RESEARCH ARTICLE

Striatal Activation by Optogenetics Induces Dyskinesias in the 6-Hydroxydopamine Rat Model of Parkinson Disease

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ABSTRACT: **Background**: Long-term levodopa (L-dopa) treatment is associated with the development of L-dopa–induced dyskinesias in the majority of patients with Parkinson disease (PD). The etiopathogonesis and mechanisms underlying L-dopa–induced dyskinesias are not well understood.

Methods: We used striatal optogenetic stimulation to induce dyskinesias in a hemiparkinsonian model of PD in rats. Striatal dopamine depletion was induced unilaterally by 6-hydroxydopamine injection into the medial forebrain bundle. For the optogenetic manipulation, we injected adeno-associated virus particles expressing channelrhodopsin to stimulate striatal medium spiny neurons with a laser source.

Results: Simultaneous optical activation of medium spiny neurons of the direct and indirect striatal pathways in the 6-hydroxydopamine lesion but L-dopa naïve rats induced involuntary movements similar to L-dopa–induced dyskinesias, labeled here as optodyskinesias. Noticeably, optodyskinesias were facilitated by L-dopa in animals that did not respond initially to the laser stimulation. In general, optodyskinesias lasted while the laser stimulus was applied, but in some instances remained ongoing for a few seconds after the laser was off. Postmortem tissue analysis revealed increased FosB expression, a molecular marker of L-dopa–induced dyskinesias, primarily in medium spiny neurons of the direct pathway in the dopamine-depleted hemisphere.

Conclusion: Selective optogenetic activation of the dorsolateral striatum elicits dyskinesias in the 6-hydroxydopamine rat model of PD. This effect was associated with a preferential activation of the direct striato-nigral pathway. These results potentially open new avenues in the understanding of mechanisms involved in L-dopa–induced dyskinesias. © 2017 International Parkinson and Movement Disorder Society

Key Words: dyskinesias; optogenetics; Parkinson disease; medium spiny neurons; L-dopa

L-dopa–induced dyskinesias (LIDs) in Parkinson disease (PD) continue to be a troublesome complication for a large proportion of patients undergoing chronic treatment.[1] Striatal dopamine depletion secondary to degeneration of the substantia nigra *pars compacta* (SNpc) neurons combined with nonphysiological dopaminergic stimulation caused by high L-dopa dosing are the principal pathogenic factors commonly associated with LIDs.[2]

In the classic pathophysiological model activation of medium spiny neurons (MSNs), the direct striato-nigral projection neurons, which express dopamine receptor 1 (DR1), facilitate movement, whereas activation of the indirect striato-pallidal pathway, which expresses dopamine receptor 2 (DR2), inhibits movement. Modern studies with optogenetics confirmed this general notion.[3] However, this dual-opposing physiological effect is under revision because there is growing experimental evidence indicating that both pathways must be activated during normal movement.[4-6] L-dopa acts on

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both pathways with opposite effects, and both pathways appear implicated in the pathophysiology of LIDs. Experimental evidence using molecular markers of MSN activation and dopamine receptor knockout mice indicate a predominant role for direct pathway (DR1 mediated) neurons[7,8] in the induction of LIDs. Moreover, studies in the MPTP monkey model also support a predominant DR1 role in the origin of LIDs.[9] Recently, indirect pathway (DR2 mediated) neurons were also implicated, exhibiting an abnormal increase in dendritic spines after L-dopa administration.[10,11] Importantly, all relevant dopaminergic antiparkinsonian drugs so far used in PD patients activate DR2 receptors, and all can induce dyskinesias at different degrees.

The pathophysiology of LIDs has classically been based on dopamine-synaptic and receptor-mediated mechanisms.[12] In addition to abnormal activation of denervated dopamine receptors by L-dopa,[13] changes in receptor density, membrane internalization, and coupling of dopamine D1 receptors as well as reduced dopaminergic synaptic plasticity[14] have been profusely ascertained and discussed as the origin of LIDs.[2]

Here we have used a widely validated LIDs model, the 6-hydroxydopamine (6-OHDA) hemiparkinsonian rat, combined with optogenetic striatal activation to show that abnormal movements similar to LIDs can be elicited by direct activation of striatal projection neurons, bypassing the dopaminergic synapsis and receptor mechanisms traditionally associated with LIDs. Direct and specific induction of dyskinesias by laser stimulation (henceforth optodyskinesias) may allow us to better understand the circuits and mechanisms of LIDs.

