Striatal overexpression of ΔJunD resets L-DOPA-induced dyskinesia in a primate model of Parkinson disease

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Abstract

Background—Involuntary movements, or dyskinesia, represent a debilitating complication of dopamine replacement therapy for Parkinson disease (PD). The transcription factor ΔFosB accumulates in the denervated striatum and dimerizes primarily with JunD upon repeated L-DOPA administration. Previous studies in rodents have shown that striatal ΔFosB levels accurately predict dyskinesia severity, and indicate that this transcription factor may play a causal role in dyskinesia sensitization process.

Methods—We asked whether the correlation previously established in rodents extends to the best non-human primate model of PD, the MPTP-lesioned macaque. We used Western Blotting and quantitative PCR to compare ΔFosB protein and mRNA levels across 2 subpopulations of macaques with differential dyskinesia severity. Second, we tested the causal implication of ΔFosB in this primate model. Serotype 2 Adeno-Associated Vectors (AAV2) were used to overexpress, within the motor striatum, either ΔFosB, or ΔJunD, a truncated variant of JunD lacking a transactivation domain, and therefore acting as a dominant negative inhibitor of ΔFosB.

Results—A linear relationship was observed between endogenous striatal levels of ΔFosB and the severity of Dyskinesia in Parkinsonian macaques treated with L-DOPA. Viral overexpression of ΔFosB did not alter dyskinesia severity in animals previously rendered dyskinetic, whereas the overexpression of ΔJunD dramatically dropped the severity of this side effect of L-DOPA, without altering the antiparkinsonian activity of the treatment.

Conclusion—These results establish a mechanism of dyskinesia induction and maintenance by L-DOPA and validate a strategy, with strong translational potential, to de-prime the L-DOPA-treated brain.

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Financial Disclosure
All the authors declare that they have no competing financial interests.
Keywords
L-DOPA; Dyskinesia; LID; Parkinson Disease; ΔJunD; ΔFosB; AAV

Introduction
Involuntary movements, or dyskinesia, represent a debilitating complication of L-3,4-dihydroxyphenylalanine (L-DOPA) therapy for Parkinson disease (PD), experienced, after long-term treatment, by the vast majority of patients. Once it develops, L-DOPA-induced dyskinesia (LID) can be triggered persistently by a single dose of L-DOPA, even after several weeks of treatment cessation. The concept of priming refers to the chain of neurobiological events establishing and maintaining this long-lasting sensitized response (1,2). Understanding the basic mechanisms of priming is crucial to overcome LID, the main limitation of L-DOPA treatment. In this context, the activator protein-1 (AP-1) family of transcription factors, composed primarily of Fos-Jun heterodimers, has received a particular attention (3–6). Indeed, one of the best replicated findings in the field is the dramatic induction of the transcription factor ΔFosB by chronic L-DOPA administration in the denervated rodent striatum (7–9).

ΔFosB is a well-characterized truncated splice variant of the FosB gene (6,10,11). The C-terminal 101 amino acids truncation in ΔFosB includes several domains important for destabilization of the protein, and results in a drastic increase in its half life. Consequently, ΔFosB protein accumulates in a region-specific manner upon repeated L-DOPA administration, until it eventually becomes the most prevalent Fos isoform (12–14). Following its expression, ΔFosB protein persists in the striatum for relatively long periods of time after cessation of L-DOPA treatment, a biochemical profile, which makes it a plausible mediator of dyskinesia maintenance. DNA-binding studies conducted in rodent striatum, under dyskinesiogenic conditions, indicate that ΔFosB/JunD containing complexes account for most of the activity at AP-1 sites (12). Under these conditions a strong correlation is observed between the number of neurons expressing ΔFOSB in the striatum and the severity of LID (4,15).

To date, two independent studies have tested, in rodents, the causal implication of striatal FosB-like proteins in the development of LID (15,16). In both studies, knockdown of the fosb gene products, using antisense oligonucleotides, inhibited the occurrence of the rodent analog of LID. These converging positive results underlined the clinical potential of interventions aimed at reducing ΔFosB activity. On the other hand, a possible confound of these previous studies was that the knockdown was not selective for ΔFosB, but also resulted in altered levels of full-length FosB, a protein with an opposite effect compared with ΔFosB on many AP-1 target genes. Finally, although elevated levels of ΔFosB have also been reported in the striatum of L-DOPA-treated PD patients examined at autopsy (17), the clinical relevance of rodent studies remains tempered by important species-specific differences in the mechanisms and phenotypic expression of LID.

