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Supplementary Information for

Role of striatal ΔFosB in L-Dopa-induced dyskinesias of parkinsonian non-human primates

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SI Materials and Methods

Animal Preparation. Nine adult cynomolgus macaques (*Macaca fascicularis;* 6-8 kg of weight) of both genders were used in this study in accordance with the *National Institute of Health Guide for the Care and Use of Laboratory Animals*. All animals were rendered parkinsonian by systemic weekly administration of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP, 0.2-0.4 mg/kg i.v.) until meeting the criteria for moderate parkinsonism (usually in 1-3 months) according to motor disability score (MDS), as measured with the standardized Motor Disability Scale for MPTP-treated non-human primates. MPTP treatment ended if MDS stabilized for a period of at least four weeks. MDS indicative of moderate parkinsonism was targeted in order to maintain animals without L-Dopa or other antiparkinsonian treatments. After stabilization, animals were evaluated for their baseline motor response to L-Dopa up to a maximum of 3 tests (one s.c. injection per week) if necessary for a clear response with consistent scores. L-Dopa methyl ester plus benserazide (Sigma Aldrich, St. Louis, MO) was used for s.c. injections. Due to the limited number of baseline tests included in the protocol, the L-Dopa dose for each animal was adjusted based on the scores in the 3 baseline tests so that in the "on" state (reversal of parkinsonism) there was a 50% reduction of MDS in all animals. These tests demonstrated the absence of dyskinesias prior to AAV injections in all animals.

Production of recombinant AAV2/5 viral vectors. In brief, pAAV-ΔFosB and pAAV-eGFP vectors were constructed as follows: the mouse ΔFosB cDNA or eGFP cDNA was cloned into pAAV-MCS plasmid (Stratagene, San Diego, CA) to generate pAAV-ΔFosB or pAAV-eGFP, respectively. To generate AAV2/5 viral particles, HEK293T cells were

co-transfected with pAAV-ΔFosB, or pAAV-eGFP along with pAAV-Helper and pAAV-RC5 (VPK-425: Cell Biolabs, Inc., San Diego, CA) using a standard calcium phosphate method, and 72 h after transfection the cells were harvested. The crude recombinant adeno-associated virus (rAAV) supernatants were obtained by repeated freeze/thaw cycles and centrifugation at 10,000 g for 10 min. Effective rAAV2/5-ΔFosB expression in HEK293T cells was detected by Western blotting with primary antibody against FosB/ΔFosB (sc-7203: Santa Cruz Biotechnology, Dallas, TX). High-titer rAAV2/5-ΔFosB (2.8 x 10^{13} GC/ml) was prepared by Vector BioLabs (Philadelphia, PA). The number of genome copies was determined by quantitative PCR within the cytomegalovirus promoter region of the vector using primers 5'-GACGTCAATAATGACGTATG-3' and 5'-GGTAATAGCGATGACTAATACG-3'. The generated viral vector particles were finally prepared for injection into the monkey brain at a dilution of 1 x 10^{12} GC per ml.

Overexpression of ΔFosB in primates. rAAV-ΔFosB or rAAV-eGFP as control was injected into the striatum of parkinsonian monkeys. Animals were randomly assigned to each treatment group (ΔFosB or control virus, n=5 and n=4, respectively). Five animals received the virus injections directly following craniotomies under stereotaxic surgery (see below) and were used for behavioral assessment. Four animals (2 in each virus group) received the virus injections through the recording chambers that were surgically implanted in preparation for neural recordings along with the behavioral assessment. All surgeries were performed under general anesthesia and followed stereotaxic coordinates for direct virus injections or for implants of bilateral recording chambers according to

previously reported procedures. The protocol for virus delivery included multiple injections covering an extended area of the primate striatum (mostly the putamen) that resulted in on-target injections in all animals following either procedure. In the 5 animals injected with virus following stereotaxic guidance under surgery, coordinates were taken from the stereotaxic atlas targeting most central areas of the sensorimotor putamen to reduce the margin of error, which is already small for a large striatal region as the putamen. In the 4 animals with recording chambers, electrophysiologic mapping of the basal ganglia was used to direct the injections into the striatum. Virus injections were performed using Hamilton syringes in microinfusion pumps (injection rate of 1µL/min) mounted on the stereotaxic frame in surgery, or connected to an *injectrode* system (Alpha Omega, Alpharetta, GA) in the micromanipulator for injection through the recording chamber. rAAV-ΔFosB or rAAV-eGFP were injected at equal particle doses (1×10^{12} vg/ml) into the striatum bilaterally. A total of 104 µl was injected in each hemisphere, and the volume was distributed in 16 sites using 1 or 2 depths of injection in each site into the putamen (90 µl) and the caudate (14 µl). Target areas were the posterolateral putamen and the posterior portion of the caudate body. All animals remained free of antiparkinsonian treatment during the 4-week period post virus injection and before initiation of testing.