Material and Methods

Animals and Stereotactic Procedure

All experimental procedures were approved by the Committee on Animal Care of the HM-CINAC and were in accordance with the National Research Council's Guide for the Care and Use of Laboratory Animals (RD 53/2013). A total of 14 adult, male, Sprague-Dawley rats (300-350 g) were housed individually with reverse light cycle (lights on: 9:00 PM-9:00 AM), and all experiments were performed during their active cycle (dark period).

To deplete dopaminergic terminals, rats (n = 9) were anesthetized with a mixture of ketamine (100 mg/kg) and xylazine (10 mg/kg) i.p., and 6-OHDA (20 μg [4 μl, 5 mg/ml]) was injected at a flow rate of 1 μl/min in the right medial forebrain bundle following the coordinates from lambda: AP, + 4.2 mm; ML, −1.2 mm; DV, −7.5 mm (Fig. 1A). Dopaminergic depletion was assessed in all animals after finishing the behavioral studies with TH immunostaining in the coronal brain sections. Only animals with a TH loss higher than 95% were included in the study (Fig. 1B).

The viral vector carrying the channelrhodopsin (1.5 μl; AAV2-CaMKIIa-hChR2(H134R)-EYFP, 2.46×1013 particles/μl; Vector Core, North Carolina University, Chapel Hill, North Carolina) was injected on the right dorsolateral striatum (bregma: AP, + 0.5 mm; ML, −3.6 mm; DV, −4.5 mm) at a flow rate 0.1 μl/min. Optic fiber (length: 2.5 mm; diameter: 200 μm; NA: 0.37; Thorlabs, Newton, New Jersey) was placed 300 μm right above the virus injection side (see Fig. 1A,B). This was anchored to the animal skull with a small screw (JI Morris, Southbridge, Massachusetts) and dental cement (Lang Dental Manufacturing Co., Inc., Wheeling, Illinois).

A group of control rats, non-dopamine-depleted but expressing ChR2, (n = 5) was also used.

Optogenetic Experiments

At 3 weeks after the surgery, the animals were handled and habituated to the experimental room for a week. After this period, the experiments started following 2 stimulation protocols. A blue laser source (300 mW, 473 nm; CNI, Qingdao City, China) was calibrated daily before every experiment, measuring the power at the end of the tip of the fiber before and after every session (10 mW). The experiments were performed in the animal's cage. After 10 minutes, basal time was established before starting the video recording, which lasted for the length of the stimulation session.

We established 2 different stimulation protocols to characterize the temporal dynamics of optodyskinesias and to determine the minimal stimulus duration needed to evoke a dyskinetic movement: (1) a basal period of 3 minutes (prelaser) followed by continuous optogenetic stimulation during 3 minutes (laser ON) and 3 minutes with laser off (postlaser stimulation), and (2) a basal period of 3 minutes followed by continuous optogenetic stimulation during 10 seconds followed by a 30-second laser off period (poststimulus) that was repeated 9 times.

L-Dopa Administration

Animals with 6-OHDA lesions that did not respond to the first laser protocol (n = 3) were treated daily (1 ml/kg; i.p.) for 5 days with a mixture of L-dopa (25 mg/kg, methyl L-dopa hydrochloride; Sigma-Aldrich) and benserazide (10 mg/kg, benserazide hydrochloride; Sigma-Aldrich).

Dyskinesia Scoring

The abnormal movements (optodyskinesias/LIDs-like behavior) were evaluated by placing the animals in their home cages surrounded by a mirror to facilitate the visualization of the animal behavior and were rated by a trained observer (Fig. 1C). The stereotyped

