Time course of striatal DeltaFosB-like immunoreactivity and prodynorphin mRNA levels after discontinuation of chronic dopaminomimetic treatment.

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SHORT COMMUNICATION
Time course of striatal ΔFosB-like immunoreactivity and prodynorphin mRNA levels after discontinuation of chronic dopaminomimetic treatment

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Abstract

ΔFosB-like proteins are particularly stable transcription factors that accumulate in the brain in response to chronic perturbations. In this study we have compared the time-course of striatal FosB/ΔFosB-like immunoreactivity and prodynorphin mRNA expression after discontinuation of chronic cocaine treatment to intact rats and chronic L-DOPA treatment to unilaterally 6-hydroxydopamine (6-OHDA) lesioned rats. The animals were killed between 3 h and 16 days after the last drug injection. In both treatment paradigms, the drug-induced FosB/ΔFosB immunoreactivity remained significantly elevated in the caudate putamen even at the longest withdrawal period examined. The concomitant upregulation of prodynorphin mRNA, a target of ΔFosB, paralleled the time-course of ΔFosB-like immunoreactivity in the 6-OHDA-lesion/L-DOPA model, but was more transient in animals treated with cocaine. These results suggest that ΔFosB-like proteins have exceptional *in vivo* stability. In the dopamine-denervated striatum, these proteins may exert sustained effects on the expression of their target genes long after discontinuation of L-DOPA pharmacotherapy.

Introduction

ΔFosB encodes for stable transcription factors of 33, 35 and 37 kDa (Chen *et al.*, 1997), which are induced in a region-specific manner in the brain as a response to various chronic perturbations (Hope *et al.*, 1994; Doucet *et al.*, 1996; Hiroi *et al.*, 1997). In a rat model of Parkinson's disease, ΔFosB-like immunoreactivity provides a cellular marker to map neuronal systems that become activated by chronic, dyskinesiogenic treatment with L-DOPA (Andersson *et al.*, 1999). Preliminary data suggest that such a marker can be used also on human post-mortem brain tissue (Cenci *et al.*, 2002). The study of gene or protein expression in human post-mortem material can, however, be hampered by a large variability in some crucial parameters. One such parameter is the time intervening between the last drug exposure and the collection of the tissue. Animal studies documenting the decay dynamics of ΔFosB-like proteins are therefore warranted.

In the present study, we have examined the levels of striatal FosB/ΔFosB-like immunoreactivity following a chronic course of treatment with cocaine in intact rats, and L-DOPA in 6-OHDA lesioned rats. Cocaine-treated animals were killed at 3 h, 2 or 8 days after the last drug injection. L-DOPA-treated animals were killed at slightly longer post-injection intervals (i.e. 2, 8 or 16 days), as a comparison between 3 h and 2 days of L-DOPA withdrawal has already been carried out in this experimental paradigm (Cenci *et al.*, 1999; see also Fig. 4). In addition to FosB/ΔFosB immunoreactivity, we have studied the levels of prodynorphin (PDyn) mRNA, which is co-induced with ΔFosB-like proteins in striatal neurons of the 'direct pathway' upon chronic dopamine (DA)-agonist treatment (Andersson *et al.*, 1999; Graybiel *et al.*, 2000; Westin *et al.*, 2001).

Materials and methods

This study was conducted on female Sprague–Dawley rats (BK Universal, Sweden; ≈225 g) in order to conform to the standard procedures used in our previous studies. The animals were housed under a 12-h light/dark cycle with free access to food and water. The study comprised 28 intact rats and 29 rats with unilateral 6-OHDA lesions. Unilateral DA-denervating lesions were carried out ≈4 months before the onset of L-DOPA treatment by 6-OHDA injection in the ascending nigrostriatal bundle, as described by Cenci *et al.* (1998). Briefly, 6-OHDA was dissolved in 0.02% ascorbate/saline at the concentration of 3 μg/μl, and was injected in two deposits of 6 and 7.5 μg, respectively. Surgery was performed on rats anesthetized with a mixture of Hypnorm (Janssen Pharmaceuticals) and Dormicum (Hoffman-La Roche). Analgesic treatment (Temgesic, Apoteksbolaget AB) was given ∼20 mins before the rats woke up. The extent of DA denervation was verified using an amphetamine-induced rotation test (2.5 mg/kg D-amphetamine i.p., 90 min testing). Only rats showing >5 full turns/min ipsilateral to the lesion were selected for the study (Winkler *et al.*, 2002).