Behavioral Assessment. Following the 4-week period post virus injection, behavioral tests began to assess the time course of development of L-Dopa-induced dyskinesia (LID). L-Dopa responses were tested only once weekly to avoid the chronic daily exposure that leads rapidly to LID appearance in this model. In these weekly tests, L-Dopa was given at

the predetermined dose (s.c.) and the animal's motor behavior was assessed for the whole duration of the response until returning to the "off" state (baseline parkinsonian disability) score. Scores were taken before L-Dopa injection ("off" state) and thereafter every 20 min interval during the "on" state. Weekly responses were tested for a period of 12 weeks. Similarly, in the 4 animals prepared for electrophysiology, motor scores were obtained in the "off" state, and at the same intervals post-L-Dopa injection after rapid transfer to the cage at the end of collecting neural data (i.e. by the second interval of the "on" state). As the first interval after L-Dopa injection (30 min) was scored in the primate chair, scores of this interval were used for behavioral-SPN activity correlation, but they were not computed in the behavioral analysis of all animals together. Experiments were conducted in the morning when animals display the highest MDS. All behavioral tests were performed in the same testing cage, and animals were also videotaped for deferred scoring by a second blind investigator. Animals were euthanized at the end of the 12^{th} week of testing for analysis of ΔFosB expression and other LID markers in the striatum.

Electrophysiology. The animal was transferred to the recording suite in a primate chair for head restraining, and motor behavior was continuously monitored through videotaping. Similar to the virus injections, references for recordings in the sensorimotor putamen (posterolateral area) were provided by the preceding basal ganglia mapping. Standard techniques were used for striatal recordings. The activity of single cells was recorded with tungsten microelectrodes (FHC Inc, Bowdoin, ME; customized microelectrodes original impedance 2-4 MΩ at 1 kHz, reconditioned to 0.1-0.3 MΩ to improve the isolation of SPNs). Electrodes were lowered to the target area through an

electronically controlled microdrive (NAN Instruments Ltd, Nazaret Illit, Israel). Signals were amplified and high band-pass filtered at 0.8 kHz (sampling frequency = 30-40 kHz; Plexon Inc, Dallas, TX, and Blackrock Microsystems LLC, Salt Lake City, UT). Real-time sorting was used to isolate single cells and monitor their activity during testing. Typical firing patterns of tonically active cholinergic interneurons and fast-spiking GABAergic interneurons that could be recognized online by their waveform and activity pattern were excluded, and the search for units compatible with SPNs (1) continued for data collection in the "off" state. The final classification of units was always performed with offline analysis (see below). Before proceeding with an isolated SPN to record activity changes before and after L-Dopa injection, three to four different SPNs were recorded (3 min segments) in the "off" state as a measure of changes in baseline firing frequency throughout the course of the 12^{th} week period. Subsequently, the last isolated SPN was monitored for several minutes before recording the baseline segment ("off" state) and proceeding with L-Dopa injection at the predetermined dose (s.c.) for the subsequent recording segments. As described in Fig. 3A, after 3-min of data collection for the "off" state and subsequent L-Dopa injection, the isolated SPN was continuously recorded while monitoring the animal's behavior for changes indicating the transition to the "on" state (rapid movements of the eyes, increased blinking, occasional yawning, and limb stretching). The onset of the "on" state began approximately 15-20 minutes after the s.c. injection of L-Dopa. The SPN activity was collected again for 3 min corresponding to the "on" state following recognition of clear behavioral changes. The LID state usually developed 15-20 minutes after turning "on" (30-40 min after L-Dopa injection) and was indicated by the presence of typical movements unique to each animal but most

commonly choreiform and dystonic movements of the legs and arms. After clear dyskinesias were observed, the SPN activity was collected again for 3 min for the dyskinesia state. However, as animals were not exposed to regular daily L-Dopa treatment, LID was generally not present in the rAAV-eGFP group. In the absence of LID, data were stored from 37-40 min after L-Dopa injection for an equivalent time period of 3 min. The stored segments were always ≥ 3 min. If the baseline activity was held throughout the total duration of the experiment, the offline analysis yielded 1 or 2 units per experiment.