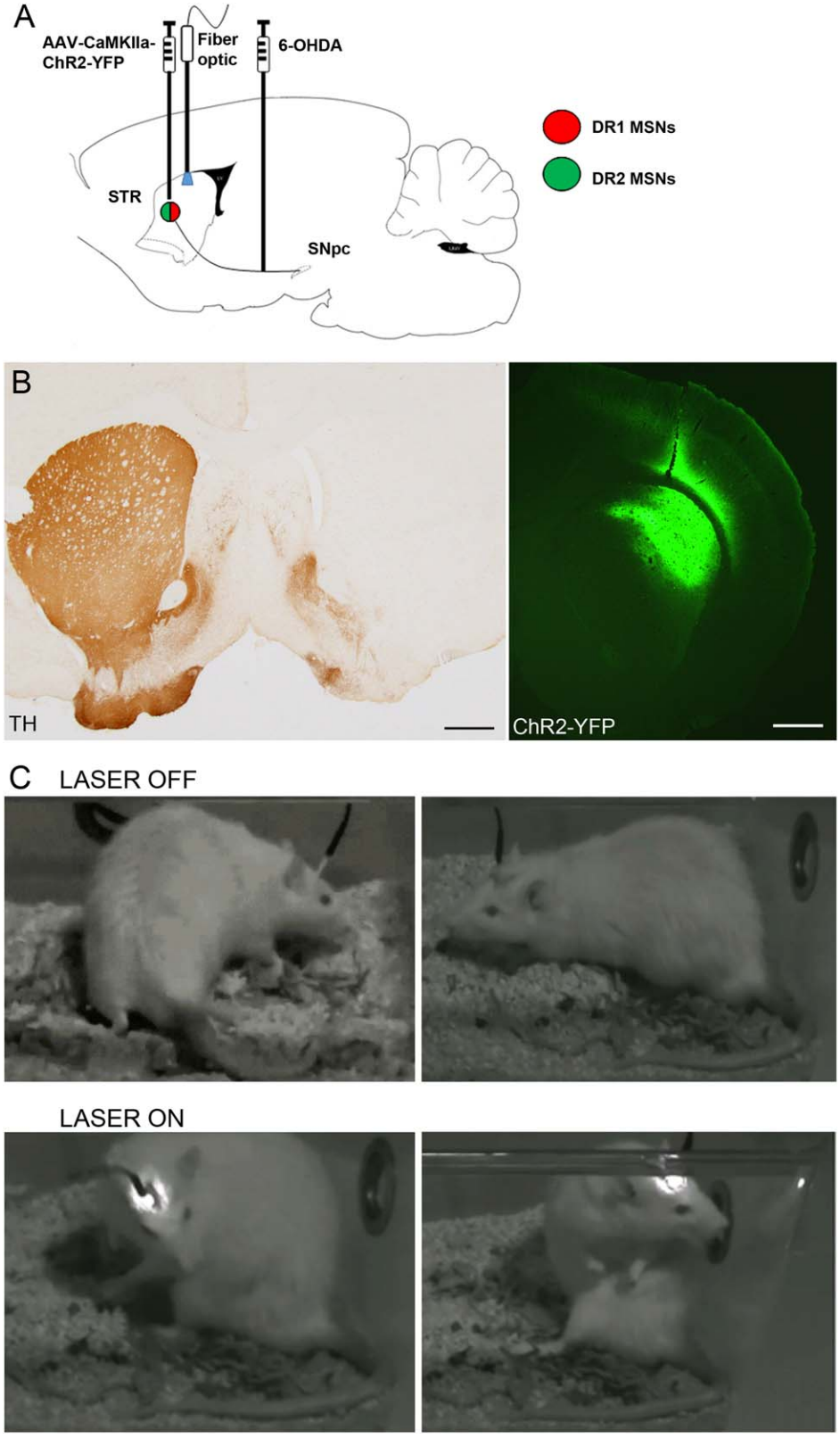

FIG. 1. Striatal optogenetic activation elicits dyskinesias in a PD model. (**A**) Schematic representation of the AAV2-CaMKIIa-hChR2-(H134R)-EYFP and 6-hydroxydopamine (6-OHDA) administration sites. (**B**) Immunostaining for Tyrosine hydroxylase (TH) showing striatal dopamine depletion (left) and dorsolateral striatum expression of Channelrhodopsin-2 (ChR2) in the dopamine depleted striatum (right). Scale bar 1 mm. (**C**) Photograms of 2 rats with laser OFF (basal) and ON, latter photograms displaying abnormal movements: optodyskinesias. STR, striatum; SNpc, Substantia Nigra pars compacta; DR1, dopamine receptor 1; DR2, dopamine receptor 2; MSNs, medium spiny neurons.