Here, we first assessed whether the correlation between ΔFosB levels and LID severity previously established in rodents extends to the best non-human primate model of PD, the MPTP-lesioned macaque (18,19). To this end, we examined the intensity of LID, on one hand, and striatal levels of ΔFosB on the other, in a cohort of 8 macaques with stable parkinsonian symptoms, and variable degrees of LID. Second, we tested the causal implication of ΔFosB in this model. To test whether increased ΔFosB signaling in this region augments the severity of LID, we overexpressed ΔFosB using an AAV2 viral vector. To test the complementary hypothesis, that reduced activity of endogenous ΔFosB may
disrupt long-term maintenance of LID, and thereby ameliorate them, we opted for a dominant-negative strategy, by overexpressing ΔJunD, a truncated mutant encoding a JunD protein devoid of transactivation domain (20). The effects of ΔFosB and ΔJunD overexpressing vectors were compared to a control vector expressing hrGFP only. Daily behavioral measures collected “OFF” and “ON” L-DOPA, both before and after viral transductions, were used to evaluate the effect of these treatments. In addition, we conducted post mortem evaluations of prodynorphin (pDYN) mRNA levels in transduced striatal tissues, as a functional readout for the activity of the viral vectors. Up regulation of this ΔFosB target gene in the striatum of L-DOPA-treated parkinsonian animals has previously been shown to constitute a good predictor of LID severity. We reasoned that upon efficient viral treatment, expression of this gene should be upregulated in animals transduced with ΔFosB and downregulated after ΔJunD overexpression.

Methods and Material

All experiments were carried out in accordance with both the European Communities Council Directive of November 24, 1986, (86/609/EEC) for the care of laboratory animals and the Institutional Animal Care and Use Committee of UT Southwestern Medical Center in AAALAC accredited facilities.

Animals and housing

We used 22 cynomolgus monkeys (Macaca fascicularis, SAH/Xierxin, Beijing, PR of China; 16 females – 6 males). Animals were housed in individual primate cages under controlled conditions of humidity, temperature, and light (12-h light/12-h dark cycle, lights on at 8.00 am); food and water were available ad libitum. Animal care was supervised by veterinarians skilled in the healthcare and maintenance of non-human primates.

Experimental parkinsonism and dyskinesia

Experiments were conducted according to previously published procedures and methods (19,21–23). 18 monkeys received once daily i.v. injections of MPTP hydrochloride (0.2 mg/kg) until they displayed parkinsonian symptoms (mean number of injections = 15±1)(18) while 5 received vehicle only. It took an average of 8 wk for the bilateral parkinsonian syndrome to stabilize (i.e., constant disability score over 2 consecutive wk). Among the 18 MPTP-treated monkeys, 6 monkeys were kept without any dopaminergic supplementation (MPTP group: “parkinsonian”), while 12 were treated chronically with twice-daily administration of Modopar (Roche, Basel, Switzerland, L-DOPA/carbidopa, ratio 4:1) for 6 months at a tailored dose designed to optimally reverse the parkinsonian features of each animal. 8 of these 12 monkeys developed dyskinesia (DYSK (+) group: “parkinsonian, dyskinetic”), while 4 did not (DYSK(−) group: “parkinsonian, non-dyskinetic”).

All monkeys, except 2 MPTP and 4 DYSK (+) animals that were kept alive for further behavioral experiments (see below), were killed by sodium pentobarbital overdose (150 mg/kg, i.v.) 1 h after the last vehicle or L-DOPA/carbidopa dose. Brains were removed quickly and divided into the two hemispheres. The right hemisphere was immediately frozen by immersion in isopentane and then stored at −80°C. The left hemisphere was dissected for isolating the striatum (combining caudate nucleus, putamen and nucleus accumbens, across the rostrocaudal extent) that was then frozen in isopentane and stored at −80°C(24).