In the "off" state, the activity of multiple SPNs was collected in every weekly session before L-Dopa injection for a total of 14 to 36 neurons per week in each rAAV-eGFP (n=2) and rAAV-ΔFosB (n=2) group. A total of 624 SPNs during "off" state were analyzed from week 1 to 12. A total of 181 SPNs were recorded continuously in complete experiments of L-Dopa injection ("off", "on" and "LID" states) from week 1 to 12.

Immunohistochemistry. Animals were euthanized following IACUC guidelines and perfused through the heart with saline solution. The brain was collected, blocked, and each hemisphere randomly assigned to fresh dissection of the striatum for immunoblotting (IB) or post-fixation for immunohistochemistry (IHC). Brain tissues from a total of 6 randomly selected animals, 3 monkeys injected with rAAV-ΔFosB and 3 monkeys injected with rAAV-eGFP, were used for IHC. Brain blocks were fixed overnight in 4% paraformaldehyde in PBS, and then immersed in PBS containing 10% and 20% sucrose for 24 h, respectively. Coronal sections (40-μm) were cut serially using a freezing microtome, and free-floating sections were washed in PBS containing 0.05%

Triton X-100 (PBS-T) and then incubated for 30 min with 0.3% H_2O_2 to quench endogenous peroxidase activity. Sections were soaked with blocking agents and then incubated with primary antibodies dissolved in dilution reagent at 4°C for 24 h. Normal Goat Serum Blocking Solution (S-1000, Vector Laboratories, Burlingame, CA) was used for blocking. A rabbit polyclonal antibody against FosB (1:100, sc-48, Santa Cruz Biotechnology, Dallas, TX) was used as the primary antibody. Goat biotinylated anti-rabbit immunoglobulin (BA-1000, Vector Laboratories) was used as the secondary antibody. After incubating sections for 60 min with VECTASTAIN® ABC reagent, reaction products were visualized using DAB substrate kit (PK-6100 and SK-4100, both reagents from Vector Laboratories). For double IHC, two primary antibodies were combined, including antibodies against FosB (1:100, rabbit polyclonal, Santa Cruz Biotechnology) and NeuN (1:100, mouse monoclonal, MAB 377, Millipore, Burlington, MA). Alexa Fluor® 488 goat anti-rabbit IgG (H + L) antibody (A-11008, ThermoFisher Scientific, Waltham, MA) and Alexa Fluor® 568 goat anti-mouse IgG (H + L) antibody (A-11004, ThermoFisher Scientific) were used as the secondary antibodies. Images were obtained using ECLIPSE E800 (Nikon, Tokyo, Japan). For cell counting, 10 images were obtained using a digital camera connected to a microscope (20× objective) in each animal, and FosB-positive cells were counted (2).

Immunoblotting. Immediately after harvesting the brains, the striatum was dissected and rapidly stored at -80 °C until processing. Striatal tissue was homogenized in 1% SDS in PBS containing phosphatase inhibitor cocktail set II (Calbiochem, La Jolla, CA) and protease inhibitor cocktail set V (Calbiochem). Lysates (50 µg) were mixed with lithium

dodecyl sulfate-sample loading buffer (Life Technologies, Grand Island, NY), electrophoresed on a NuPage 4–20% (GenScript, Piscataway, NJ), and separated proteins were transferred to a polyvinylidine fluoride membrane (Bio-Rad, Hercules, CA). Membranes were blocked with 5% BSA (Sigma-Aldrich, St. Louis, MO) in Tris-buffered saline and 0.1% Tween-20 before probing with antibodies. ECL Plus (Perkin-Elmer, Waltham, MA) was used to develop immunoblots, and band intensity was quantified by ImageJ. Primary antibodies used were: FosB and pERK (Cell Signaling Technology, Danvers, MA); DARPP-32, p-T34-DARPP-32, and Cdk5 (Santa Cruz Biotechnology, Dallas, TX); and β-actin (Sigma-Aldrich, St. Louis, MO).

Data Analysis and Statistics. Behavioral data were compared across animal groups of virus injections (rAAV-ΔFosB or rAAV-eGFP) and time. Data were processed to obtain the following parameters; 1) total dyskinesia score, 2) peak dyskinesia score, 3) itemized scores, i.e.: dyskinesia observed in neck and face (cephalic), upper limbs, or lower limbs, and 4) total MDS scores at 0 min ("off" state) and 70 min after L-Dopa injection ("on" state). These scores were calculated for each animal pre-virus injection (baseline score) and at each of the 12 weeks of testing post virus injection. All data composed continuous variables (decimal points in score values) and were analyzed with ANOVA for repeated measures and *post-hoc* Fisher's test if the ANOVA *F* value was significant.