behavior that we quantified in this study was: i) abnormal movement of the forelimb contralateral to the dopamine depletion side (right) and ii) axial torsion, an abnormal neck/trunk torsion towards contralateral to the dopamine depletion side (Fig. 1C). We quantified the duration in seconds spent displaying each behavior during the stimulation period. Scores were obtained for both the ON and OFF laser stimulation periods. Protocol A was represented as total time (in seconds) spent exhibiting each behavior, and in protocol B data were shown as the percentage of time exhibiting each behavior during the ON and OFF periods. We also measured the intensity in the ON laser stimulation periods as follows: (a) for forelimb dyskinesia, 1 = repetitive paw movements, 2 = abnormal extension of the limb; (b) for axial torsion, 1 = head and neck torsion in 45° to 90° angle, 2 = head, neck torsion, and upper trunk in a 60° to 90° angle, 3 = upper trunk torsion and neck torsion > 90°.[15] For the L-dopa–treated animals, the same abnormal movements were quantified during 3 minutes for the 30 minutes immediately after the L-dopa injection (see also ref. [16]).

Brain Tissue Collection and Processing

90 minutes after the last laser stimulation, the animals were deeply anaesthetized and transcardially perfused (sodium pentobarbital, 100 mg/kg) using saline (NaCl al 0.9%, 250 ml) followed by 4% PFA (PB 0.1 M; pH 7.3; 250 ml) at a constant rate (38 ml/min). The brains were removed and stored in a cryoprotectant solution (PB 0.1 M, azida 0.1%, glycerol) until they sank. The striatum was cut into coronal sections (30-μm thick) in a freezing microtome (Leica Microsystems, Spain). Tissue was stored at 4 °C in PB solution (0.1 M, pH 7.3, 0.02% azida) until immunohistochemical or immunofluorescence procedures were performed.

Immunostaining

Immunohistochemistry was performed on free-floating coronal brain sections as described previously,[17,18] with the following rabbit antisera: tyrosine hydroxylase (TH, 1:1000; Millipore, Massachusetts) and FosB (1:7500; Santa Cruz Biotechnology, Santa Cruz, California). For the immunofluorescence experiments, we used Alexa fluor 488-conjugated secondary antibody (1:1000; Invitrogen, Carlsbad, California).

Quantification of FosB immunoreactivity was carried out using Image J (1.42°; National Institutes of Health, Bethesda, Maryland).[19] Immunostaining intensity and the number of immunolabeled nuclei/cells were determined for all animals in each group using 5 serial rostrocaudal sections per animal from both hemispheres: the dopamine-depleted side and the control side (intact). Digital images were obtained under a Leica microscope using a 10 × objective. The data are presented as the number of stained nuclei per square millimeter in the following 3 regions of interest: striatal regions (dorsomedial, dorsolateral, and ventral) from dopamine-depleted and control striatum. Each region of interest was 1.18 mm^2.

Double-Labeling Immunohistochemistry

Double-labeling immunohistochemistry studies were performed as described previously.[8,20] Briefly, immunostaining was carried out in the free-floating coronal brain sections (30-μm thick) using a standard avidin-biotin immunocytochemical protocol with the following rabbit antisera: dynorphin-B (1:10,000; Serotec, Oxford, United Kingdom) and FosB (1:7500; Santa Cruz Biotechnology, Santa Cruz, California). The two-color dual antigen DAB immunostaining was carried out because both antibodies of interest were developed in the same species. Immunostaining was obtained by sequential primary antibody reaction. Dynorphin-B immunostaining was developed using nickel-intensified DAB (purplish gray) and after avidin-biotin blocking, FosB was developed using the DAB-alone (brown) staining reaction.

Statistical Analysis

Behavioral data were analyzed by 2-way repeated-measures analysis of variance followed by the Tukey post hoc test. Immunohistochemistry (FosB) data were evaluated by analysis of variance followed by a Fisher post-hoc test. The analysis was performed with GraphPad Prism 6 (La Jolla, California). Data are expressed as the mean ± standard error of the mean (SEM) unless stated otherwise. The minimum significance level was $P \leq .05$.