Twenty-three DA-denervated rats received a 9-day treatment with L-DOPA methyl ester (10 mg/kg/day) mixed with the peripheral DOPA-decarboxylase inhibitor, benserazide-hydrochloride (15 mg/kg/day), according to Cenci *et al.* (1999). The two drugs (purchased from Sigma–Aldrich, Sweden) were dissolved in saline and administered by single daily i.p. injections. Rats were allocated to three groups, to be killed at either 2, 8, or 16 days after the last injection ($n = 6$–9 per time point). The groups were matched with respect to the animals'

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dyskinesia scores. Two L-DOPA-treated rats that had showed no dyskinesia in response to L-DOPA were excluded from the study (Cenci *et al.*, 1999). An additional six rats with 6-OHDA lesions were given daily injections of physiological saline for 9 days and killed at 2, 8, or 16 days after the last injection ($n = 2$ per time point).

Twenty-one intact rats were injected i.p. with 30 mg/kg cocaine/HCl (Apoteksbolaget, Sweden) twice daily (at 10.00 and 17.00 h) for 3 days, followed by an additional injection in the morning of day 4. This treatment regimen was chosen because it induced higher striatal levels of FosB/ΔFosB immunoreactivity and PDyn mRNA than did single daily injections of the same drug dose for up to 12 days (data not shown). The rats in this experiment were allocated to three groups, which were matched with respect to the degree of cocaine-induced stereotypic movements. The rats were killed at 3 h, 2 days or 8 days after the last injection ($n = 6$–8 per time point). An additional seven intact rats were injected with saline according to the same administration regimen and killed at 3 h, 2 days or 8 days after the last injection ($n = 2$ or 3 per time point).

All animals underwent behavioral testing at least twice during the drug treatment period. A rat dyskinesia rating scale was used in the animals treated with L-DOPA (Cenci *et al.*, 1998); while a motor activity and stereotypy scale was used in the cocaine-treated rats (Creese & Iversen, 1973). Rats were deeply anesthetized with sodium pentobarbitone (240 mg/kg, i.p., Apoteksbolaget) and killed by decapitation. Brains were rapidly removed and frozen on dry ice. Sections through the striatum (16 μm thick) were cut using a cryostat, thaw-mounted onto microscope slides (SuperFrost Plus; Menzel Glazer, Germany), and stored at −20 °C. Immunohistochemistry was performed using a standard peroxidase-based method (Vectastain Elite ABC Kit, Vector Laboratories Inc., Burlingame, CA, USA) as described by Andersson *et al.* (1999). The primary antiserum was purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA) and used at a dilution of 1 : 15000. This antibody was raised against an N-terminal peptide that is common to both full-length FosB and ΔFosB. We have previously shown that chronic treatment with cocaine or L-DOPA at the regimens used in this study induces FosB isoforms with a molecular mass of 32–37 kDa (Cenci *et al.*, 1999; Andersson *et al.*, 2001), i.e. the reported molecular weights of ΔFosB-like proteins, but not full-length FosB (Chen *et al.*, 1997).

In situ hybridization histochemistry was performed using 48-mer oligonucleotides complementary to PDyn mRNA, which had been labelled at the 3′ end with [α-^{35}S]dATP, as described previously (Cenci *et al.*, 1998; Andersson *et al.*, 1999).