Electrophysiology data were analyzed first with offline spike-sorting of each recording segment separately using principal component analysis (PCA; Plexon Offline Sorter). After sorting, specific waveform parameters in the clusters were applied to classify units (1, 3). In addition, we applied the established criteria for striatal unit

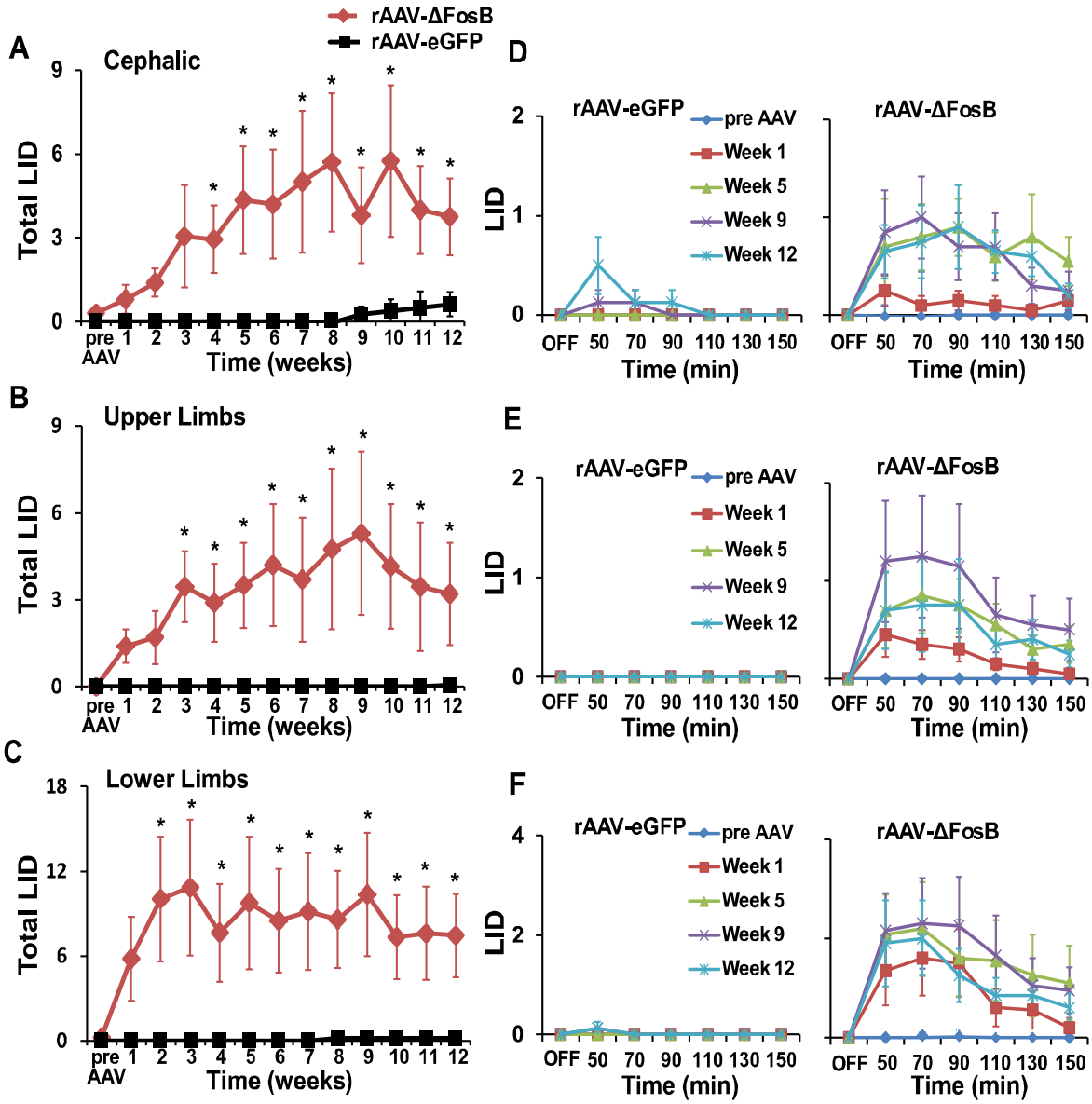
classification according to activity parameters as previously described (1). Units classified as TANs and FSIs or unclassified units (ambiguous parameters) were excluded from further analysis. Classified SPNs in the "off" state were followed in subsequent segments of the experiment for comparison, verifying consistency of cell isolation through all segments. The obtained sorted and classified data were then minimally post-processed using Matlab (MathWorks, Natick, MA). Spike trains for each segment (180 seconds at a minimum) were analyzed for firing frequencies binned at 1 second. Mean frequencies of pools of units recorded in the "off" state throughout the 12-week period were compared between virus groups with two-way ANOVAs. Mean frequencies of each unit in the "off", "on", and dyskinesia states were compared with one-way ANOVAs for repeated measures. *Post-hoc* Bonferroni's test was applied when the ANOVA *F* value was significant ($p < 0.01$). SPNs with significantly increased or decreased firing frequency in the "on" state ($p < 0.01$) were separated accordingly, i.e. increase or decrease response in the "on" state. Only three cells with no significant firing rate change in the "on" state ($p > 0.05$) were excluded from further analysis. We did not use a predetermined threshold of change in the "on" state for inclusion, which would be arbitrary; instead, we included all units with changes that were statistically significant. In each SPN, frequency changes in the dyskinesia state determined if the increased or decreased firing rate in the "on" state was stable or not. Unstable responses were defined by statistically significant ($p < 0.01$) firing rate changes in the dyskinesia state in the opposite direction to the changes developed in the onset of the "on" state, as described in Liang et al. 2008. The progression of unstable responses to L-Dopa was also analyzed over the 12-week period in each group of rAAV and then correlated with the