Assessment of dopaminergic lesions

DA transporter binding using (125I)-(E)-N-(3-iodoprop-2-enyl)-2β-carboxymethyl-3β-(4′-methylphenyl)-nortropane (PE2I; Chelatec, France) was measured as previously described (18,25).
immunohistochemistry, counterstaining with cresyl violet (Nissl staining) and cell counts (Mercator, Explora Nova, La Rochelle, France) were performed as previously described (18,23,25).

**In situ hybridization histochemistry**

The *in situ* hybridization procedure was performed as previously described (21,26) with probes designed to recognize prodynorphin (pDYN) (27) and preproenkephalin (pENK) mRNAs (28), 2 genes whose regulation in the striatum is classically being used as predictor of dyskinesia severity and treatment response respectively.

**Stereotactic surgery**

A total volume of 40 μl of virus was injected to each animal (20 μl per side at 3 different rostro-caudal sites). Two MPTP and 4 DYSK(+) monkeys were used to assess whether viral-mediated overexpression of ΔFosB or ΔJunD in motor striatum modulates existing LIDs. Horsley-Clarke stereotaxic technique coupled with ventriculography were used to determine the actual position of left and right putamen (22,29,30,31). Intracerebral injection of AAV-hrGFP in 2 MPTP/vehicle animals, of AAV-ΔFosB-hrGFP in 2 MPTP/dyskinetic animals, and of AAV-ΔJunD-hrGFP in 2 MPTP/dyskinetic animals were performed bilaterally with a 10 μl Hamilton syringe mounted into a Kopf microinjector system, at 3 sites extending from 2 mm anterior to AC (AC0 mm) to 4 mm caudal to AC (AC-4 mm) (Figure 3).

**Post-hoc verification of viral transgenes expression using immunofluorescent detection of hrGFP**

To confirm proper targeting and maintenance of transgene expression 3 months after virus infusions, brains were collected after completion of behavioral studies and were immunolabelled for hrGFP. We reasoned that quantification of the immunostaining for hrGFP would provide the most reliable and unbiased estimation of viral vectors activity. First, hrGFP is expressed by all 3 vectors and can be assessed with a single antibody. Second, in contrast to FosB and JunD antigens, hrGFP is not expressed endogenously. Twenty μm-thick sections were postfixed with 4% paraformaldehyde in phosphate buffer saline (PBS) for 10 minutes and processed using a 1/500 dilution of a rabbit anti-hrGFP primary antibody (Stratagene). hrGFP positive area was drawn manually and determined for each sections using an image analysis system (Mercator, Explora Nova, La Rochelle, France). Serial quantifications of the numbers of hrGFP immunopositive cells were used to estimate hrGFP expression volume (mm³).

**Behavioral experiments**

Monkeys’ behavioral responses to their tailored dose of L-DOPA/carbidopa (administration at 8.00 am) were defined prior to AAV intracerebral injections. 4 weeks post-AAV injection, animals started (in the AAV-hrGFP MPTP/vehicle group) or resumed (in the AAV-ΔFosB-hrGFP MPTP/dyskinetic and AAV-ΔJunD-hrGFP MPTP/dyskinetic groups) their once daily L-DOPA treatment. They were then were daily assayed for behavioral responses to L-DOPA. Parkinsonian condition (and its reversal) was assessed on a parkinsonian monkey rating scale using videotape recordings of monkeys as previously described (19,22). A score of 0 corresponds to a normal animal and a score above 6 to a parkinsonian animal. The severity of dyskinesia was rated using the Dyskinesia Disability Scale: 0, dyskinesia absent; 1, mild, fleeting, and rare dyskinetic postures and movements; 2, moderate, more prominent abnormal movements, but not interfering significantly with normal behavior; 3, marked, frequent and, at times, continuous dyskinesia intruding on the normal repertoire of activity; or, 4, severe, virtually continuous dyskinetic activity replacing...
normal behavior and disabling to the animal. Mean parkinsonian and dyskinetic scores (± SD) were calculated for the peak-of-dose effect of L-DOPA, i.e. between 60 and 120 min post L-dopa administration.