Quantitative analysis of the markers under investigation was carried out by a blinded investigator separately in the medial and the lateral half of the CPu at mid-rostrocaudal levels (i.e. 0.2–1 mm rostral to bregma; Paxinos & Watson, 1997). A quantification of FosB/ΔFosB immunostaining was carried out using a cell count program that also yields information on the optical density (OD) of each counted object (NIH IMAGE 1.61). Two areas (0.54 mm^2 on the section) were sampled in each the medial and the lateral half of the CPu in two sections per animal. In the 6-OHDA-lesioned group, measurements were carried out only on the DA-denervated side; as previously reported, no group differences are found on the side of the striatum contralateral to the lesion with this regimen of L-DOPA administration (Cenci *et al.*, 1999). The amount of staining per neuron was expressed as noncalibrated OD units using the formula: area of the neuron in pixel × average OD/pixel. In order to measure PDyn mRNA, the hybridized sections were exposed to Fuji imaging plates (Fujifilm, Sweden) for 12 h. Plates were scanned in a BAS-5000 phosphorimager (Fujifilm) to obtain digitized autoradiographs. The photo-stimulated luminescence emitted by the hybridized sections was calibrated against radioactivity levels (kBq/g) using simultaneously exposed ^{14}C standards (Amersham Biosciences Ltd, UK). The hybridization signal was analyzed on two sections per animal using the program TINA (Fujifilm).

Statistical comparisons were performed using one-factor analysis of variance (ANOVA) and posthoc Newman–Keul's test. The null hypothesis was rejected when $P < 0.05$. In each of the two experimental paradigms, control animals that had been treated with saline were pooled together in one group irrespective of their survival time.

Results

After 2 days of withdrawal from chronic L-DOPA treatment, the number of FosB/ΔFosB-immunoreactive neurons was elevated above control levels by about 200% in the medial part and 800% in the lateral part of the DA-denervated CPu (Fig. 1A and B; $P < 0.05$ vs lesion-only controls in both comparisons; compare Fig. 3A and D). The amount of staining per neuron had increased by 124% and 57% in the medial and the lateral part of the CPu, respectively ($P < 0.05$ vs controls; Fig. 1C–D). At longer withdrawal periods (8 and 16 days) the levels of ΔFosB-like immunoreactivity were overall reduced compared to the 2-day time-point, but remained elevated above control levels (photomicrographs are shown in Fig. 3A–D). After 16 days of L-DOPA withdrawal, the number of FosB/ΔFosB-positive neurons was elevated above control levels by 120% in the medial CPu (Fig. 1A; $P < 0.05$ vs controls; $P > 0.05$ vs the 2-day interval), and by 270% in the lateral CPu (Figs 1B and 3C; $P < 0.05$ vs both the controls and the 2-day group, and Fig. 3C). At the same survival period, the average OD/cell was increased by 88% above controls in the medial CPu (Fig. 1C; $P < 0.05$ vs both controls and 2-day group), but had returned to basal (unstimulated) levels in the lateral CPu (Fig. 1D; $P < 0.05$ vs 2 days survival).

The temporal and spatial expression pattern of PDyn mRNA closely mimicked that of ΔFosB-like immunoreactivity (Fig. 1E and F, and photomicrographs in Fig. 3E–H). Two days after the last injection of L-DOPA, PDyn mRNA levels were significantly upregulated in both the medial and the lateral part of the DA-denervated CPu (Fig. 1E and F), but this effect was much more pronounced in the latter region (≈ 450% increase above controls, $P < 0.05$; Figs 1F and 3E). The upregulation of PDyn mRNA induced by L-DOPA showed a decline at longer survival periods, although it remained clearly detectable for 16 days of drug withdrawal (compare Fig. 3G vs H). At this survival period, PDyn mRNA levels were increased by ≈100% above control values in both the medial and the lateral CPu (Fig. 1E and F; $P < 0.05$ for 16-day group vs controls in both regions; $P < 0.05$ for 16-day vs 2-day in lateral CPu).

