cAMP Response Element-Binding Protein Is Required for Dopamine-Dependent Gene Expression in the Intact But Not the Dopamine-Denervated Striatum

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The cAMP response element-binding protein (CREB) is believed to play a pivotal role in dopamine (DA) receptor-mediated nuclear signaling and neuroplasticity. Here we demonstrate that the significance of CREB for gene expression depends on the experimental paradigm. We compared the role of CREB in two different but related models: L-DOPA administration to unilaterally 6-hydroxydopamine lesioned rats, and cocaine administration to neurologically intact animals. Antisense technology was used to produce a local knockdown of CREB in the lateral caudate–putamen, a region that mediates the dyskinetic or stereotypic manifestations associated with L-DOPA or cocaine treatment, respectively. In intact rats, CREB antisense reduced both basal and cocaine-induced expression of *c-Fos*, *FosB*/Δ*FosB*, and prodynorphin mRNA. In the DA-denervated striatum, CREB was not required for L-DOPA to induce these gene products, nor did CREB contribute considerably to DNA bind-

ing activity at cAMP responsive elements (CREs) and CRE-like enhancers. Δ*FosB*-related proteins and JunD were the main contributors to both CRE and AP-1 DNA–protein complexes in L-DOPA-treated animals. In behavioral studies, intrastriatal CREB knockdown caused enhanced activity scores in intact control animals and exacerbated the dyskinetic effects of acute L-DOPA treatment in 6-OHDA-lesioned animals. These data demonstrate that CREB is not required for the development of L-DOPA-induced dyskinesia in hemiparkinsonian rats. Moreover, our results reveal an unexpected alteration of nuclear signaling mechanisms in the parkinsonian striatum treated with L-DOPA, where AP-1 transcription factors appear to supersede CREB in the activation of CRE-containing genes.

Key words: Parkinson's disease; immediate-early genes; direct pathway; opioid precursor; psychostimulant; sensitization; protooncogenes; motor stereotypies

Drugs that stimulate dopamine (DA) receptors have the potential to produce long-lasting behavioral and neural alterations. L-DOPA pharmacotherapy of Parkinson's disease is associated with a high incidence of motor complications, such as wearing-off fluctuations and dyskinesias, the development of which is closely paralleled by changes in striatal gene expression (for review, see Calon et al., 2000). Animal studies have shown that both L-DOPA-induced dyskinesia and DA-dependent stereotypies are associated with an induction of prodynorphin (PDyn) and *fos* family genes in striatal projection neurons of the "direct pathway" (for review, see Graybiel et al., 2000). Multiple lines of evidence suggest that such an induction is mediated by cAMP response element-binding protein (CREB) phosphorylation through activation of D₁ receptors and protein kinase A (PKA) (Cole et al., 1994, 1995; Simpson and McGinty, 1995; Konradi et al., 1996). Phosphorylated CREB has been detected in the DA-denervated rat striatum after treatment with L-DOPA (Cole et al., 1994) and in the intact striatum after psychostimulant drug administration

(Cole et al., 1995; Konradi et al., 1996). Moreover, amphetamine-induced striatal *c-Fos* expression can be blocked by knockdown of CREB mRNA (Konradi et al., 1996). Furthermore, in striatal protein extracts, CREB accounts for DNA binding activity at cAMP responsive element (CRE) sites (Cole et al., 1995; Konradi et al., 1996), which are present in both the *c-fos* (Curran et al., 1987) and the PDyn promoter (Douglass et al., 1994). These and other findings have prompted the notion of CREB as a critical mediator of drug-induced neuroplasticity in the striatum (for review, see Pierce and Kalivas, 1997; Calon et al., 2000). However, the requirement of CREB for DA receptor-induced cellular and behavioral plasticity may not hold true in all experimental paradigms.

Here, we have used antisense technology to assess whether L-DOPA-induced changes in *fos* and PDyn gene expression, and/or the concomitant dyskinetic manifestations in hemiparkinsonian rats (Cenci et al., 1998; Andersson et al., 1999), can be blocked by CREB knockdown. We have also performed a parallel experiment to investigate whether CREB is required for both basal and cocaine-induced expression of the same genes in neurologically intact animals. The information provided by antisense experiments was complemented by an analysis of DNA–protein interactions in striatal protein extracts. In agreement with currently held notions, CREB was found to be essential for cocaine-induced gene expression in the intact striatum, where it accounted for most protein binding activity to CRE-like elements. By contrast, in the DA-denervated striatum, CREB was not required for L-DOPA to induce *c-fos*, *fosB*, and PDyn gene

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Table 1. Treatment groups and number of animals

	Drug dose	Oligonucleotide	Number of animals
6-OHDA-lesioned rats			
Saline		No	20
Acute L-DOPA	3 mg/kg	No	3
Acute L-DOPA	5 mg/kg	No	5
Acute L-DOPA	5 mg/kg	CREB AS	5
Acute L-DOPA	5 mg/kg	Control oligo	5
Acute L-DOPA	8 mg/kg	No	17
Acute L-DOPA	8 mg/kg	CREB AS	16
Acute L-DOPA	8 mg/kg	Control oligo	16
Chronic L-DOPA	8 mg·kg ⁻¹ ·d ⁻¹	No	9
Chronic L-DOPA	8 mg·kg ⁻¹ ·d ⁻¹	CREB AS	9
Chronic L-DOPA	8 mg·kg ⁻¹ ·d ⁻¹	Control oligo	9
Intact rats			
Saline		No	15
Saline		CREB AS	7
Saline		Control oligo	6
Acute cocaine	15 mg/kg	No	7
Acute cocaine	15 mg/kg	CREB AS	19
Acute cocaine	15 mg/kg	Control oligo	19
Acute cocaine	30 mg/kg	No	5
Acute cocaine	30 mg/kg	CREB AS	6
Acute cocaine	30 mg/kg	Control oligo	8
Chronic cocaine	30 mg·kg ⁻¹ ·d ⁻¹	No	5

expression, and both CRE- and AP-1-binding activity was mainly accounted for by Δ FosB-related proteins and JunD. The present results show that CREB is not a critical mediator of L-DOPA-induced gene expression changes in the parkinsonian striatum, where AP-1 transcription factors appear to supersede CREB in the activation of CRE-containing genes.

MATERIALS AND METHODS

Subjects. Female Sprague Dawley rats (BK Universal, Stockholm, Sweden; ~225 gm body weight at the beginning of experiment) were housed under a 12 hr light/dark cycle with *ad libitum* access to food and water. The number of animals and experimental groups in the present study is shown in Table 1.

Dopamine denervating lesions. All rats receiving L-DOPA treatment had sustained unilateral injections of 6-hydroxydopamine (6-OHDA-HCl; Sigma-Aldrich Sweden AB) into the right ascending DA fiber bundle at least 4 weeks before drug treatment, as described previously (Cenci et al., 1998). Briefly, 6-OHDA was dissolved in 0.02% ascorbic acid/saline at a concentration of 3 μ g/ μ l and injected at the following coordinates (in mm, relative to bregma and the dural surface): (1) anterior (A) = -4.0, lateral (L) = -0.8, ventral (V) = -8.0, toothbar = +3.4 (6 μ g deposit); and (2) A = -4.4, L = -1.2, V = -7.8, toothbar = -2.3 (7.5 μ g deposit).