Viral vectors

Construction and packaging of AAV-ΔFosB-hrGFP and AAV-ΔJunD-hrGFP vectors has been described previously (32,33). cDNAs for ΔFosB and ΔJunD149, were inserted into a recombinant AAV2 (AAV serotype 2) vector (see (32) for cloning details), downstream of a CMV promoter in pAAV-IRE5-hrGFP vector (Stratagene). Choice of the AAV2 serotype was guided by the substantial evidence from in vivo and in cell culture studies indicating that, among all AAV serotypes, AAV2 vectors show one of the most focalized transduction pattern in the brain, with virtually no expression in glia. In the mouse striatum, a recent study showed that ~97% of cells infected by AAV2 are immunopositive for the neuronal marker NeuN whereas less than 5% are immunopositive for the glial marker GFAP(34). The plasmid was packaged into an AAV virus using a triple-transfection, helper-free method (33). The virus was then titered using an AAV ELISA kit (Progen) and evaluated for infectivity in HT1080 cells. Titer was 1–2 × 10^{11} infectious particles/ml.

Western Blotting

FosB/ΔFosB proteins were detected by western blot using a rabbit polyclonal antiserum raised against a middle region of both ΔFosB and full-length FosB (amino acids 75-150, SC7203, SantaCruz Biotechnology, Santa Cruz, CA). Brain regions were isolated by obtaining punches from 400um thick frozen sections of the motor striatum, dissected on a cryostat. Western blotting, was performed according to previously published procedures (35). To specifically identify FosB vs ΔFosB bands, immunoblots from brain samples were compared to protein extract from Rat adrenal pheochromocytoma (PC12) cells (Clontech, Mountain View, CA, USA) transduced either with FosB- or ΔFosB-expressing viral vectors (31).

Quantitative PCR

RNA was prepared using the RNAeasy Micro Kit (QIAGEN). cDNA was obtained using a first-strand synthesis kit (Invitrogen). All PCR experiments were conducted in triplicate using SYBR green PCR mastermix (ABI) and the data were analyzed by using the DDCt method and were normalized to measures of GAPDH mRNA.

Results

Striatal ΔFosB protein predicts the severity of LID in Parkinsonian macaques after repeated L-DOPA administration

Dopaminergic denervation (MPTP) and L-DOPA administration induced an overall increase in striatal levels of ΔFosB protein measured by western blot. The induction of ΔFosB protein after repeated administration of L-DOPA was significantly higher in the subset of animals developing a dyskinetic response (Figure 1A and 1B). There was a significant main effect of treatment, (ANOVA F_{3,13} = 5.97, p<0.01). Post hoc comparisons with Fisher LSD test indicate that normalized levels of ΔFosB protein were significantly increased in dyskinetic animals (mean ± sem: 109.24 ± 13.44), when compared to unlesioned controls (62.40 ± 2.40; p<0.01) or parkinsonian non-dyskinetic macaques (85.42 ± 9.33; p<0.05).

ΔFosB mRNA expression (Figure 1C) was also modified by treatment (ANOVA main treatment effect F_{3,12} = 5.80, p<0.01) but there was no significant difference between dyskinetic and non-dyskinetic parkinsonian macaques. On the other hand, striatal pDYN mRNA level (Figure 1D) was significantly altered (ANOVA main treatment effect F_{3,12} =
4.01, p<0.05). Post hoc comparisons, using Fisher LSD test, showed that there was a sensitized induction of pDYN in dyskinetic (1.87 ± 0.61) compared to non-dyskinetic animals (0.56 ± 0.56; p<0.05). Regression analyses (Figure 1E–G) demonstrate a significant linear relationship between ΔFosB protein levels and the severity of Dyskinesia in L-DOPA treated animals (p<0.05, r^2 = 0.6741) as well as between ΔFosB protein levels and pDYN mRNA (p<0.05, r^2 = 0.79).

**Neither ΔFosB nor ΔJunD striatal overexpression alters the antiparkinsonian activity of L-DOPA**

On the basis of these observations, we wondered what would be the behavioral impact of overexpressing ΔFosB and of functionally inhibiting ΔFosB in already dyskinetic animals compared to L-DOPA-naïve MPTP-treated monkeys (Figure 2A). Parkinsonism of all groups (OFF scores) was improved by L-DOPA treatment (Figure 2B) (ON scores: mean parkinsonian score between 60 and 120 min post L-DOPA administration ± SD). Areas under the curve (AUC) were not different between groups (Two-tailed unpaired t test: p>0.5 between groups).