development of LID.

Data availability

The data that support the findings of this study are available from the corresponding author upon request.

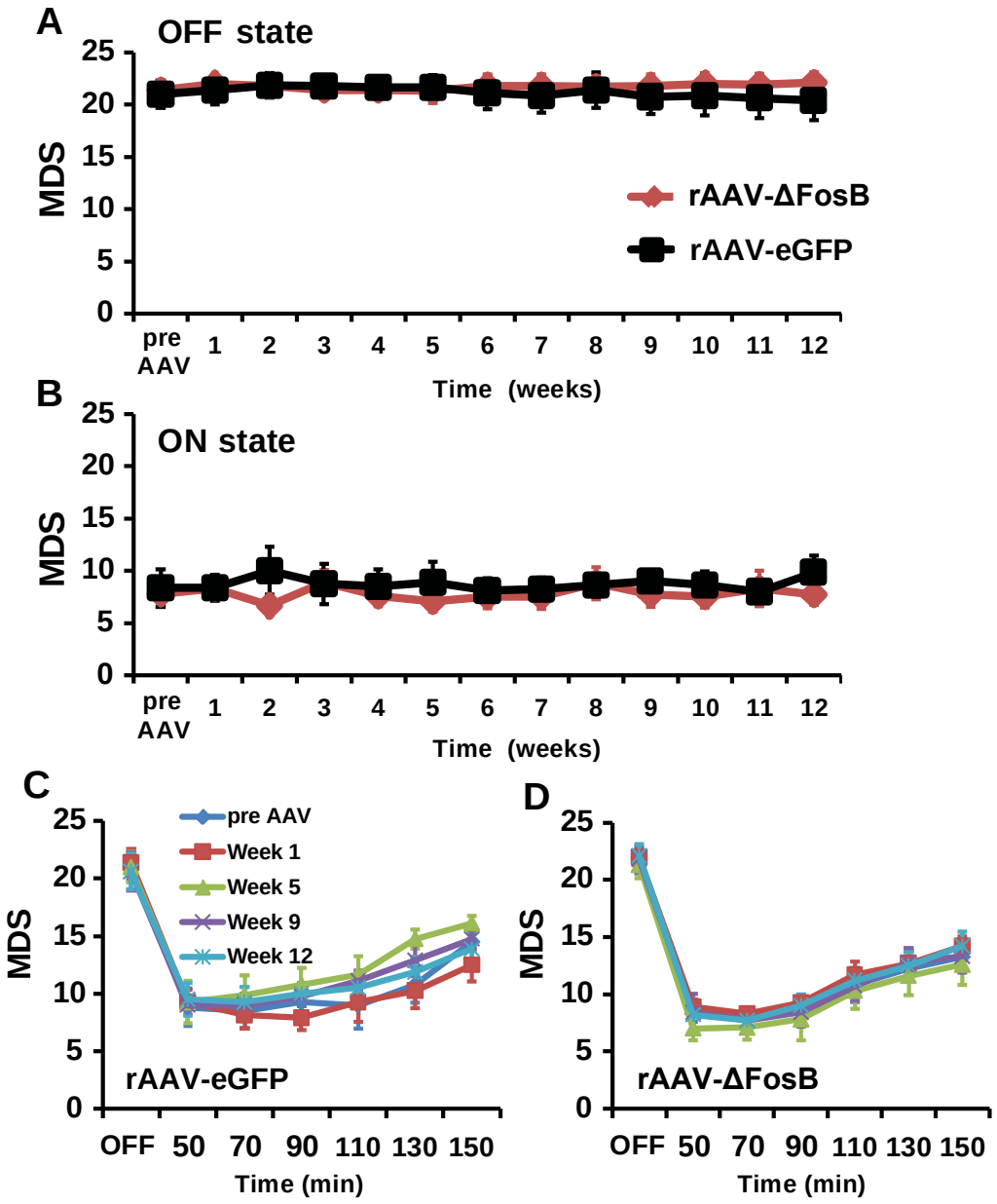
Fig. S1.