To evaluate the extent of DA denervation, the rats were tested for amphetamine-induced rotation at 2 weeks after lesion. Turning behavior was recorded in an automated rotometer over a 90 min period after an injection of 5 mg/kg dexamphetamine sulfate (Apoteksbolaget Sweden AB). Only rats that showed more than seven full turns per minute ipsilaterally to the lesion (corresponding to >97% DA depletion in the caudate-putamen) (Schmidt et al., 1982) were selected for the study.

Drugs. DA-denervated rats received L-DOPA methyl ester (3–8 mg/kg, i.p.; Sigma-Aldrich) mixed with the peripheral DOPA-decarboxylase inhibitor, benserazide hydrochloride (15 mg/kg; kindly provided by Hoffman-La Roche, Basel, Switzerland). Neurologically intact rats were injected with cocaine (15 or 30 mg/kg, i.p.; Apoteksbolaget Sweden AB). All drugs were dissolved in physiological saline and injected immediately. Animals were killed at 3 hr after injection. This survival period was chosen on the basis of previous literature showing that upregulation of

Fos family proteins and PDyn mRNA can be detected in the striatum at this time after DA agonist administration (Hurd and Herkenham, 1992; Andersson et al., 1999; Cenci et al., 1999).

Antisense experiments. The CREB antisense (AS; 5'-TGGTCATC-TAGTACCCGGTG-3') and CREB sense (5'-CACCGGTGACTAG-ATGACCA-3') sequences were published previously (Konradi et al., 1994). The "scrambled" control sequence was a random assortment of the bases that composed the AS (5'-TGCTGAACCTGTGCGCCAGTG-3'). This sequence showed no homology to any known mammalian gene. All oligonucleotides were partially phosphorothioate modified (sulfur substitutions at both the 5' and 3' terminal phosphodiester linkage) and were purchased from Scandinavian Gene Synthesis.

For single injections, oligonucleotides were dissolved in sterile PBS at a concentration of 2.5 mM. One microliter of the solution was injected over 2 min (the cannula being left in place for an additional 2 min) at the following coordinates (in mm, relative to bregma and the dural surface): A = +0.6, L = $\pm$ 3.5, V = -4.7, tooth bar at -2.3. Surgery was performed on rats anesthetized with a mixture of Hypnorm (Janssen Pharmaceutical) and Dormicum (Hoffman-La Roche) that was dissolved in sterile water (1:1:2 solution; 2.7 ml/kg, i.p.). Analgesic treatment (Temgesic, Apoteksbolaget AB; 0.167 ml/kg body weight, s.c.) was given ~20 min before the rats awakened.

For continuous infusions, oligonucleotides were dissolved in sterile PBS at 0.2 mM concentration and infused through Alzet osmotic minipumps (Model 2002, 14 d duration; Alza Corporation, Palo Alto, CA) into the striatum at a rate of 0.5 μ l/hr (2.5 nmol in 12 μ l per day). Implantation surgery was performed on anesthetized rats as described above. The injection cannulas were implanted in the lateral caudate-putamen at the following coordinates: A = +0.6, L = +4.0, V = -5.0, tooth bar at -2.3. The cannulas were secured to the skull using screws and dental acrylic. The reservoirs were inserted subcutaneously in the neck. Rats were treated with antibiotics (Borgal, 1:500 in the drinking water; Hoechst) from 1 d before to 6 d after pump implantation.

Dyskinesia and stereotypy rating. An investigator who was completely unaware of the rat group membership (experimentally blinded) performed all behavioral testing. L-DOPA-induced abnormal involuntary movements (AIMs) were rated using a rat dyskinesia scale that we introduced recently (Cenci et al., 1998; Andersson et al., 1999; Lee et al., 2000). This scale only takes into account purposeless movements that are clearly abnormal for the rat, whereas enhanced manifestations of normal motor activities, such as grooming, gnawing, rearing, and sniffing, are not included in the rating. Briefly, rats were observed individually for 1 min every 20 min after an injection of L-DOPA. Each rat was scored on a severity scale from 0 to 4 on each of four subtypes of dyskinetic-like movements (axial, orolingual, forelimb, and locomotive AIMs).

Motor activity and stereotypies were rated according to Creese and Iversen (1973). Rats were observed for 30 sec every 10 min after an injection of cocaine or saline. Every rat was scored on a scale from 0 to 6 (0 = asleep or stationary; 1 = active; 2 = very active with bursts of intense sniffing; 3 = stereotyped sniffing along a fixed path in the cage; 4 = stereotyped sniffing or rearing in a fixed location; 5 = stereotyped behavior in one location with bursts of gnawing and licking; and 6 = continual gnawing and licking of the cage bars in a fixed location). Note that scores of 0–2 in this scale reflect generic motor activation, whereas scores $\geq$ 3 reflect a clearly stereotypic behavior (i.e., repetition of single or multiple movements according to an inflexible pattern). Dyskinesia and activity–stereotypy ratings were performed at 60–120 min after drug injection, corresponding to the peak of the time–action curve for both L-DOPA (Lee et al., 2000) and cocaine (our unpublished data).

Histological procedures. The rats were deeply anesthetized with sodium pentobarbitone (240 mg/kg, i.p.; Apoteksbolaget AB) and killed by decapitation. The brains were rapidly removed and frozen on dry ice. Coronal sections (16 μ m thick) were cut through the striatum using a cryostat, thaw-mounted onto microscope slides (SuperFrost Plus; Menzel Glazer), and stored at -20°C.

Screening for unspecific tissue damage after oligonucleotide delivery to the striatum. In the antisense study, one series of sections per animal was stained using cresyl violet to verify the location of the cannula track and the extent of the surrounding inflammation. The amount of monocyte infiltration at the injection site did not differ between the three oligonucleotide sequences used in this study. A total of eight animals showing severe inflammation and hemorrhage were excluded from the study.

To assess ongoing neuronal death, another series of sections was stained using Fluoro-Jade B histochemistry according to Schmued and Hopkins (2000). Briefly, sections were treated with 1% NaOH in 80%

ethanol for 5 min, rinsed sequentially in ethanol and water, transferred to a solution of 0.06% potassium permanganate for 10 min, and rinsed in water. Thereafter, the sections were incubated for 20 min with Fluoro-Jade B (Histo-Chem Inc., AR; 0.0004% in 0.1% acetic acid/water), rinsed repeatedly, and dried in a slide warmer. Cells along the needle track in the striatum and the cortex were clearly fluorescent at 32 hr after a single oligonucleotide injection. In contrast, no fluorescent neurons could be detected after 14 d of continuous oligonucleotide infusion, indicating that the oligonucleotides per se did not exert cytotoxic effects during the infusion time.

Immunohistochemistry. Immunohistochemistry was performed using a standard peroxidase-based method (Vectastain Elite ABC Kit, Vector Laboratories, Burlingame, CA) with 3,3'-diaminobenzidine (Sigma-Aldrich Sweden AB) as a chromogen.

The following primary antisera were used at the indicated dilutions: CREB (1:5000; rabbit polyclonal from Upstate Biotechnology, Lake Placid, NY); c-Fos (1:1000, sheep polyclonal from Sigma-Genosys); FosB/ Δ FosB (1:15000, rabbit polyclonal from Santa Cruz Biotechnology, Santa Cruz, CA); neuron-specific nuclear protein (NeuN, 1:100, mouse monoclonal from Chemicon, Temecula, CA); and JunD (1:5000, rabbit polyclonal from Santa Cruz Biotechnology). Before immunohistochemistry, cryostat sections were fixed by a 10 min immersion in phosphate-buffered 4% paraformaldehyde at 4°C, pH 7.4, rinsed three times in

potassium PBS, and incubated with an avidin–biotin blocking kit (Vector Laboratories; 1 hr incubation in each solution, avidin and biotin).