**AAV-mediated overexpression of ΔJunD resets LID whereas ΔFosB has no effect**

While in AAV-ΔFosB-hrGFP injected macaques, dyskinesia scores remained unchanged after surgery (Figure 2C), in AAV-ΔJunD-hrGFP injected animals, dyskinesia scores dropped significantly from day 1 until day 14 post surgery, when compared to pre-surgery scores (Figure 2C). Dyskinesia in this latter group resumed on Day 14 of L-DOPA treatment, and re-developed with kinetics fully comparable to the one of L-DOPA-naïve animals, infused with the control AAV-hrGFP virus (black). Accordingly, AUC for LID scores of AAV-ΔJunD-hrGFP infused animals was similar to control AAV-hrGFP infused animals (Two-tailed unpaired t test: p<0.5) (Figure 2C - inset), while the AAV-ΔFosB-hrGFP animals were significantly more dyskinetic from the 2 other groups (Two-tailed unpaired t test: * p<0.01 vs. 2 other groups).

**L-DOPA-induced changes in striatal neuropeptides**

We then ensured that all MPTP-treated groups were comparably lesioned as previously established (23). Measures of dopamine transporter (DAT) binding in the striatum were used as index of the severity of lesions induced by MPTP. All groups exhibited comparable decreases DAT binding (Figure 3A; Two-tailed unpaired t tests: * p<0.05 versus unlesioned control controls).

pENK mRNA levels measured by in situ hybridization were used as a functional marker of the extent of lesion. Decreases in pENK mRNA levels compared to drug naïve MPTP-treated macaques is known to correlate with antiparkinsonian response to L-DOPA (26). As expected from behavioral experiments, all three groups showed a significant decrease in pENK mRNA compared to drug naïve MPTP-treated macaques (Figure 3B; Two-tailed unpaired t test: p<0.05) that correlated with antiparkinsonian response to L-DOPA (p<0.001, r^2 = 0.841).

Finally, changes in pDYN mRNA levels measured by in situ hybridization were used as a functional readout for the influence of ΔJunD overexpression on ΔFosB transcriptional activity. Both the control AAV-hrGFP and the AAV-ΔFosB-hrGFP groups showed the expected increase in pDYN mRNA (Figure 3C, data expressed as percentage of expression in drug naïve MPTP-treated macaques; Two-tailed unpaired t test: * p<0.05 versus drug naïve MPTP-treated macaque). On the contrary, the AAV-ΔJunD-hrGFP group exhibited pDYN levels not different from drug naïve MPTP-treated macaques (Figure 3C) suggesting a functional blockade of ΔFosB binding sites.

_Biol Psychiatry_. Author manuscript; available in PMC 2010 February 20.
Verifications of viral vector targeting in striatum

Accurate localizations of injection tracks and volumes of transduced tissues were determined post-mortem. All injection tracks were localized in the striatum (Figure 4A). Immunohistochemical detection of hrGFP (Figure 4A) was used to define the volume of transduced striatal tissue. No difference was observed in transduced volumes across the three experimental groups (Figure 4B).