Striatal overexpression of ΔFosB induces generalized L-Dopa-induced dyskinesias including face and neck (cephalic), and upper and lower limbs. (A-C) Total LID scores developed during the course of 12 weeks after virus injection in cephalic segment (A), upper limbs (B), and lower limbs (C). Animals infused with rAAV-ΔFosB into the striatum showed LID in each part of the body from the first s.c. L-Dopa injection after

virus injection (week 1). Animals infused with control rAAV-eGFP virus into the striatum started to develop only mild LID in cephalic and lower limbs 9 weeks after virus injection (only two animals exhibited dyskinesia). (D-F) Time course of total LID scores in cephalic segment (D), upper limbs (E), and lower limbs (F) in animals infused with rAAV-eGFP (left) or rAAV-ΔFosB (right) before rAAV injection (pre-AAV, blue) and during the weeks following rAAV injection (weeks 1 (red), 5 (green), 9 (purple), or 12 (light blue). In each part of the body, animals infused with rAAV-ΔFosB showed higher LID scores compared with animals infused with rAAV-eGFP. OFF = before s.c. injection of L-Dopa. Data are means ± SEM. * $p < 0.05$, two-way ANOVAs for repeated measures followed by Fisher's PLSD test.

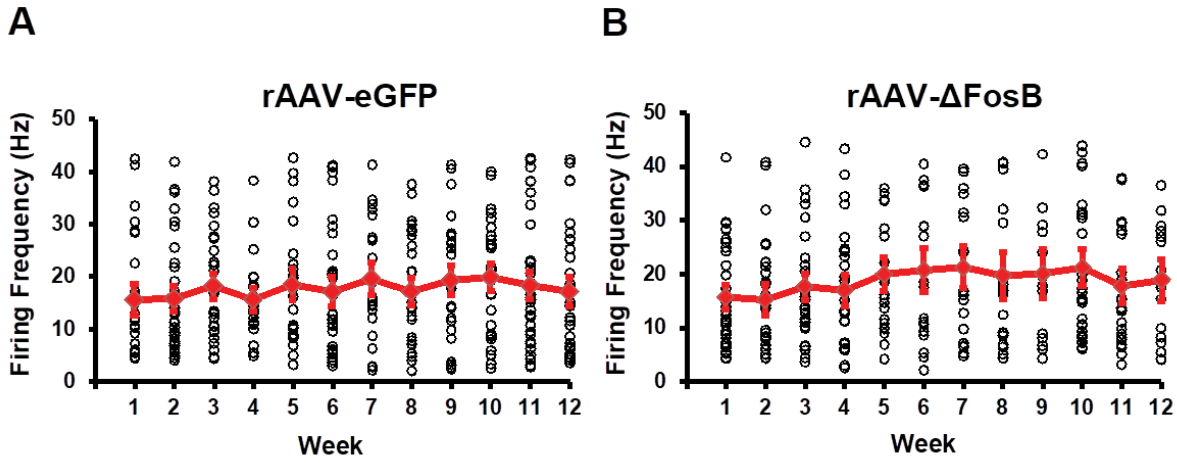
Fig. S2.



Motor disability scores (MDS) showing no changes in parkinsonism or the anti-parkinsonian action of L-Dopa after virus infusion. (A, B) MDS in the OFF state (A) and the ON state (70 min after s.c. L-Dopa injection; B) in animals infused with rAAV-ΔFosB (red) or rAAV-eGFP (black) before (pre-AAV) and after virus infusion (from week 1 to week 12). There is no difference in MDS in both the OFF and ON states