In situ hybridization histochemistry. *In situ* hybridization histochemistry (ISHH) was performed using synthetic oligomers complementary to nucleotides 934–982 of the cloned PDyn gene (Civelli et al., 1985), nucleotides 322–360 of the cloned preproenkephalin (PPE) cDNA (Howells et al., 1984), nucleotides 1286–1333 of feline glutamic acid decarboxylase cDNA (GAD67; Kobayashi et al., 1987), nucleotides 767–803 of the dopamine D1 receptor cDNA (Monsma et al., 1990), and nucleotides 1587–1629 of an orphan opiate receptor cDNA (Wang et al., 1994). Oligonucleotides (0.2 μ M) were labeled at the 3' end with 4 μ M [α - 35 S]dATP (>37 TBq/mmol; Amersham Pharmacia Biotech) using 15 U of terminal deoxynucleotidyltransferase (Amersham Pharmacia Biotech) for 2 hr at 37°C. The labeled probes were purified by spin-column chromatography (Chroma Spin Columns, Clontech Laboratories, Palo Alto, CA) to specific activities of >10⁹ cpm/ μ g. Specificity of the oligomers used had previously been determined by Northern blot analysis, homology screens of the sequences in GenBank, and comparisons of the anatomical distribution of the hybridization signal with previous studies attempting to localize the same mRNA transcripts. Specificity of hybridization was determined by competition experiments in which the 35 S-labeled probe was diluted with an excess of either the same unlabeled oligonucleotide or an unlabeled unrelated oligonucleotide.

Figure 1. Different methods of CREB antisense delivery produce different extents of CREB knockdown in the striatum. The effects of single oligonucleotide injections are shown in *A–D*, whereas those produced by a 14 d oligonucleotide infusion are shown in *E–H*. The number of CREB-immunoreactive cells in the region flanking the injection cannula is shown in *A* (single injection) or *E* (continuous infusion), and the corresponding photomicrographs are shown in *A'* or *E'* (antisense group), and *A''* or *E''* (control oligonucleotide). NeuN-immunoreactive neurons within the same regions are shown in *B–B''* and *F–F''*. Camera lucida drawings illustrate the regional distribution of CREB knockdown after single oligonucleotide injection (*C*, antisense; *D*, control) or continuous oligonucleotide infusion (*G*, antisense; *H*, control). #*p* < 0.01; *n* ≥ 7 in all groups; values give means + SEM. Asterisks in *A'*, *A''*, *B'*, *B''*, *E'*, *E''*, *F'*, and *F''* mark the cannula track. AS, CREB antisense; Ctrl, control oligo. Scale bars in *B''* and *F''*, 200 μ m.

The slide-mounted sections were air dried and incubated with the hybridization mixture, which comprised 50% formamide (deionized), 4× SSC (1× SSC = 0.15 M NaCl and 0.015 M sodium citrate), 1× Denhardt's solution (0.02% Ficoll, 0.02% polyvinyl pyrrolidone, 10 mg/ml of RNase-free bovine serum albumin), 1% sarcosyl, 10% dextran sulfate, 500 µg/ml sheared and denatured salmon sperm DNA, 25 µl/ml tRNA, 200 mM dithiothreitol, 0.2 mg/ml heparin, and 10^7 cpm/ml of 35 S-labeled oligonucleotide probe. Forty microliters of hybridization mixture were added to each section (240 µl per slide). Slides were coverslipped with Parafilm and incubated for 18 hr at 42°C in a humid chamber. After hybridization, the Parafilm coverslips were floated off in 1× SSC at 55°C, and the sections were given four 15 min washes in 1× SSC at 55°C, plus a final wash beginning at 55°C; they were then cooled down to room temperature. The slides were then rinsed twice in distilled water, dehydrated in 70 and 95% ethanol (2 min each). The slides were exposed to Fuji imaging plates (see below) and subsequently dipped in photographic emulsion (LM-1, Amersham Pharmacia Biotech). Emulsion-coated sections were exposed for 2–7 weeks at –20°C, developed in Kodak D-19 at 18°C, fixed, and coverslipped with D.P.X. mountant (BDH).

Image analysis and cell counts. Quantitative analyses of ISHH or immunohistochemical specimens were performed by an investigator who was completely unaware of the rat group membership (experimentally blinded). An analysis of the antisense effect on various cellular markers was performed on two striatal sections per animal spanning rostrocaudal levels –0.2 to +0.70 mm relative to bregma, according to Paxinos and

Watson (1997). Measurements were performed in two 0.54-mm²-large rectangular areas flanking the cannula track. Additional measurements were taken in control areas, having the same coordinates, on the contralateral side.

To measure ISHH labeling, the hybridized sections were exposed to Fuji imaging plates (Fujifilm Sweden AB) for 1–12 hr. The plates were scanned in a BAS-5000 phosphorimager (Fujifilm Sweden AB) to obtain digitized autoradiographs. The photo-stimulated luminescence emitted by the hybridized sections was calibrated against radioactivity levels (kilobecquerels per gram) using simultaneously exposed 14 C standards (Amersham Pharmacia Biotech). The hybridization signal was analyzed using the program TINA (Fujifilm Sweden AB).

Counts of immunostained cells were performed using the program NIH Image 1.61. Sample areas (0.54 mm² in size) were digitized through a ProgRes3012 video camera (Kontron) connected to an Olympus microscope (20× magnification). Data are expressed as number of positive cells per square millimeters.

Electrophoretic mobility shift assay. Anesthetized rats were killed by decapitation; the brains were rapidly extracted and immediately frozen on dry ice. Striata were dissected at –20°C in a cryostat, and left and right striata were collected separately. Whole-cell protein extracts were prepared by Dounce homogenization in ice-cold homogenization buffer (20 µl/mg tissue; 20 mM HEPES, 25% glycerol, 1.5 mM MgCl₂, 0.4 mM EDTA, 1 mM DTT, 0.5 mM PMSF). The homogenate was centrifuged at 10,000 × g for 3 min at 4°C. The preparation was homogenized once more

Figure 2. L-DOPA regulates c-Fos, FosB/ΔFosB, and prodynorphin independently of CREB in 6-OHDA-lesioned rats. The effects of acute doses of L-DOPA on the number of c-Fos (A) or FosB/ΔFosB (B) immunoreactive cells and on the levels of PDyn mRNA (C) were measured on the cross-sectional area of the caudate–putamen at mid-rostrocaudal levels. L-DOPA induced levels of Fos family proteins (A, B) and PDyn (C) mRNA dose dependently. A single injection of CREB antisense oligonucleotides did not prevent the induction of c-Fos (D), FosB/ΔFosB (E), or PDyn mRNA (F) by L-DOPA. CREB antisense-treated animals are marked as AS + and represented using bars with diagonal stripes, whereas animals receiving control oligonucleotides are marked as AS – and represented using bars with no texture. Antisense effects were measured in 0.54-mm²-large areas next to the injection track. **p* < 0.01 versus lesion-only; #*p* < 0.05 versus 5 mg/kg; and \$*p* < 0.05 versus 3 mg/kg L-DOPA; *n* = 3–5 per group in A–C, and ≥5 per group in D–F; values give means + SEM.

and centrifuged at 10,000 $\times$ g for 10 min at 4°C. The supernatant was collected and protein concentration was determined using the Bio-Rad Protein Assay (Bio-Rad Laboratories, Hercules, CA).