Discussion

To further establish the translational potential of targeting ΔFosB in LID, we first evaluated whether the correlation previously established in rodents between ΔFosB levels and severity of LID extend to the best non-human primate model of PD, the MPTP-lesioned macaque (18,19). We first confirmed by cloning and sequencing that identical splicing of the FosB gene occurs in the macaque brain as reported previously from human and rodent tissues. We found that sequences of FosB and ΔFosB mRNA are 98% conserved with the human variants, and we established a quantitative PCR method to selectively quantify FosB and ΔFosB mRNA from macaque tissues (supplemental figure S1). We next examined the relationship between the intensity of LID, on one hand, and ΔFosB mRNA and protein levels in striatum, on the other. In clinical settings, only a subset of patients receiving optimized doses of L-DOPA develop dyskinesia within the first months of treatment and similar heterogeneity is also observed among non-human primates (19,23). In a cohort of 8 macaques with stable parkinsonian symptoms, and receiving optimal doses of L-DOPA for 6 months, we observed a significant increase in striatal levels of ΔFosB protein only in the subset of animals showing overt LID (Figures 1A and 1B). A linear regression analysis conducted in this cohort indicates that, about 70% of the interindividual variability in dyskinesia severity can be predicted from ΔFosB protein levels (Fig. 1E). Although an overall increase in ΔFosB mRNA expression with L-DOPA treatment was observed, there was no significant difference in ΔFosB mRNA levels between dyskinetic and non-dyskinetic animals at the same time point (Fig. 1C). On the other hand, sensitized induction of pDYN mRNA was observed in dyskinetic compared to non-dyskinetic animals (Fig. 1E). As predicted from previous rodent (3,6,8,15,36) and primate (26) studies, mRNA levels of pDYN, a well-validated target gene of ΔFosB, correlated highly with both ΔFosB protein levels and dyskinesia scores (Fig. 1D–F), but not with ΔFosB mRNA levels. Together, these results suggest that enhanced ΔFosB levels in dyskinetic monkeys likely reflect pulses of protein synthesis and accumulation, rather than a sustained increase in ΔFosB mRNA stability, gene transcription or splicing. Having demonstrated that the link between ΔFosB protein levels and dyskinesia severity extends to our primate experimental model, we next tested the causal role of ΔFosB signaling in the long-term maintenance of LID.

Recent clinical studies have indicated that gene therapy using AAV vectors is a well-tolerated approach which holds great promise in PD patients (37). We adopted a similar approach to manipulate ΔFosB function locally within the macaque motor striatum. To test whether increased ΔFosB signaling in this region augments the severity of LID, we overexpressed ΔFosB using an AAV2-ΔFosB-IRES-hrGFP viral vector. To test the complementary hypothesis, that reduced activity of endogenous ΔFosB may disrupt long-term maintenance of LID and thereby ameliorate them, we opted for a dominant-negative strategy, by overexpressing a truncated form of the JunD protein (20). The choice of this strategy was based on previous data indicating that JunD, a major in vivo partner for ΔFosB (11,35), is the most prevalent Jun family protein found, under dyskinesiogenic conditions, in ΔFosB-containing, AP-1 dimers (5,12,38). To create an AAV-JunD-hrGFP vector, a cDNA encoding an N terminal truncated JunD, devoid of transactivational activity (32,39,40), was inserted into pAAV2-IRES-hrGFP. The effects of ΔFosB and ΔJunD overexpressing vectors were compared to a control vector expressing hrGFP only. In all
viral vectors, hrGFP was expressed as a second open reading frame translated from an internal ribosome entry site (IRES) and was used as an internal standard to ascertain the infection efficiency in each animal at the end of the experiment. Based on preliminary infusions and immunohistological evaluations of hrGFP expression, we determined that complete antero-posterior coverage of the macaque motor striatum could be achieved with an overall viral volume of 20 μl per side (5 μl at 4 different anteroposterior locations in the putamen). Our post mortem estimations, conducted on the brains collected after the completion of the behavioral study, indicated that hrGFP expression was detected across similar volumes of approximately 60 mm3 with each of the 3 vectors.

In previous rodent studies, manipulations of ΔFosB levels have been conducted solely in drug naïve animals, prior to any L-DOPA treatment (15,16). This experimental design is not the most clinically relevant as patients do not usually seek dyskinesia relief before starting L-DOPA treatment. In addition, in our preliminary studies, both AAV-ΔFosB-hrGFP or AAV-ΔJunD-hrGFP viruses have proved inefficient when administered prior to the beginning of L-DOPA treatment (data not shown).

Therefore, to directly address the influence of ΔFosB signaling on long-term maintenance of LID, AAV-ΔFosB-hrGFP or AAV-ΔJunD-hrGFP viruses were stereotaxically infused into the motor striatum of parkinsonian macaques, previously rendered dyskinetic by daily administration of L-DOPA for 3 months. In assigning animals to ΔFosB or ΔJunD conditions, particular care was taken to establish balanced groups for pre-infusion severity of both parkinsonian (Fig. 2A) and LID (Fig. 2B) symptoms. The control group was composed of parkinsonian macaque infused with AAV-hrGFP, and was previously naïve to L-DOPA. Four weeks after stereotactic delivery of the viral vectors, L-DOPA treatment was resumed in all groups.