Results

Optogenetic Activation of Striatal MSNs Causes Abnormal Movements in a PD Model

Animals were tested with a continuous pulse of blue laser stimulation (protocol A, 10 mW, 3 minutes ON) that was preceded and followed by a period without stimulation (3 minutes OFF). Laser stimulation generated abnormal movements on the animal's contralateral side to the dopamine depletion mainly in the forepaw and neck/trunk (Fig. 2A). In the forepaw, the movements mainly consisted in repetitive, forceful flexion/extension limb movements, whereas the neck/trunk was twisted and deviated toward the contralateral side to the dopamine-depleted hemisphere. These optodyskinesias highly resembled the movements evoked by L-dopa in hemiparkinsonian rats (Fig. 1C and Supplementary Videos). The same experimental stimulation protocol did not cause any motor behavior or abnormal motor manifestations in non-dopamine-

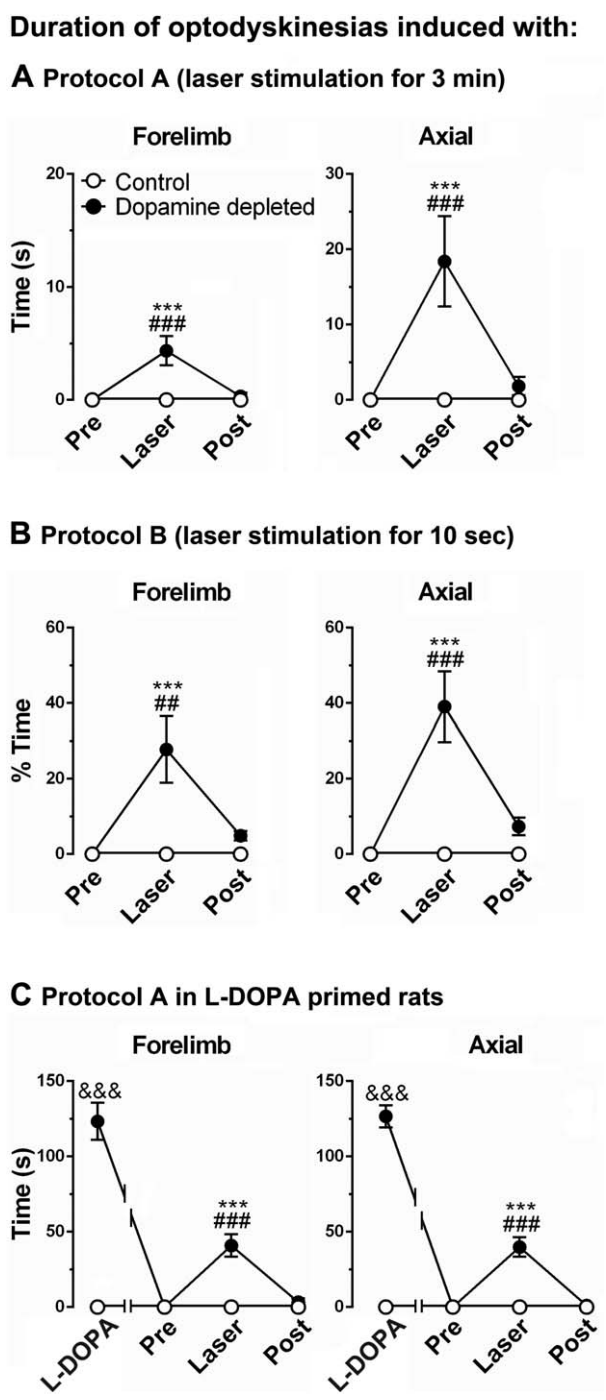

FIG. 2. Striatal optical stimulation in hemiparkinsonian animals induces abnormal involuntary movements. Duration of the optodyskinetic behavior is displayed for axial and forelimb movements. (**A**) Different behaviors analyzed before, during, and after laser stimulation in control and dopamine-depleted animals using protocol A. (**B**) Different behaviors analyzed before, during, and after laser stimulation in control and dopamine-depleted animals using protocol B. (**C**) Animals that did not initially respond to laser stimulation were treated with L-dopa and then responded to laser stimulation (protocol A; mean ± standard error of the mean). ***P <.001 versus prelaser; ###P <.001 versus post; &&&P <.001 versus laser.

depleted (control) animals (Fig. 2A). No rotations or orofacial abnormal behavior was observed in any animal.