Oligonucleotides were synthesized with two G-overhangs and annealed in the presence of 20 mM NaPO₄, 1 mM EDTA, and 100 mM KCl. Double-stranded oligonucleotides were radioactively labeled with ³²P-dCTP by reverse transcription (Moloney murine leukemia virus reverse transcriptase, Promega, Madison, WI). The dynCRE3 used (nucleotides –1562 to –1522 of the rat PDyn promoter; 5'-CCTGCTGCGT-CAGAGCATGA-3') (Douglass et al., 1994) has been shown to be important for CREB-regulated PDyn gene expression in various cell culture systems (Collins-Hicok et al., 1994; Messersmith et al., 1994). The dynAP-1 element is a noncanonical AP-1 site mapping to oligonucleotides –257 to –249 of the PDyn promoter (5'-TTTGAAGTGACAAACAGCGCT-3') (Naranjo et al., 1991). Two additional sequences were used for comparison, namely, the consensus sequence for the palindromic CRE/ATF site, here called consensus CRE (Konradi et al., 1993)(5'-GCTGACGTCAGGG-3'), and a canonical AP-1 sequence from the human collagenase promoter (5'-TTCCGGCTGACTCATCAAGCG-3') (Vallone et al., 1997).

To examine DNA–protein interactions, protein extracts (3 μ g) were preincubated on ice for 10 min in gel-shift buffer (10 mM HEPES, 10% glycerol, 0.1 mM EDTA, 67 mM KCl, 150 ng poly(dI-dC), 5 mM MgCl₂, 2 mM DTT, and 100 mM PMSF). After preincubation, 1 ng of ³²P-labeled double-stranded oligonucleotide was added, and the samples were incubated for 10 min at room temperature. DNA–protein complexes were separated on a 4% nondenaturing polyacrylamide gel (29.5:1 acrylamide/bisacrylamide) in 0.25 $\times$ TBE buffer (1 $\times$ TBE contains 0.045 M Tris-borate and 0.002 M EDTA). The gel was dried onto a filter paper and exposed to autoradiographic films or phosphorimager plates (Fujifilm Sweden AB) that were later used for computer-assisted measurements of the optical density of shifted bands.

Cold competition assays were performed using two different mixes of protein extracts, pooled from the striatum either ipsilateral (lesioned side) or contralateral (intact side) to the 6-OHDA lesion of three chronically L-DOPA-treated rats. Samples of 3 μ g of pooled protein extracts were incubated with nonradioactively labeled consensus CRE or canonical AP-1 oligonucleotides at 5 $\times$ and 50 $\times$ the concentration of the radioactively labeled probe.

In supershift assays, the preincubation was extended in the presence of antisera overnight, and proteinase inhibitors were added [500 μ M 4-(2-aminoethyl)-benzenesulfonylfluoride, 150 nM aprotinin, 1 μ M leupeptin, 0.5 mM EDTA, 1 μ M E-64; Proteinase Inhibitor Cocktail Set I, Calbiochem].

Western immunoblotting. Striatal protein extracts were prepared as described above. Proteins (7.5 μ g) were loaded onto a 12% acrylamide resolving gel/4% stacking gel for SDS-PAGE and electroblotted onto polyvinylidene difluoride membranes (Amersham Pharmacia Biotech). Membranes were blocked in TBS-T (20 mM Tris, pH 7.6, 0.9% NaCl, 0.01% Tween 20) containing 3% non-fat dry milk and then incubated overnight at 4°C with antiserum. After four 15 min rinses in TBS-T, membranes were incubated with horseradish peroxidase-conjugated anti-rabbit antibodies (Amersham Pharmacia Biotech; 1:2000 in TBS-T) for 1 hr at room temperature. The membranes were washed four times for 15 min each in TBS-T and developed with the enhanced chemoluminescence method (ECL; Amersham Pharmacia Biotech), and exposed to Hyperfilm-ECL (Amersham Pharmacia Biotech) for 5–45 min. The following antisera were used at the indicated dilutions: FosB/ Δ FosB (1:10 000, rabbit polyclonal from Santa Cruz Biotechnology), FosB/

Δ FosB (1:7500, rabbit polyclonal from Research Biochemicals International, Natick, MA), and panFos (1:5000, kind gift of Dr. E. Vaudano, Lundbeck A/S).

Statistical analysis. Image-analysis data from antisense-treated and control rats were compared using Student's two-tailed t test. Statistical comparisons between more than two groups were performed using one-factor ANOVA and *post hoc* Newman–Keuls test. Correlations were estimated using simple regression analysis. Group comparisons of dyskinesia and stereotypy rating scores were performed using the Mann–Whitney U test. The null hypothesis was rejected when $p < 0.05$.

RESULTS

Regional specificity, time dependence, and dose dependence of antisense-mediated CREB knockdown

In agreement with previous studies (Konradi et al., 1994; Konradi and Heckers, 1995), we found that a single antisense injection produced a downregulation of striatal CREB immunoreactivity, which was maximal at ~32 hr after injection. At this time, a single dose of CREB antisense (2.5 nmol) had reduced the number of CREB-immunoreactive neurons by $23.4 \pm 9.5\%$ in a region extending up to 300 μ m around the injection site ($p < 0.01$ vs control group) (Fig. 1*A–A'*,C). By comparison, the number of cells immunoreactive for the neuron-specific marker NeuN was the same in the AS and control (Ctrl) groups (Fig. 1*B–B'*).

Continuous infusion of CREB antisense over 14 d (2.5 nmol/d) produced a dramatic downregulation of CREB-immunoreactive cell numbers ($-80 \pm 5\%$ vs Ctrl group) (Fig. 1*E–E'*). The knockdown of CREB was still evident at 1 mm distance from the cannula (compare AS with Ctrl in Fig. 1, *G* and *H*, respectively). The antisense effect was not caused by unspecific cell death, because there was no difference in the number of NeuN-immunoreactive neurons between the AS and Ctrl groups (Fig. 1*F–F'*). In addition, the expression of constitutively expressed transcription factors (JunD) or mRNA transcripts (PPE, GAD, D1-receptor, and an orphan opiate receptor mRNA) did not differ between the two groups ($p > 0.27$ in AS vs Ctrl oligo group; data not shown). Moreover, using Fluoro-Jade B histofluorescence as a method to detect degenerating neurons (Schmued and Hopkins, 2000), we did not find ongoing cell death in the region adjoining the chronic oligonucleotide infusion, although we saw a number of fluorescent neurons after acute oligonucleotide injections (data not shown).

CREB is not required for L-DOPA-induced changes in c-fos, fosB, and prodynorphin gene expression in the DA-denervated striatum

Before studying the effects of CREB antisense, we set out to determine whether single doses of L-DOPA, which lie within the therapeutic range for the treatment of Parkinson's disease (3, 5,

Table 2. Continuous CREB antisense infusion does not affect L-DOPA-induced changes in gene expression

Gene expression markers	Acute L-DOPA		Chronic L-DOPA	
	CREB AS	Control oligo	CREB AS	Control oligo
Number of c-Fos+ cells	144 $\pm$ 31	106 $\pm$ 31	9 $\pm$ 4	8 $\pm$ 3
Number of FosB+ cells	42 $\pm$ 9	63 $\pm$ 18	140 $\pm$ 32	170 $\pm$ 62
PDyn mRNA (kBq/gm)	976 $\pm$ 89	1061 $\pm$ 71	2250 $\pm$ 325	1881 $\pm$ 541

Data were collected from animals sustaining continuous oligonucleotide infusion in the lateral caudate–putamen for 14 d. L-DOPA (8 mg/kg per injection, i.p.) was administered either on day 14 (acute treatment) or on days 2–14 (chronic treatment). The three genes under investigation show a differential response to the effects of chronic versus acute L-DOPA treatment: higher levels of PDyn mRNA and FosB-like proteins are seen after chronic treatment, and higher levels of c-Fos expression are found after acute treatment. However, none of the observed changes is affected by CREB antisense.