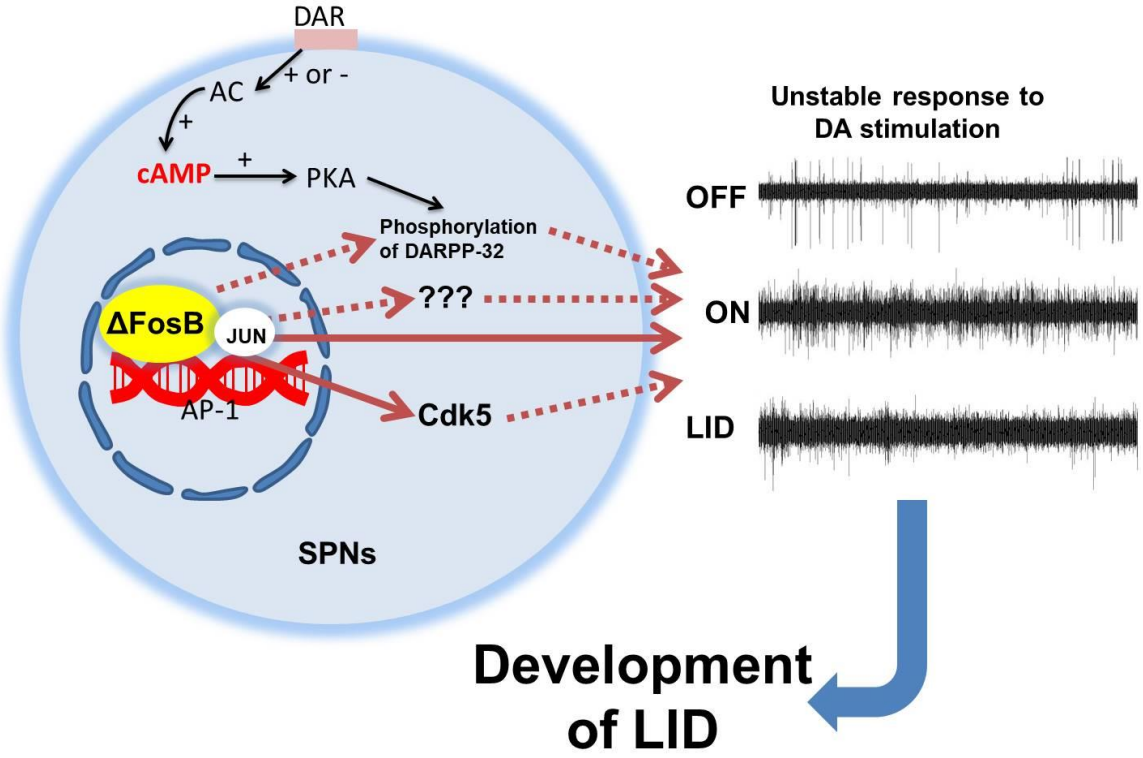
between animals infused with the two rAAV vectors and throughout the testing period of 12 weeks. (C, D) Time course of absolute MDS following s.c. L-DOPA injection in animals infused with rAAV-eGFP (C) or rAAV-ΔFosB (D) before virus injection (pre-AAV) and at weeks 1, 5, 9, and 12 after virus injection.

Fig. S3.



Baseline firing frequencies of SPNs showing no difference following virus infusion in the striatum. (A, B) Baseline firing frequencies (Hz) of each individual SPN recorded in OFF state (before s.c. injection of L-Dopa) are plotted as small circles at each time point (weeks 1-12) in the group of animals infused with rAAV-eGFP (A) or rAAV-ΔFosB (B). The "off"-state SPN firing frequency was variable within the same range in the two viral vector groups, ranging from 2 to 44 Hz in these moderately parkinsonian animals where hyperactivity may not be as largely distributed across units as in animals with more advanced parkinsonism. Average of baseline SPN firing frequencies at each time point shown as red lines ranged from 16.3 to 19.6 Hz in the rAAV-eGFP group and 15.5 to 19.6 Hz in the rAAV-ΔFosB group. Striatal infusion of rAAV-eGFP or rAAV-ΔFosB resulted in similar baseline activities of SPNs (averages and variance) over the time period of recordings (12 weeks). In each group, baseline SPN firing frequencies also remained unchanged from week 1 to 12, which is congruent with no changes in the severity of parkinsonism expressed by MDS in the "off" state.

Fig. S4.



Schematic diagram of molecular mechanisms underlying LID. Over-expressed ΔFosB heterodimerises with Jun family proteins and translocates to the nucleus of striatal projection neuron (SPN). Then, the ΔFosB-Jun heterodimer undergoes complex formation with activator protein-1 (AP-1) on DNA. Thereafter, this complex induces transcriptional changes regulating a variety of genes (kinases, phosphatases and other molecules) which directly or indirectly, via upregulation of Cdk5 (Cyclin dependent kinase 5), phosphorylation of DARPP-32 (DA- and cAMP-regulated phosphoprotein, 32 kDa) or changes in other signaling molecules, lead to unstable changes of the SPN firing frequency in response to dopamine (DA). This altered dopamine response across SPNs is associated with the appearance of L-Dopa-induced dyskinesia (LID). DAR; dopamine receptor, AC; adenylyl cyclase, cAMP; cyclic adenosine monophosphate, PKA; protein kinase A.