Chronic cocaine treatment to intact rats had an overall weaker inductive effect on FosB/ΔFosB in the striatum than did L-DOPA treatment to DA-denervated rats (compare Fig. 1A and B vs Fig. 2A and B; see also Fig. 4). Levels of FosB/ΔFosB immunostaining were hardly detectable in the striatum of intact rats injected with saline (empty bars in Figs 2A and B, photomicrograph in Fig. 3L). Chronic cocaine treatment induced FosB/ΔFosB-immunoreactive neurons in both the medial and the lateral CPu, as seen at a survival period of 3 h after the last injection ($P < 0.05$ vs controls; Fig. 2A and B). The average staining intensity per neuron was also significantly elevated in both regions at the same survival period ($P < 0.05$ vs controls; Fig. 2C and D). In the medial CPu, the number of FosB/ΔFosB-positive neurons remained elevated by about 600% above control levels for up to 8 days of cocaine withdrawal (Fig. 2A; $P < 0.05$ vs both controls and 3-h survival group, photomicrograph is shown in Fig. 3K). The remaining FosB/ΔFosB-immunoreactive neurons were, however, as pale as the neurons in the control group (Fig. 2C; $P < 0.05$ vs 3-h survival, compare Fig. 3K and L). In the lateral CPu, the number of FosB/ΔFosB-immunoreactive neurons and their staining intensity had

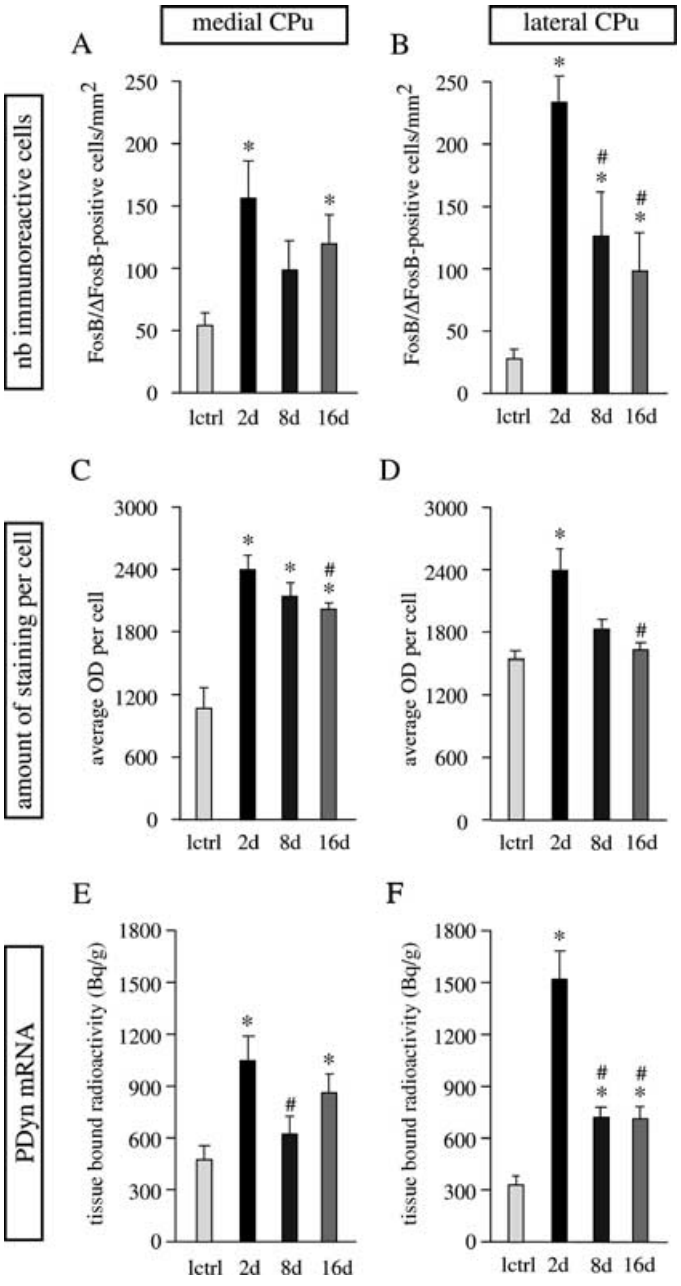

FIG. 1. Results from 6-OHDA-lesioned rats treated chronically with L-DOPA. The number of FosB/ΔFosB-like-immunoreactive cells (A-B), their average optical density (OD; C and D) and the levels of PDyn mRNA (E–F) were measured separately in the medial (A, C and E) and the lateral (B, D and F) half of the CPu on the side ipsilateral to the lesion. Values give mean ± SEM, $n = 6$–9 per group, $^{*}P < 0.05$ vs lesion-only saline-treated control (lctrl) group, $\#P < 0.05$ vs 2-day group.