Figure 3. CREB knockdown differentially affects the induction of c-Fos (*A, B, G, H*), FosB/ΔFosB (*C, D, I, J*), or PDyn mRNA (*E, F, K, L*) by L-DOPA or cocaine. In the absence of CREB, L-DOPA was able to induce all gene expression markers in the 6-OHDA-lesioned striatum (compare antisense group in *A, C*, and *E* with control group in *B, D*, and *F*), whereas

Figure 4. Loss of CREB increases abnormal involuntary movements (orolingual, limb, and axial AIMS) in 6-OHDA-lesioned rats treated with L-DOPA. In the acutely L-DOPA-treated rats (*A*), CREB antisense produced levels of AIM scores that were similar to chronic L-DOPA-treated rats (compare *AS* group in *A* with *Ctrl* group in *B*). A trend toward more severe dyskinesias in the CREB AS group was also seen during chronic L-DOPA treatment (*B*). AIMS scores were collected during a single testing session in *A* and during four testing sessions in *B* (the AIM score in the last of these sessions is shown in the diagram). Each dot in the scattergram represents one single animal; the group median is shown as a horizontal bar. $^{\#}p < 0.01$.

and 8 mg/kg), were sufficient to induce changes in the expression of the genes under investigation. Drug-induced expression of c-Fos and FosB was monitored at the protein rather than mRNA level because the interval after injection that was chosen in this study (3 hr) is beyond the peak of mRNA induction.

L-DOPA was found to induce c-Fos, FosB/ΔFosB, and PDyn mRNA expression in the DA-denervated striatum in a dose-dependent manner (Fig. 2*A–C*). Doses of 5 and 8 mg/kg L-DOPA induced a significant increase in c-Fos and FosB/ΔFosB immunoreactivity (Fig. 2*A, B*), as well as in PDyn mRNA levels (Fig. 2*C*), whereas the lowest dose tested did not produce any detectable effects.

Surprisingly, a single injection of CREB antisense had no effect on the ability of either 5 or 8 mg/kg L-DOPA to induce c-Fos, FosB/ΔFosB, or PDyn in striatal neurons (Fig. 2*D–F*). We therefore asked whether a more pronounced downregulation of CREB would be required to block the effects of L-DOPA. CREB antisense was continuously infused into the DA-denervated striatum for 14 d, during which time the rats received either acute (on day 14) or chronic treatment (on days 2–14) with L-DOPA (8 mg/kg per injection). Continuous CREB antisense infusion neither blocked nor attenuated the effects of acute or chronic L-DOPA treatment on striatal gene expression (Table 2, Fig. 3*A–F*).

Behavioral testing showed that continuous CREB antisense infusion exacerbated the dyskinetic effects of L-DOPA (Fig. 4). After intrastriatal CREB knockdown, acute L-DOPA injections

cocaine was unable to induce the same gene products in the intact striatum (*G, I, K*, antisense group; *H, J, L*, control group). Photomicrographs were taken under bright-field (*A–D, G–J*) or dark-field illumination (*E, F, K, L*) from animals that sustained intrastriatal oligonucleotide infusion and acute drug injections. Asterisks mark the cannula track. Scale bar, 100 μm.

Figure 5. Cocaine-induced expression of c-Fos, FosB/ΔFosB, and PDyn mRNA is dependent on CREB. Acute treatment with cocaine produced a dose-dependent increase in the number of c-Fos (*A*) and FosB/ΔFosB immunoreactive cells (*B*), and in the levels of PDyn mRNA (*C*), as measured on the cross-sectional area of the caudate–putamen at mid-rostrorocaudal levels. Knockdown of CREB prevented the cocaine-stimulated increase in c-Fos (*D*), FosB/ΔFosB (*E*), and PDyn mRNA (*F*). CREB antisense-treated animals are marked as AS + and represented using bars with diagonal stripes, whereas animals receiving control oligonucleotides are marked as AS – and represented using bars with no texture. The data presented here are derived from two sets of experiments. Animals injected with 15 mg/kg cocaine received a single intrastriatal injection of CREB AS, whereas animals injected with 30 mg/kg cocaine or saline received a 14 d intrastratial oligonucleotide infusion. * $p < 0.05$ versus saline; § $p < 0.01$ versus 15 mg/kg cocaine; # $p < 0.05$ versus control oligo; $n \geq 5$ in each group; values give means + SEM.

produced severe AIMs of the trunk, limb, and orofacial region, i.e., body parts that are somatotopically related to the lateral striatal subregion targeted by the antisense ($p = 0.003$ in AS vs Ctrl group) (Fig. 4*A*). A trend toward more severe dyskinesias after CREB AS infusion was also seen when L-DOPA was given chronically on days 2–14 after pump implantation (but $p = 0.112$) (Fig. 4*B*). Thus, in contrast with our initial hypothesis and that of others (Calon et al., 2000), these data demonstrate that CREB is not required for the development of dyskinesia and that its knockdown causes greater sensitivity to the dyskinesigenic effects of L-DOPA.

CREB is essential for both basal and cocaine-induced c-fos, fosB, and prodynorphin gene expression in the intact striatum

The failure of CREB antisense to block gene expression changes produced by L-DOPA was not compatible with the proposed critical role of CREB in DA-dependent nuclear signaling. This prompted us to examine the requirement for CREB as a mediator of DA-dependent gene expression in the intact striatum using our knockdown paradigm. Previous studies had shown that CREB antisense could block amphetamine-induced striatal c-Fos expression in normal rats (Konradi et al., 1996). However, no data were available on the role of CREB in PDyn and fosB gene induction by DA agonists in the striatum *in vivo*.

A single, intrastriatal injection of CREB AS oligonucleotides

attenuated the inductive effect of 15 mg/kg cocaine on c-Fos and FosB/ΔFosB immunoreactivity (Fig. 5*A,B,D,E*) and caused a ~40% reduction in PDyn mRNA levels within the targeted striatal subregion (Fig. 5*F*). The latter effect represents a down-regulation of basal PDyn gene expression, because 15 mg/kg cocaine did not have a significant inductive effect on the PDyn transcript [Fig. 5*C* (compare black and shaded bars)]. We thus tested a higher dose of cocaine (30 mg/kg, i.p.), which produced a marked induction of all the genes under investigation (Fig. 5*A–C*, white bars). In this experiment, CREB AS oligonucleotides were delivered chronically into the striatum for 14 d using osmotic minipumps. c-Fos, FosB/ΔFosB, and PDyn mRNA induction by 30 mg/kg cocaine was completely blocked by CREB AS infusion (Fig. 5*D–F*; photomicrographs are shown in Fig. 3*G–L*). An analysis of animals injected with saline instead of cocaine on the 14th day of antisense infusion confirmed that CREB was required to maintain basal expression of PDyn mRNA in the striatum (Fig. 5*F*, shaded bars).