Table S1.

Subjects and clinical data

Monkey	Age (yr)	Sex	Virus	Chamber	MDS OFF	MDS ON	IHC	IB
#1	6	M	eGFP	+	22	9.5	YES	YES
#2	7	M	eGFP	+	20	6.5	YES	YES
#3	6	F	eGFP	-	17	10.5	YES	YES
#4	6	F	eGFP	-	20.5	5.5	NO	YES
#5	5	M	ΔFosB	+	21	5.5	YES	YES
#6	6	M	ΔFosB	+	20	8	YES	YES
#7	7	F	ΔFosB	-	21	9.5	YES	YES
#8	6	M	ΔFosB	-	16.5	9	NO	YES
#9	7	M	ΔFosB	-	18	10.5	NO	YES

Monkeys #1-4 received rAAV-eGFP injections, and monkeys #5-9 received rAAV-ΔFosB injections. In monkeys #1, 2, 5, and 6, chambers were surgically implanted in preparation for electrophysiological experiments. MDS = motor disability score in the "OFF" or "ON" state. F = female; M = male. IHC = immunohistochemistry. IB = immunoblotting.

Table S2.

Distribution of SPN responses to dopamine

A. rAAV-eGFP group

	Stable change				Unstable change			
Increase in ON	(n)	OFF	ON	DYS	(n)	OFF	ON	DYS
Month 1	9	13.5	22	25.7	3	10.5	20.6	10.8
Month 2	10	13.8	21.4	25.2	6	14.9	22.8	14
Month 3	8	19.1	26.1	29.8	8	16	30.9	21.9

	Stable change				Unstable change			
Decrease in ON	(n)	OFF	ON	DYS	(n)	OFF	ON	DYS
Month 1	14	23.3	16.5	13.4	2	30.3	20.4	27.6
Month 2	8	22.4	14.9	13.9	9	23.3	14.6	22.4
Month 3	12	21.4	14.8	11.9	9	24.4	16.9	24.9

B. rAAV-ΔFosB group

	Stable change				Unstable change			
Increase in ON	(n)	OFF	ON	DYS	(n)	OFF	ON	DYS
Month 1	4	13.7	20.2	24.1	13	15.8	25.3	17.4
Month 2	2	5.4	13.1	24.2	8	11.7	18.4	10.3
Month 3	2	10.3	13.2	14.1	6	18.4	26.8	16.4

	Stable change				Unstable change			
Decrease in ON	(n)	OFF	ON	DYS	(n)	OFF	ON	DYS
Month 1	3	22.1	17.4	14.4	9	18.7	9.2	17.4
Month 2	9	21	12.8	9.6	9	23.8	15.3	23.1
Month 3	7	20.8	11.7	8.1	11	18.9	11.7	18.8

Averages of firing frequencies (Hz) of SPNs grouped according to the type of DA response (increase or decrease in the ON state followed by stable or unstable response in the dyskinesia [DYS] state) in both animal groups (rAAV-eGFP or rAAV-FosB). Data were generated in weekly recordings (weeks 1-12) in each animal. Monthly averages are presented for each group for comparison (Month 1-3). Results correspond to data in Figs. 5C-F and 6C-F. Frequency changes in the transition to motor states in individual neurons are $p < 0.001$. n = number of SPNs that were analyzed.

Table S3.

Topographic distribution of LID

	Transgenic model (the present study)	Models of chronic L-Dopa therapy (previous studies)
	(n = 5)	(n = 5)
Cephalic (Neck & Face)	4.4 ± 1.6	6.1 ± 2.3
Upper extremities	3.6 ± 1.9	6.7 ± 2.7
Lower extremities	8.7 ± 2.9	12.3 ± 3.1

LID scores in the transgenic model obtained at week 12 post-virus injection and in models of chronic L-Dopa treatment after stabilization of consistent dyskinesias. For details of animals exposed to chronic (daily) L-Dopa treatment, please refer to our previous study (Beck et al., Mov Disord 2018, ref #27). In both groups (n=5, each group), LID was similarly distributed in body segments, and was most prominent in lower extremities. As expected, fully developed LID reached higher scores in all body segments in the group of animals prepared with more severe parkinsonism and maintenance on regular L-Dopa treatment for typical LID studies. Data are shown as means ± SEM.

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