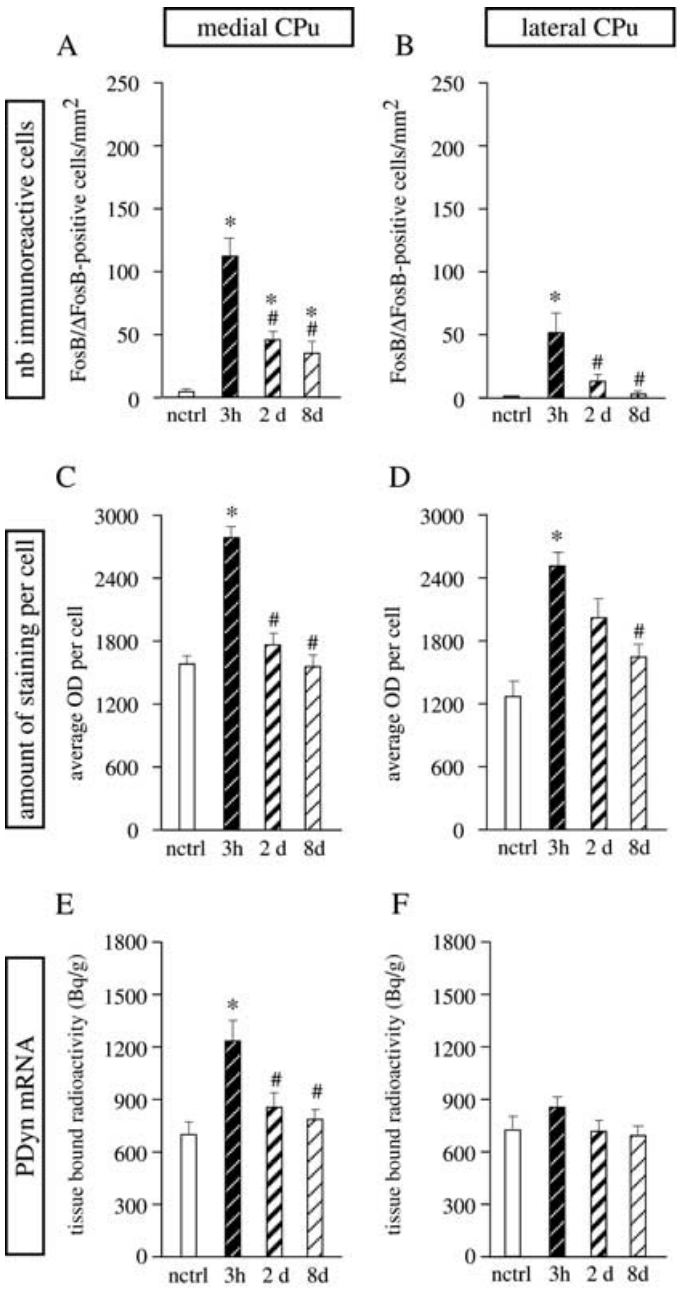

FIG. 2. Results from intact rats treated chronically with cocaine. The number of FosB/ΔFosB-like-immunoreactive cells (A and B), their average optical density (OD; C and D) and the levels of PDyn mRNA (E and F) were measured separately in the medial (A, C and E) and the lateral (B, D and F) half of the CPu. Values give mean ± SEM, $n = 6$–8 per group, $^{*}P < 0.05$ vs normal controls (nctrl), $\#P < 0.05$ vs 2-day group.

completely returned to baseline by 8 days after the last drug injection (Fig. 2B and D; $P < 0.05$ for 2 and 8 days vs 3-h survival).

