes. Other observed changes in AP-1 DNA binding, though, are presumably due to post-transcriptional events. For example, Fos-B and Jun-D levels within AP-1 DNA-binding complexes are increased in the absence of corresponding changes in their mRNA levels. It is well known that phosphorylation regulates Jun-D-mediated AP-1 transcriptional activation (37), and it is presumably this type of protein modification event that regulates the levels of Jun-D and Fos-B present in AP-1 DNA-binding complexes after cocaine treatment. Thus, cocaine appears to modulate both striatal and cerebellar AP-1 DNA binding through both transcriptional and post-transcriptional (presumably protein phosphorylation) processes. Lastly, preliminary experiments (data not shown) using various combinations of antisera in gel retention analyses further suggest that cocaine is able to alter AP-1 heterodimer formation and levels in a brain region-specific fashion. Striatal extracts from cocaine-treated animals appear to display an enrichment of c-Fos/Jun-B, Fos-B/Jun-B, and Fos-B/Jun-D heterodimers, whereas cerebellum appears to exhibit a cocaine-induced increase in c-Fos/Jun-D, c-Fos/c-Jun, and Fos-B/Jun-D heterodimer levels.

Perhaps the most important question that remains to be answered involves identification of those striatal and cerebellar AP-1-regulated target genes whose short and long term expression may be affected by changes in the AP-1 system after administration of cocaine. Previous studies have shown that genes encoding a diverse array of proteins are transcriptionally altered in specific brain regions after either acute or chronic cocaine administration. Such a list includes transcripts encoding the rat dopamine transporter in the substantia nigra (38), prodynorphin and substance P in the dorsal striatum (39), and neuropeptide Y in the cortex and nucleus accumbens (40). Identification of additional target genes, and the dissection of molecular mechanisms linking alteration of the AP-1 system to transcriptional modulation of those target genes, will certainly increase our understanding of the molecular events underlying both short and long term cellular changes associated with psychomotor stimulant drugs.

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