Behavioral testing showed that CREB AS infusion significantly enhanced the activity/stereotypy scores recorded from control animals injected with saline (Fig. 6*A*) ($p = 0.020$ for AS vs Ctrl). On single monitoring periods, CREB AS rats reached scores of 2, whereas the highest scores recorded from control rats never exceeded 1 [note that scores of 1–2 in the Creese and Iversen (1973) scale indicate motor activation rather than stereotypy; see

Figure 6. Loss of CREB increases activity/stereotypy scores in intact rats challenged with an injection of saline (*A*) but not cocaine (*B*). CREB AS was infused continuously for 14 d before the acute challenge. The rats were neurologically intact. Each dot in the scattergram represents one single animal; the group median is shown as a horizontal bar. $^{\#}p < 0.01$.

Materials and Methods]. CREB AS, however, did not produce a significant effect on cocaine-induced stereotypies (Fig. 6*B*) ($p = 0.243$; maximal stereotypy scores per monitoring period amounted to 4–5 in CREB AS rats and 3–4 in the controls).

DNA binding activity in the DA-denervated striatum: effects of L-DOPA treatment

In agreement with previous studies (Cole et al., 1995; Carlezon et al., 1998), our antisense data showed that the PDyn gene is an important target of CREB in the intact striatum, but indicated that transcription factors other than CREB regulate PDyn expression in the parkinsonian striatum treated with L-DOPA. We were therefore interested in studying striatal DNA binding activity at two enhancer elements from the PDyn promoter: the noncanonical dynCRE3 element (Collins-Hicok et al., 1994; Douglass et al., 1994; Messersmith et al., 1994; Cole et al., 1995) and the noncanonical dynAP-1 (Naranjo et al., 1991). Both enhancer elements have been shown to mediate induction of PDyn in different cell culture systems. We compared binding at these two elements with binding at a consensus CRE (Konradi et al., 1993) and at a canonical AP-1 element (Vallone et al., 1997).

The specificity of DNA binding activity at dynCRE3 and dynAP-1 was verified by cold competition with nonradioactively labeled consensus CRE or canonical AP-1 oligonucleotides, using protein extracts prepared from both the DA-denervated and the intact striatum of L-DOPA-injected animals. The canonical AP-1 and consensus CRE oligonucleotides competed with both PDyn-enhancer elements in a dose-related manner (Fig. 7*A,B*). By contrast, an unrelated AP-4 oligonucleotide did not compete for but rather enhanced the specific dynCRE3 and dynAP-1 binding (Fig. 7).

In line with previous studies (Hope et al., 1994; Doucet et al., 1996; Kashiwara et al., 1996), DA denervation alone produced a slight induction of DNA binding to the consensus AP-1 on the lesioned side (Fig. 8*A*). In addition, we found a small increase of binding to the dynCRE3 (Fig. 8*C*, compare lanes 1, 2).

Both acute and chronic L-DOPA treatment produced a further increase in binding of striatal proteins from the lesioned side to all the promoter elements tested, particularly to the dynCRE3 and the canonical AP-1 (Fig. 8*A,C*, compare L-DOPA, lanes 3

Figure 7. Gel mobility shift assay and competition experiment determine the specificity of DNA–protein complexes formed with dynCRE3 (*A*) and dynAP-1 (*B*). Unlabeled consensus AP-1, CRE, and the unrelated AP-4 sequences were used to compete for proteins bound to radiolabeled dynCRE3 (*A*) and dynAP1 (*B*) promoter elements. Striatal samples were pooled from several L-DOPA-treated rats. The experiment was repeated three times.

and 5, and saline, lane 1). The increase in DNA binding activity induced by chronic L-DOPA treatment was associated with the development of abnormal involuntary movements during the drug treatment period (Fig. 8*B,D*). Animals that did not develop dyskinesias showed levels of DNA binding activities comparable to those found in saline-injected (lesion-only) controls (Fig. 8*A,C*, compare nondyskinetic rat in lane 7 and dyskinetic rat in lane 5).

In addition, both acute and chronic L-DOPA treatment induced striatal DNA binding activity to the dynAP-1 (Fig. 8*E*) and to the consensus CRE probe (Fig. 8*F*).

FosB-related proteins account for striatal CRE- and AP-1-binding activity in DA-denervated animals treated with L-DOPA

Striatal protein extracts from DA-denervated and chronically L-DOPA-treated animals were incubated with antibodies against CREB or FosB/ΔFosB-related antigens (before the addition of a radioactive oligonucleotide probe) to determine which of these proteins interacted with the enhancer elements under investigation. The FosB/ΔFosB antibody was particularly interesting to test, because of previous findings in our lab demonstrating that

A canonical AP-1 **B canonical AP-1** **C dynCRE3** **D dynCRE3** **E dynAP-1** **F consensus CRE**

Figure 8. Acute and chronic treatment with L-DOPA induces striatal DNA binding activity to the canonical AP-1 (*A*), the dynCRE3 (*C*), the dynAP-1 (*E*), and the consensus CRE (*F*). Optical densities of specific bands are expressed as the fold induction on the lesioned over the intact side. * $p < 0.05$ versus saline group; § $p < 0.05$ versus intact side; values give means $\pm$ SEM; $n \geq 5$ in all groups but chronic *n-dys* (i.e., chronically L-DOPA-treated but nondyskinetic case). As shown in *B* and *D*, only one animal remained nondyskinetic (0 cumulative dyskinesia scores) during chronic L-DOPA treatment. In *B* and *D*, levels of binding to the canonical AP-1 (*B*) and dynCRE3 (*D*) in the chronic L-DOPA cases are plotted on the cumulative axial, limb, and orolingual AIM scores recorded from the same animals. The probability value (p) and correlation coefficient (R) of the corresponding simple regression equation are given in the bottom right corner. L, 6-OHDA-lesioned side; I, contralateral intact side.

Figure 9. FosB/ΔFosB-related proteins are the predominant transcription factors bound to CRE and AP-1 enhancers in L-DOPA-treated rats. To study the contribution of CREB- or FosB/ΔFosB-related proteins to the DNA binding activity induced by L-DOPA (A, C, E, G) or cocaine (B, D, F, H), supershift assays were performed using antisera against CREB or FosB/ΔFosB. Antibodies were preincubated with striatal protein extracts before addition of the radioactively labeled promoter elements dynAP-1 (A, B), canonical AP-1 (C, D), dynCRE3 (E, F), and consensus CRE (G, H). The experiments were repeated at least three times with samples from different rats. In the *left-hand pictures*, s1 and s2 are protein extracts from intact striata of two chronically L-DOPA-treated rats, whereas s1' and s2' are extracts from the corresponding 6-OHDA-lesioned side. Rats treated with acute L-DOPA showed comparable results. In the *right-hand pictures*, s, a, and c are protein extracts from animals treated with saline, acute cocaine, or chronic cocaine, respectively.

intrastratial infusion of *fosB* antisense prevents PDyn gene induction by L-DOPA (Andersson et al., 1999). In the DA-denervated and L-DOPA-treated striatum, FosB/ΔFosB-related antigens were the main contributors to binding activity at each of

the four enhancer elements under investigation (Fig. 9A,C,E,G). The CREB antiserum had no effect on the binding activity to the dynAP-1 and canonical AP-1 probes (Fig. 9A,C) and reduced the specific dynCRE3- and consensus CRE-binding to a much lesser

Figure 10. Like FosB/ΔFosB-related proteins, JunD can bind at AP-1 and CRE enhancers in 6-OHDA-lesioned, L-DOPA-treated rats. To characterize possible binding partners to FosB/ΔFosB proteins in the L-DOPA-induced dynCRE3 complex, supershift assays were performed with JunD or JunB antisera. *s1* and *s2* are samples from the lesioned striata of two L-DOPA-treated rats. This experiment was repeated with samples from additional animals, and with the dynAP-1 probe, it obtained similar results.

extent than the FosB antiserum did (Fig. 9*E,G, lesion*). This was in contrast to cocaine-treated animals, in which CREB was the major protein binding at the enhancer elements under investigation (Fig. 9*D,F,H*) (see results below).