In the medial part of the CPu, PDyn mRNA levels were upregulated by 85% at 3 h after the last administration of cocaine (Fig. 2E; $P < 0.05$ vs controls; compare Fig. 3M and P). In the same striatal region, PDyn gene expression was no longer different from baseline values at 2 and 8 days of cocaine withdrawal (Fig. 2E; $P < 0.05$ for 2 and 8 days vs 3-h survival; Fig. 3N and O). In the lateral CPu, chronic cocaine treatment did not induce a detectable up-regulation of PDyn mRNA (Fig. 2F; $P > 0.05$ vs controls).

Discussion

In this study, we have measured both the number of FosB/ΔFosB-immunoreactive cells in the striatum and their staining intensity at different withdrawal periods after chronic dopaminomimetic treatment. This quantitative analysis was carried out separately in the medial and the lateral part of the CPu because the rat striatum shows a functionally important medio-lateral topography (Andersson *et al.*, 1999), which is also reflected in various drug-specific patterns of gene induction (Moratalla *et al.*, 1996; Andersson *et al.*, 1999; Cenci *et al.*, 1999; Saka *et al.*, 1999).

In the DA-denervated CPu, the levels of FosB/ΔFosB-like immunoreactivity showed a slow decline after discontinuation of chronic L-DOPA treatment. However, a prominent upregulation of ΔFosB-like proteins persisted for up to 16 days of drug withdrawal. This persistence was seen as an elevation in the number of immunopositive neurons and/or in their average staining intensity relative to the values found in saline-injected, 6-OHDA lesioned controls. Cocaine treatment to intact animals produced a weaker increase in striatal FosB/ΔFosB immunoreactivity that was overall more transient and short-lived than that seen in the DA-denervated CPu after L-DOPA treatment (for a discussion about the clinical relevance of the drug doses used in this study see Andersson *et al.*, 2001). However, a residual increase in the number FosB/ΔFosB-immunoreactive cells was detected in the medial part of the CPu for up to 8 days of cocaine withdrawal. Thus, in both treatment paradigms, drug-induced ΔFosB-like immunoreactivity showed similar decay dynamics (Fig. 4). The slow decay of