The duration of the stimuli was modified to characterize the temporal dynamics of optodyskinesias and determine the minimal stimulus duration needed to evoke a dyskinetic movement. We decreased the laser stimulation to a 10-second pulse of continuous light at 10 mW followed by a 30-second off period (protocol B). This protocol was sufficient to induce optodyskinesias, similar to those evoked by protocol A, showing the axial and forelimb abnormal movements (Fig. 2B).

The severity of these opotdyskinetic behaviors was assessed for axial and forelimb movements. The intensity evoked by the longer stimulation protocol (A) was higher than the one induced by the shorter protocol (B). In any case, these behaviors showed lower severity than those induced by the laser in L-dopa pretreated animals (Fig. 3A-C).

L-Dopa Primes Optodyskinesias

An additional group of dopamine-depleted rats (n = 3) that initially did not respond to the laser using protocol A were then treated with L-dopa (25 mg/kg; i.p. daily for 5 days) developing typical LIDs. Subsequently, after the L-dopa priming, optodyskinesias were triggered in the same animals with laser stimulation only (Fig. 2C). However, no difference in the degree of dopamine depletion was found in the TH staining between L-dopa–primed and nonprimed rats. In addition, nondenervated animals did not exhibit any behavioral response to either L-dopa treatment or laser stimulation (Fig. 2C).

Optodyskinesias Share Common Molecular Markers With LIDs

The expression pattern of FosB, which is a well-recognized molecular marker associated with LIDs[7], was significantly up-regulated after laser stimulation in the dopamine-depleted side. However, as expected, FosB expression showed no change on the contralateral side (intact hemisphere) and in the control group (non-dopamine-depleted animals; Fig. 4A-C). Furthermore, double immunostaining combining FosB with dynorphin B, a marker of direct-pathway neurons (DR1) showed that FosB was primarily expressed in dynorphin-containing neurons, as known to occur in LIDs[8] (Fig. 4D).

Discussion

The data presented here indicate that activation of MSNs and, therefore, striatal efferent pathways, generates abnormal involuntary movements (optodyskinesias) similar to those evoked by L-dopa administration in dopamine-depleted animals. Importantly, in our protocol, this optodyskinetic behavior was evoked only in animals with striatal dopamine depletion, supporting the classic understanding of LIDs pathophysiology in

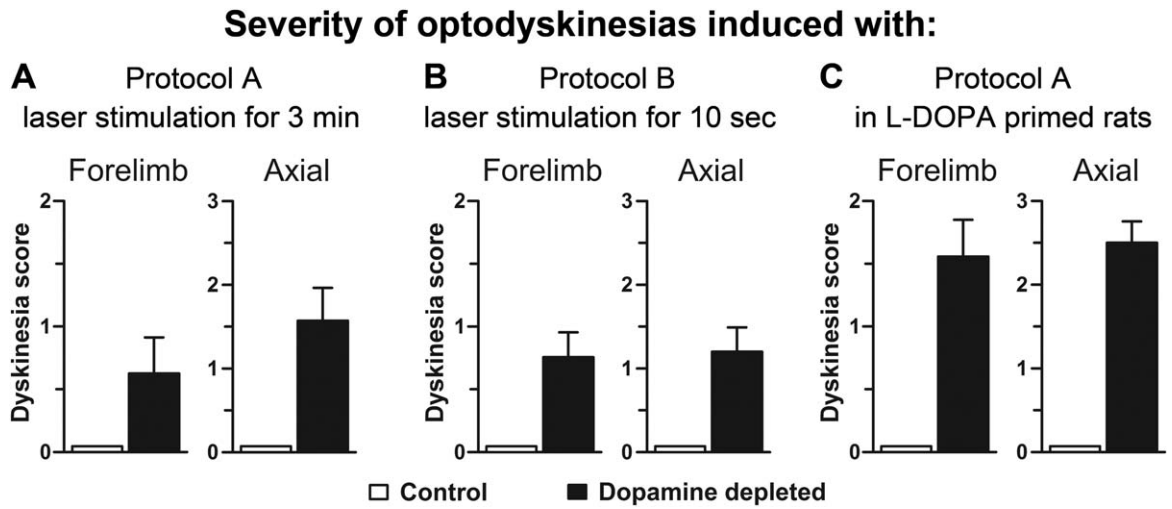

FIG. 3. Severity of the optodyskinetic behavior induced by laser stimulation. Graphs show the severity of the optodyskinetic behavior obtained using the different stimulation protocols: (**A**) longer stimulation (protocol A), (**B**) shorter stimulation (protocol B), and (**C**) protocol A in L-dopa–primed rats (mean ± standard error of the mean).

which dopamine depletion triggers a set of molecular and physiological changes in the motor circuit that facilitate the generation of dyskinesias in response to dopaminergic drug treatments.[21] Indeed, the abnormal movements elicited by L-dopa treatment and laser stimulation in this study are highly similar from the behavioral point of view, reinforcing the fact that both optodyskinesias and LIDs share similar mechanisms and pathways.