In AAV-ΔFosB transduced animals, the therapeutic (i.e., antiparkinsonian) activity of L-DOPA (Fig. 2A), as well as dyskinesia severity (Fig. 2B) were maximal from day 1, and unaltered in comparison to pre-infusion scores (Fig. 2A–B, left side). In contrast, the overexpression of ΔJunD produced a dramatic reduction of dyskinesia severity compared to pre-infusion scores. This effect coincided with a lack of pDYN upregulation by L-DOPA in transduced tissues (Figure 3), a result we interpret as functional readout of efficient ΔFosB antagonism by ΔJunD (3,6,36). Despite continuous transgene expression (Figure, AAV-ΔJunD-hrGFP treatment did not permanently prevent the reappearance of dyskinesia with repeated L-DOPA administration. Interestingly, the kinetics of the reappearance of LID in AAV-ΔJunD-hrGFP injected animals overlapped with the one of naïve AAV-hrGFP animals experiencing their first L-DOPA course. In both of these conditions, dyskinesia developed from day 8 onwards with a gradual worsening until stabilization on day 18 (Fig. 2B).

Our interpretation of these results is that overexpression of ΔFosB in the motor striatum of dyskinetic animals treated with L-DOPA is devoid of behavioral effect, whereas overexpression of ΔJunD resets dyskinesia-related striatal networks to a drug-naïve state, putting the animals back into the so-called “honeymoon” period of L-DOPA treatment. Importantly, the anti-dyskinetic effect of ΔJunD occurs without any alteration in the efficacy of L-DOPA on parkinsonian symptoms (Fig. 1A). Dominant-negative inhibition of endogenous ΔFosB activity in the motor striatum, through expression of ΔJunD, thus appears to provide a novel and valuable avenue to interfere with the long-term maintenance of dyskinesia, potentially offering patients a return to the honeymoon period. Our interpretation is that this activity is mediated primarily through an antagonism of endogenous ΔFosB activity. The possibility that ΔJunD may alter additional protein-protein interactions by competing for other partners of endogenous JunD protein cannot be excluded. It will be important in further studies to determine why the antidyskinetic activity
of ΔJunD is not sustained. A possibility is that accumulating levels of ΔFosB ultimately supersede ΔJunD antagonism. On the other hand, since levels of viral transgenes were evaluated at a single time point, after completion of the behavioral experiment, we cannot exclude the possibility that these levels may correspond to a fraction of the peak intensity occurring earlier in the study. A full time-course analysis of transgene expression during the length of L-DOPA treatment will be necessary to address this question. The fact that expression of viral transgenes may have dropped during the experiment, could also explain the absence of effect of ΔFosB overexpression in dyskinetic monkeys. This lack of effect, however, was not totally unexpected, as previous studies suggest that full occupancy of AP-1 sites, by endogenous ΔFosB containing complexes, may occur under dyskinesiogenic conditions (5,12,38). Further characterization of the mechanisms underlying ΔJunD de-priming activity are required, as well as ways to extend the period of ΔJunD’s efficacy in reducing dyskinetic responses to L-DOPA.

Supplementary Material
Refer to Web version on PubMed Central for supplementary material.

Acknowledgments
We thank Akshay Bhonsle, Li Hao, Li Jun and Baishen Ren for outstanding technical assistance. This work was supported by a grant to OB, EJN, and EB from the Michael J. Fox Foundation for Parkinson research.

References


FIG 1. Striatal levels of ΔFosB proteins, but not ΔFosB mRNA, predict the intensity of dyskinesia and pDYN induction in response to repeated L-DOPA treatment.