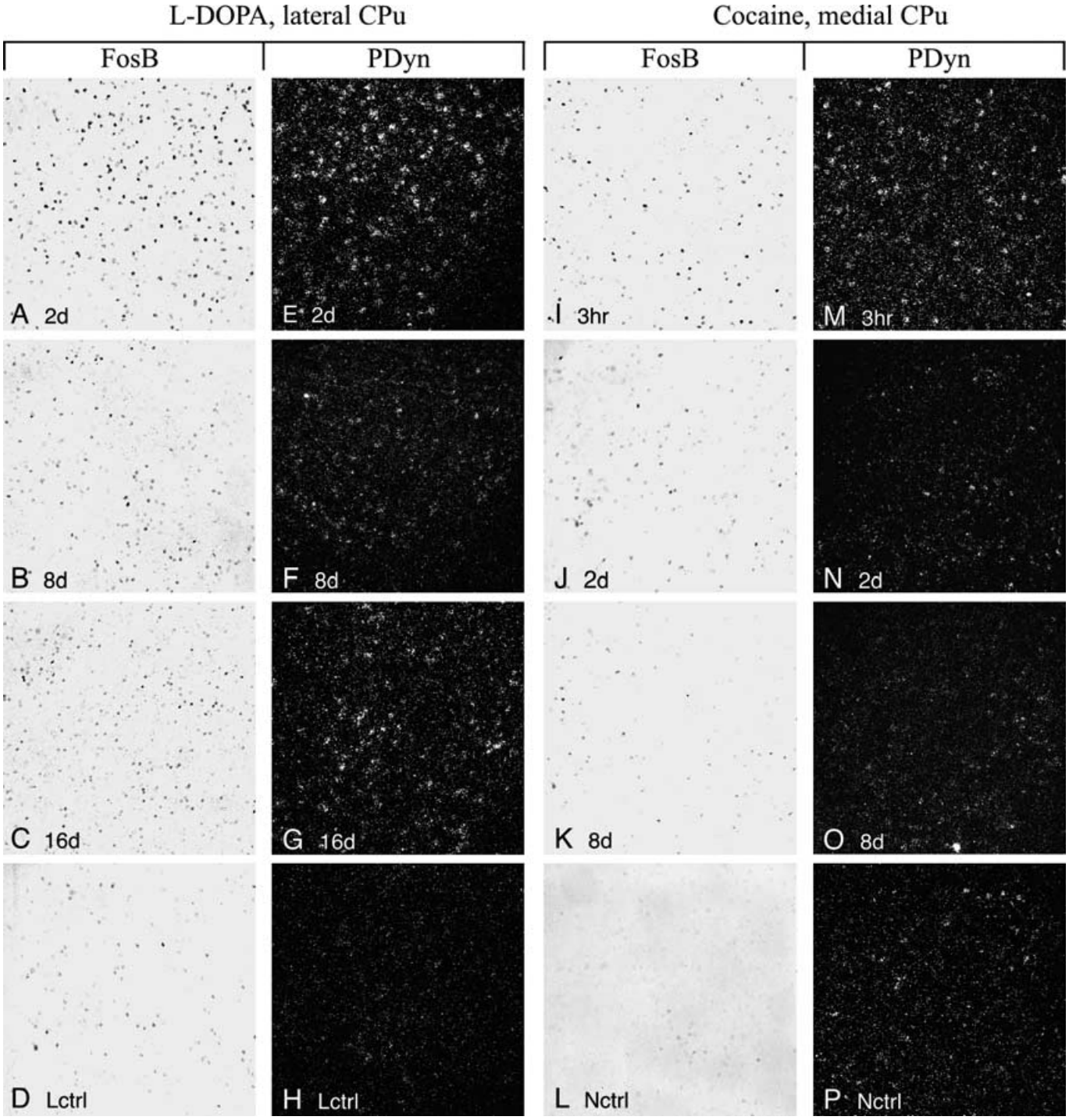

FIG. 3. Photomicrographs of ΔFosB-like-immunoreactive nuclei in the striatal subregion showing the most robust induction, i.e. the lateral CPu in 6-OHDA-lesioned rats treated with L-DOPA (A–D), and the medial CPu in cocaine-treated rats (I–L). Cellular labelling for PDyn mRNA from the corresponding parts of the CPu is shown in dark-field photomicrographs (E–H and M–P).

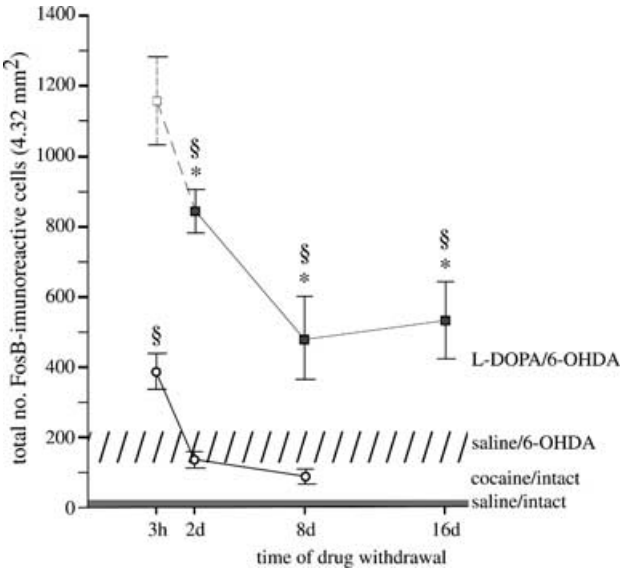

FIG. 4. Comparison of the decay dynamics of striatal FosB/ΔFosB-like-immunoreactivity after discontinuation of chronic L-DOPA treatment to 6-OHDA-lesioned rats (square symbols) or chronic cocaine treatment to intact rats (round symbols). Total number of counted ΔFosB-immunoreactive nuclei counted in both the medial and lateral CPu (eight areas $^{*}0.54\,mm^2$) is shown in the diagram. Data from the 3-h time-point after L-DOPA administration have been obtained from another group of animals treated with the same L-DOPA regimen, which were not processed simultaneously with the animals allotted to this study. The intervals defined by the mean ± SEM of controls are shown as a hatched stripe (saline-injected 6-OHDA lesioned group) or in a grey stripe (saline injected intact rats). $^{*}P < 0.05$ vs lesion-only controls, §$P < 0.05$ vs normal controls.