The fact that the laser acts on such a local area and still was able to evoke a plethora of dyskinetic-like movements is interesting. According to this local activation and time-restricted stimulation, the length and severity displayed in these movements is lower than the traditional LIDs. Despite the dopamine-depleted area being similar, the area reached by the pharmacological approach is wider in magnitude and impacts in a more dramatic way, reaching a wider striatal area. This may explain the differences in the length and intensity of the behavior observed between both methodologies to induce abnormal movements in the dopamine-depleted rat model, demonstrating why the optogenetic manipulation evokes a milder impact on the brain with the corresponding associated behavior.

The activation of MSNs induced by the laser stimulation was assessed by measuring FosB expression levels, an early gene that is a well-recognized, LID-associated molecular marker.[7] FosB expression was significantly up-regulated in MSNs on the dopamine-depleted side following laser stimulation, but there was no increase in FosB expression on the contralateral side to the dopamine depletion or in the control group of animals. These results are in agreement with previous studies that show selective FosB activation associated with LIDs in hemiparkinsonian mice.[7] In addition, using a double immunostaining technique, we further showed that FosB is expressed primarily in dynorphin-containing (DR1) neurons. These results imply that, similar to what has been described for the L-dopa–induced dyskinesias,[8] optogenetic activation induces dyskinesias, at least in part, mediated by recruiting D1-expressing MSNs.

The origin of LIDs have been classically linked to dysfunction of the dopamine-denervated striatum. However, the precise circuits involved in the generation of LIDs are not well defined, and indeed, the striatal origin of the abnormal activity leading to dyskinetic movements rest on limited direct evidence. Bateup and colleagues[22] described that inhibition of the striatal DARP-32 protein in direct projection abolished dyskinetic behavior in response to L-dopa in mice. However, the fundamental observation indicating that abnormal movement generation is mediated by the striatum comes from the observation in patients treated with dopaminergic mesencephalic fetal cell transplants. The 3 most experienced sites undertaking this procedure reported dyskinesias similar to LIDs as a direct consequence of the transplants.[23-25] Our results showing that abnormal motor behavior can be readily generated in the presence of severe striatal dopamine depletion by direct MSNs activation, bypassing the dopamine receptors and presumably without modifying any other striatal afferent or intrastriatal circuit, also serve to substantiate the fundamental involvement of the denervated striatum in the origin of LIDs. Thus, the focal but potent striatal stimulation induced by optogenetics can recruit and activate in an abnormal manner, the striatal projections mimicking the LIDs caused by L-dopa treatment in PD patients.

We admit that in addition to the dopaminergic system, LIDs are associated with disturbances in several