(A) Dopaminergic denervation (MPTP+) and L-DOPA (L-DOPA+) administration in Rhesus macaques (n=4–5/group), induce an overall increase in striatal levels of ΔFosB protein (35–37 kD) measured by western blot. Note recombinant FosB and ΔFosB protein standards collected from PC12 cells after viral overexpression. (B) Induction of ΔFosB protein is sensitized after repeated administration of L-DOPA, only in the subset of animals developing a dyskinetic response (DYSK+). Post hoc comparisons, Fisher LSD test * p<0.05; ** p<0.01 (C) Overall increase in ΔFosB mRNA expression with L-DOPA treatment. Note the lack of significant upregulation of ΔFosB mRNA in dyskinetic (DYSK+) compared to non-dyskinetic (DYSK−) animals. (D) Sensitized induction of pDYN mRNA also occurs in dyskinetic compared to non-dyskinetic animals. (E) Significant linear relationships between ΔFosB protein levels and LID severity. (F) Significant linear relationships between ΔFosB protein levels and pDYN gene expression. (G) ΔFosB mRNA levels do not correlate with pDYN gene expression.
FIG 2. AAV-mediated overexpression of ΔJunD in motor striatum resets L-DOPA-induced dyskinesia without altering L-DOPA efficacy

(A) Experimental design showing the sequence of experiments including the 4 weeks interval between surgery and behavioral observations depicted in panels B and C. (B) Parkinsonism (OFF scores) of all groups was improved by L-DOPA treatment prior to surgery (ON scores, p< 0.05; Two-tailed unpaired t test) squares on the left side of the panel). The vertical dashed red line symbolizes the 4 weeks interval between surgery and behavioral experiments. Parkinsonian scores remained significantly improved by L-DOPA after AAV stereotactic infusion. Main plot shows mean parkinsonian score evaluated between 60 and 120 min post L-DOPA administration ± SD.: AAV-hrGFP (black line; n=2): group composed of parkinsonian animals receiving vehicle prior to viral transduction, and treated with symptomatically optimal doses of L-DOPA after AAV-hrGFP infusion. AAV-ΔFosB-hrGFP (orange line; n=2): group composed of parkinsonian animals treated with L-DOPA and rendered dyskinetic prior to to viral transduction with AAV-ΔFosB-hrGFP. AAV-ΔJunD-hrGFP (purple line; n=2): group composed of parkinsonian animals treated with L-DOPA and rendered dyskinetic prior to to viral transduction with AAV-ΔFosB-hrGFP. (C) While dyskinesia scores remained unchanged after surgery in AAV-ΔFosB-hrGFP injected macaques (orange line) in AAV-ΔJunD-hrGFP injected animals (purple
line), dyskinesia scores dropped significantly from day 1 until day 14 post surgery, when compared to pre-surgery scores. Dyskinesia in this latter group resumed on Day 14 of L-DOPA treatment, and re-developed with kinetics fully comparable to the one of L-DOPA-naïve animals, transduced with the control AAV-hrGFP virus (black). Inset shows A.U.C. for LID scores. Two-tailed unpaired t test: * p<0.01 vs. the 2 other groups.
FIG 3. Post-mortem validation of lesions and L-DOPA-induced changes in the striatum

(A) Dopamine transporter (DAT) binding data in the striatum are expressed as percentage of dopamine transporter binding in non-MPTP control macaques (dashed black line; n=5; mean ± sem). Two-tailed unpaired t test: * p<0.05 versus controls. (B) Preproenkephalin-A (pENK) mRNA levels are expressed as percentage of expression in drug naïve MPTP-treated macaques (dashed red line; n=5; mean ± sem). Two-tailed unpaired t test: * p<0.05 versus drug naïve MPTP-treated macaques. (C) pDYN mRNA levels measured by in situ hybridization are expressed as percentage of expression in drug naïve MPTP-treated macaques (dashed red line; n=5; mean±sem). Two-tailed unpaired t test: * p<0.05 versus drug naïve MPTP-treated macaque.
FIG 4. Post-mortem validation of viral targeting in the striatum

(A) Post-hoc verification of infection sites shows the three rostro-caudal levels of the motor putamen (ear bars +20 mm, +17 mm and +15 mm) that received the AAV vectors. The left column shows schematic representation of basal ganglia at the targeted levels. Put: putamen, Cd: caudate nucleus, GPe: globus pallidus externus, GPi: globus pallidus internus. Circles around needle track indicate sites where hrGFP-immunostaining was detected. (B) Representative example of hrGFP-immunostaining in the outlined circles (A). Volume (mm$^3$ of striatum) estimates (bottom) showed no differences between treatment conditions (Two-tailed unpaired t test).