ΔFosB-like proteins *in vivo* matches the long half-life that has been measured *in vitro* (Chen *et al.*, 1997).

The functional implications of the long half-life of ΔFosB-like proteins remain to be explored. However, it has been shown that chronic administration of cocaine induces striatal DNA-binding activity to canonical AP-1 elements that remains high for at least 7 days after cessation of the treatment (Hope *et al.*, 1992; Hope *et al.*, 1994). The ability of ΔFosB-like proteins to form AP-1 complexes long after discontinuation of their inductive stimuli suggests that gene transcription may be influenced by ΔFosB-like proteins in striatal neurons without an ongoing stimulation of DA receptors. In 6-OHDA-lesioned rats, the induction of ΔFosB-like proteins produced by chronic L-DOPA treatment is causally linked with an upregulation of PDyn mRNA in the DA-denervated CPu (Andersson *et al.*, 1999, 2001). Moreover, the same regimen of chronic cocaine treatment used in this study has been shown to induce striatal PDyn mRNA expression in intact rats (Steiner & Gerfen, 1993). Due to these previous findings, PDyn mRNA levels were examined in this study in order to provide a marker of transcriptional effects possibly mediated by ΔFosB-like proteins. In the DA-denervated CPu, there was a perfect regional and temporal correspondence between changes in FosB/ΔFosB-like immunoreactivity and PDyn mRNA levels. Interestingly, we found a significant upregulation of PDyn mRNA for up to 16 days of L-DOPA withdrawal, which supports our hypothesis regarding an ongoing transcriptional regulation by the persistently expressed ΔFosB-like proteins. The alternative explanation, i.e. an increased stabilization of mRNA transcripts in the striatal neurons, is very unlikely (Malter, 2001).

Intact rats chronically administered with cocaine showed a significant upregulation of PDyn mRNA only in the medial CPu, i.e. the striatal subregion that showed the most robust induction of ΔFosB-like proteins. In this subregion, the levels of PDyn mRNA did not show any residual, significant elevation at survival periods longer than 3 h. In agreement with our previous studies (Andersson *et al.*, 1999; Andersson *et al.*, 2001), we suggest that the more transient drug-induced upregulation of PDyn mRNA seen in the intact CPu compared to the DA-denervated CPu reflects different underlying regulatory mechanisms. Indeed, we have shown that DA-dependent PDyn induction is mediated by the cAMP response element-binding protein (CREB) in the intact CPu (Andersson *et al.*, 2001). The transient kinetics of CREB phosphorylation (Shaywitz & Greenberg, 1999) may therefore account for the rapid decay in PDyn gene transcription displayed in this study by the intact animals treated with cocaine. We have also shown that dimers of ΔFosB-like proteins and JunD supersede CREB in the activation of PDyn gene expression in DA-denervated animals treated with L-DOPA (Andersson *et al.*, 1999; Andersson *et al.*, 2001). This switch in transcriptional regulation may account for the more persistent upregulation of the PDyn transcript in the latter model.

In conclusion, our results demonstrate the exceptional stability of ΔFosB-like proteins after discontinuation of chronic dopaminomimetic treatment *in vivo*. 6-OHDA-lesioned rats treated with L-DOPA showed a spatially and temporally coordinate upregulation of FosB/ΔFosB immunoreactivity and PDyn mRNA in the DA-denervated striatum for at least 16 days of drug withdrawal. These data suggest that the L-DOPA-induced ΔFosB-like proteins have ongoing transcriptional effects for more than 2 weeks after treatment discontinuation. Such effects may account, at least in part, for the long-lasting effects on brain function produced by L-DOPA treatment (Vallone *et al.*, 1997; Crocker *et al.*, 1998; Rascol, 2000).

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Abbreviations

CPu, caudate putamen; CREB, cAMP response element-binding protein; DA, dopamine; ISHH, *in situ* hybridization histochemistry; 6-OHDA, 6-hydroxydopamine; PDyn, prodynorphin.

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