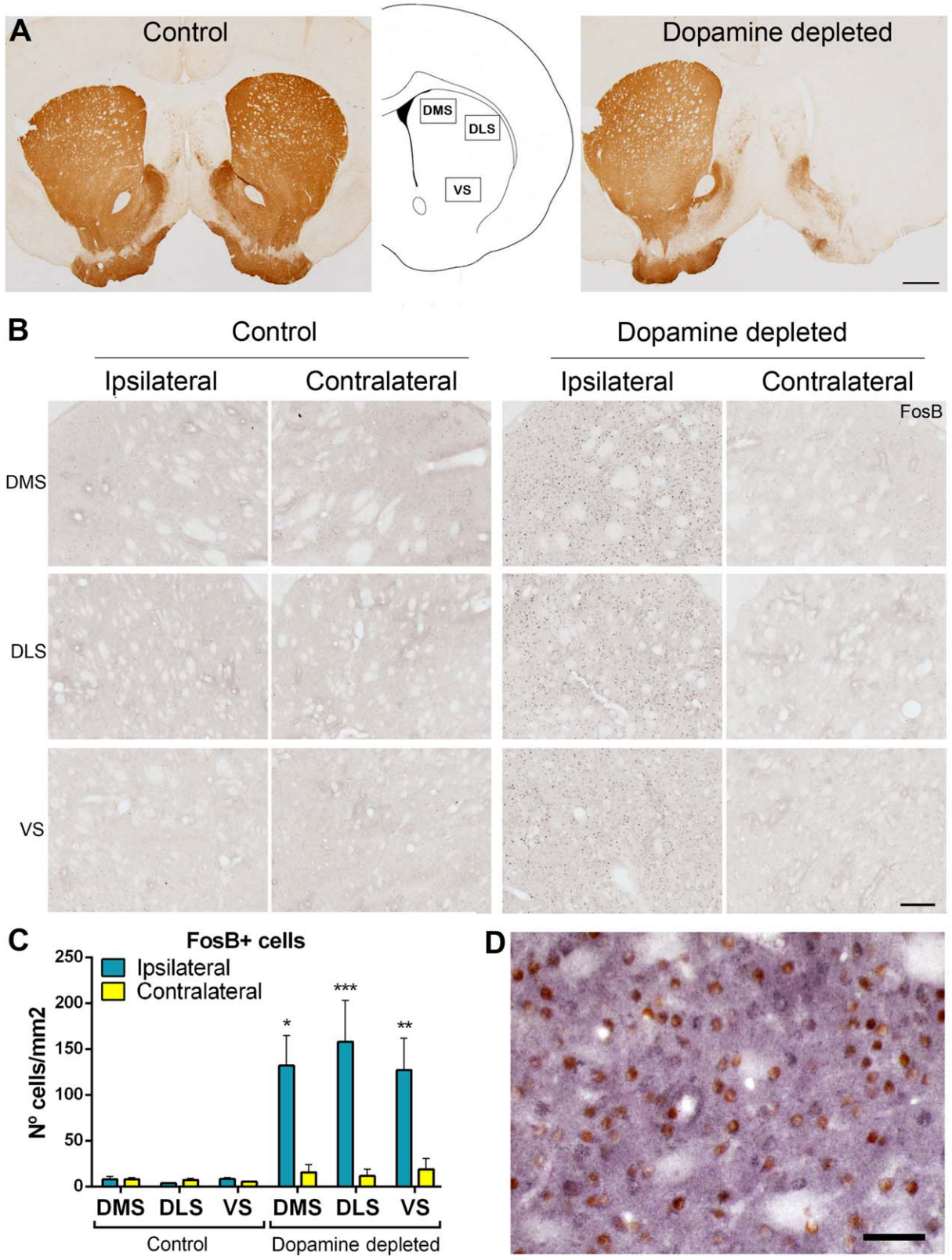

FIG. 4. Increased FosB expression after the optical stimulation in dopamine depleted animals. (**A**) TH immunostaining in a coronal section of control and dopamine-depleted rats. Diagram shows 3 areas magnified below: DMS, DLS, and VS. Scale bar 1 mm. (**B**) Inmunohistochemistry images showing FosB staining in the ipsilateral and contralateral side of the optogenetic stimulation in control and dopamine-depleted rats. Magnification 10×. (**C**) Histogram represents quantification of FosB-positive cells (mean ± standard error of the mean)/mm^2. (**D**) FosB colocalization in dynorphine-containing striatal neurons. Dynorphin (Dyn-B, purple) and FosB (brown) expression are shown under 40 × magnification (calibration bar 50 μm). DMS, dorsomedial striatum; DLS, dorsolateral striatum; VS, ventral striatum.*P <.05, **P <.01, ***P <.001.

other neurotransmitters and neuromodulators, including serotoninergic striatal activity,[26-28] cholinergic activity,[29,30] and glutamatergic cortico-striatal dysfunction in the parkinsonian state.[31] The impact of L-dopa treatment is clearly wider and less restricted than striatal optogenetics.

In sum, we present a new experimental protocol to induce dyskinesias in a PD animal model. These results open the possibility of unraveling the synaptic mechanisms and striatal microcircuits involved in the pathogenesis of LIDs and entail a powerful new approach to shed light on the mechanisms underlying LID generation and persistence. ■

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Supporting Data

Additional Supporting Information may be found in the online version of this article at the publisher's web-site.