Supershift analyses from acutely L-DOPA-treated rats produced identical results, i.e., FosB/ΔFosB-related proteins were the main contributors to AP-1 and CRE binding in the DA-denervated striatum (data not shown). Because acute L-DOPA treatment induces c-Fos in addition to FosB, an antibody against c-Fos was also tested in supershift analysis. A small supershift at the canonical AP-1 oligonucleotide could be detected after acute L-DOPA treatment; however, c-Fos proteins did not contribute to the DNA binding activity at the other enhancer elements (data not shown).

Putative dimerization partners of FosB-related transcription factors are JunB and JunD proteins (Chen et al., 1995; Vallone et al., 1997). We thus performed a supershift analysis using JunB and JunD antibodies, alone or in combination with the FosB/ΔFosB antiserum. Specific dynCRE3-containing complexes were displaced to some extent by the JunD antibody alone, and to an even larger extent by the JunD and FosB antibodies in combination (Fig. 10). In contrast, the JunB antibody did not displace any of the protein–DNA complexes, and the supershift produced by a combination of JunB and FosB/ΔFosB antibodies was entirely accounted for by the latter (Fig. 10). This experiment was repeated with the dynAP-1 enhancer element with similar results.

DNA binding activity in the intact striatum after treatment with cocaine

In agreement with previous findings (Hope et al., 1994), both acute and chronic cocaine treatment induced an approximately twofold increase in striatal protein binding to the canonical AP-1 probe (Table 3, Fig. 9*D, autoradiographs*). DNA binding activity to the dynCRE3 enhancer was induced, albeit slightly, only after acute cocaine (Table 3, Fig. 9*F*), whereas no induction was found at the consensus CRE element (Table 3, Fig. 9*H*). In agreement with previous findings (Cole et al., 1995), striatal protein binding to the dynAP-1 promoter element could not be detected (Fig. 9*B*).

The CREB antiserum supershifted the specific bands not only from the dynCRE3 and consensus CRE (Fig. 9*F,H*) but also from the canonical AP-1 enhancer element (Fig. 9*D*). The FosB/ΔFosB antibody produced only a minor displacement of the specific

AP-1 bands, which was much less pronounced than that seen in the 6-OHDA-lesioned and L-DOPA treated striatum (Fig. 9, compare *C, D*). A c-Fos antiserum was found to supershift small amounts of the AP-1 complex induced by acute cocaine treatment (data not shown).

FosB/ΔFosB proteins are expressed at a higher level in the L-DOPA-treated striatum

The data presented so far revealed dramatic differences between L-DOPA- and cocaine-induced striatal DNA binding activity, in terms of which elements within the PDyn promoter were activated and which proteins were bound to them. These data raised the question of whether the difference between the two drug treatment paradigms could be attributed to a differential induction of FosB/ΔFosB-related proteins on a quantitative or qualitative level. Striatal protein extracts therefore were analyzed by Western immunoblotting using two different FosB/ΔFosB antisera (see Materials and Methods). Both antisera showed striatal induction of FosB/ΔFosB-immunoreactive bands with the same molecular weight (i.e., 33 and 35–37 kDa) after treatment with cocaine and L-DOPA (Fig. 11*A*). However, the levels of induction were significantly higher in the L-DOPA-treated animals (Fig. 11*B*). These results were confirmed using an antiserum that recognizes all Fos family proteins (Fig. 11*C*) and were consistent with the results obtained in the quantitative immunohistochemical analysis (compare FosB-positive cell numbers after treatment with 8 mg/kg L-DOPA or 30 mg/kg cocaine in Figs. 2*B* and 5*B*).

DISCUSSION

Current knowledge of DA agonist-induced signaling pathways is mainly derived from studies performed in neurologically intact animals and cultured neurons. However, conclusions from these model systems may not be applicable to the DA-denervated striatum. Indeed, DA denervation causes profound changes in the expression, functional coupling, and state of phosphorylation of striatal DA and glutamate receptors (Narang and Wamsley, 1990; LaHoste and Marshall, 1992; Dunah et al., 2000; and see references therein), all of which are critical for DA-dependent nuclear signaling (Graybiel et al., 1990; Konradi et al., 1996).

In this study, we have used an antisense approach to verify the requirement of CREB for striatal PDyn and fos family gene induction, as produced by L-DOPA in DA-denervated rats and cocaine in neurologically intact animals. Knowing that the response to DA agonists is supersensitive in parkinsonian subjects (see below), L-DOPA was titrated to just above the threshold dose for induction of the genes under investigation. The doses of L-DOPA tested here (3–8 mg/kg) are in the range used for the treatment of Parkinson's disease. Cocaine was tested at 15 mg/kg, a typical bolus dose in "binge" cocaine studies in rats (Quinones-Jenab et al., 2000; Rosin et al., 2000), and at 30 mg/kg, a dose that is among the highest used in the literature (Steiner and Gerfen, 1993) and approximates half the LD₅₀ for cocaine in rats (Derlet et al., 1990).

In the first part of the study we used a single-injection method for intrastriatal CREB antisense delivery. Because single antisense injections may not be able to block gene expression changes by strong inducing stimuli (Konradi et al., 1994), we also examined the effects of continuous antisense infusion for 14 d. The two methods of antisense delivery produced identical results: CREB knockdown reduced basal and cocaine-induced PDyn, c-fos, and fosB gene expression in the intact caudate–putamen but had no

Table 3. Cocaine-induced DNA binding activity

Treatment	Canonical AP-1	dynCRE3	Consensus CRE
Saline	1.00 ± 0.04	1.00 ± 0.03	1.00 ± 0.04
Acute cocaine	1.97 ± 0.21*	1.42 ± 0.11*	0.96 ± 0.04
Chronic cocaine	1.70 ± 0.20*	1.25 ± 0.10	0.94 ± 0.04

Cocaine induces DNA binding activity to the canonical AP-1 and dynCRE promoter element, but not to the consensus CRE or the dynAP-1 probe (data not shown). The data are expressed as fold induction relative to saline-treated controls. This experiment was repeated twice with samples from different animals; * $p < 0.05$ versus saline group; $n = 5$ in each group.

effect on the gene expression changes produced by L-DOPA in the 6-OHDA-lesioned striatum.

A tight coupling between levels of CREB activity and PDyn gene expression in intact striatal neurons has been demonstrated previously with herpes virus-mediated gene transfer of native or mutated CREB in the nucleus accumbens (Carlezon et al., 1998). Here we demonstrate that in the sensorimotor striatum CREB is indeed required for basal PDyn expression as well as for the induction of PDyn by cocaine. In contrast, in the DA-denervated striatum, transcription factors other than CREB, such as Δ FosB-like proteins and JunD, are paramount for the regulation of PDyn.

In the rat Parkinson model, upregulation of striatal PDyn mRNA by L-DOPA is tightly associated with the development of dyskinesia (Cenci et al., 1998). Accordingly, the present study shows a close correlation between AIM scores induced by chronic L-DOPA treatment and striatal DNA binding activity at AP-1 and CRE-like sites in the PDyn promoter (note, however, that an increase in DNA binding activity was also detected in the acute L-DOPA cases, which did not exhibit significant dyskinesia). The importance of PDyn upregulation as a determinant of AIM development is further supported by the anti-dyskinetic effect of treatments that block or prevent striatal PDyn gene induction during chronic L-DOPA treatment (Andersson et al., 1999; Henry et al., 1999; Lee et al., 2000) (and our unpublished data). It is therefore not surprising that striatal knockdown of CREB, which did not block PDyn gene induction by L-DOPA, was ineffective in reducing dyskinesia. On the contrary, CREB knockdown caused an even greater sensitivity to the dyskinesia-inducing effect of L-DOPA in 6-OHDA-lesioned rats. Hyperkinetic effects of CREB knockdown occurred also in intact animals, which showed increased motor activation after a stressful stimulus (saline injection). Because this response occurred in the absence of any drug treatment, it is most likely caused by altered regulation of striatal genes that depend on CREB for their basal expression. Indeed, CREB has been shown to maintain constitutive expression of several genes in various tissues, including brain (for review, see Herdegen and Leah, 1998), and basal levels of CREB phosphorylation are found in the striatum (Sgambato et al., 1998). In intact animals injected with cocaine, CREB knockdown did not significantly augment the stereotypic behavior induced by the treatment. This lack of effect may depend on two factors. First, in the cocaine paradigm, CREB knockdown produces two parallel phenomena that are likely to affect motor behavior in opposite directions, namely, a dysregulation of constitutively expressed, CREB-dependent genes, and a blockade of cocaine-induced gene expression. Second, the motor response to cocaine in intact rats involves DA-receptor stimulation in the striata of both sides. Indeed, unilateral injections of DA agonists in the lateral CPU

have been shown to produce stereotypies half as intense as those elicited by bilateral injections (Dickson et al., 1994). Thus, bilateral knockdown of CREB may be required to fully uncover its role in cocaine-induced stereotypies.

DA-dependent expression of c-Fos and PDyn has been shown to require activation of CRE- and AP-1-like enhancers in the corresponding promoters (Naranjo et al., 1991; Konradi et al., 1994; Cole et al., 1995). We therefore performed a study of DNA-protein interactions at consensus CRE and AP-1 sites in

A FosB/ Δ FosB

B FosB/ Δ FosB levels

C PanFos

Figure 11. L-DOPA induces FosB/ Δ FosB proteins to a higher level than cocaine does. Immunoblots with an antibody to FosB/ Δ FosB are shown in *A*, and the corresponding densitometric analysis is shown in *B*. All the immunoblots used for analysis were processed simultaneously. A representative immunoblot stained with a *PanFos* antibody is shown in *C*. Arrows indicate the expected molecular weights of c-Fos (55 kDa), Δ FosB (33 kDa), and the chronic FRAs (35–37 kDa). * $p < 0.001$ versus saline, acute cocaine, and chronic cocaine group. # $p < 0.05$ versus saline controls in the cocaine paradigm. # $p < 0.01$ versus saline controls in the L-DOPA paradigm; $n \geq 5$; values give means + SEM.

the two drug treatment paradigms under investigation. Because DNA binding affinity depends not only on the DNA binding site per se but also on the adjacent bases (Ryseck and Bravo, 1991), we also used DNA probes reproducing an AP-1-like site and a CRE-like element from the PDyn promoter. Both enhancers have been found to mediate transactivation of the PDyn gene in cell cultures (Naranjo et al., 1991; Messersmith et al., 1994). Both L-DOPA and cocaine were found to increase AP-1 binding to the canonical AP-1 site, whereas increased binding to the consensus CRE was detected only in L-DOPA-treated rats. At the PDyn promoter, both cocaine and L-DOPA induced protein binding to the dynCRE3 element, although this increase was much larger in the 6-OHDA/L-DOPA model. Moreover, only L-DOPA induced any binding to the dynAP-1. An analysis of the protein composition of the induced protein–DNA complexes provides an explanation for the observed differences between drug treatment paradigms. In intact rats treated with cocaine, striatal DNA binding activity was mainly accounted for by CREB, which is constitutively expressed and is thought to bind to the DNA regardless of its phosphorylation state (for review, see Shaywitz and Greenberg, 1999). In 6-OHDA-lesioned animals treated with L-DOPA, Δ FosB-related proteins, in combination with JunD, were the main contributors to striatal AP-1- and CRE-binding activity and could target both CRE- and AP-1-like sites in the PDyn promoter.

AP-1 complexes containing FosB and JunD have been shown to bind consensus CREs *in vitro* (Ryseck and Bravo, 1991). Our data show that such a phenomenon can occur *in vivo* in a specific pathophysiological condition, i.e., the parkinsonian striatum treated with a therapeutic dose of L-DOPA. As shown by Western blot analysis, striatal expression of Δ FosB (~33 kDa) and its derivatives, the so-called chronic FRAs (~35–37 kDa) (Hope et al., 1994), was induced to a much larger extent by L-DOPA in DA-denervated rats than by cocaine in intact animals. These high levels of expression, which reflect a condition of DA-receptor supersensitivity at the gene induction level (for review, see Gerfen, 2000), seem to enable Δ FosB-like proteins to compete for and regulate consensus CREs, in addition to AP-1 sites. Our data show that JunD can also bind to these sites, presumably after dimerization with the induced Δ FosB-like proteins (Vallone et al., 1997; Pérez-Otaño et al., 1998).

We propose that, in the parkinsonian striatum treated with L-DOPA, dimers of Δ FosB-like proteins and JunD can bypass CREB in the transactivation of PDyn and fos family genes. Indeed, intra-striatal knockdown of fosB (Andersson et al., 1999) but not CREB (present study) blocks the striatal induction of PDyn by L-DOPA. A switch from CREB- to Δ FosB/chronic FRAs-driven gene activation may explain the magnitude and peculiar dynamics of the supersensitive gene induction response, which is seen in the DA-denervated striatum after treatment with DA agonists. Indeed, Δ FosB–JunD dimers have a much longer half-life than phosphorylated CREB (for review, see Herdegen and Leah, 1998) and persist at very high levels both during and after long-term treatment with L-DOPA (Westin et al., 2001) (and our unpublished data). Accordingly, DA-agonist treatment to 6-OHDA-lesioned rats has been shown to produce a persistent striatal induction of both PDyn and early response genes (Steiner and Gerfen, 1996; Westin et al., 2001). This persistent induction reflects the breakdown of homeostatic mechanisms that would normally limit the gene expression changes produced by repeated DA-agonist administration, as determined by studies performed

in intact animals (Steiner and Gerfen, 1993; for review, see Gerfen, 2000).

In conclusion, by combining *in vivo* gene induction assays and antisense technology, this study demonstrates that CREB is essential for the expression of PDyn and fos family genes in intact but not DA-denervated striatal neurons. In the latter, L-DOPA-induced Δ FosB-like proteins, in combination with JunD, can compete with CREB for binding to consensus CRE sites. Thus, these data uncover a novel and unexpected alteration of transcriptional regulatory mechanisms that occurs in the supersensitive parkinsonian striatum treated with L-DOPA.

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