Basal ganglia activity in a mouse model of dyskinesia

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Contents

Abstract

Parkinson`s disease (PD) can be successfully treated by dopamine supplement therapy. However, prolonged L-dopa treatment induces a motor complication, L-dopa induced dyskinesia (LID). To elucidate the mechanisms underlying LID, we recorded neuronal activity in a mouse model of LID. PD mice were generated by injection of 6-hydroxydopamine into the medial forebrain bundle, and L-dopa was intraperitoneally injected repeatedly until the mice develop LID. Spontaneous neuronal activity and response to cortical stimulation were recorded in the globus pallidus (GP) and substantia nigra pars reticulata (SNr) in normal, PD, “dyskinesia-off” LID (24 h after last L-dopa treatment), and “dyskinesia-on” LID (20 min after L-dopa treatment when mice develop dyskinesia) states in awake conditions. The following results were obtained: (1) Spontaneous activity of GP and SNr neurons showed a little difference between control, PD, dyskinesia-off and dyskinesia-on states. (2) Cortically evoked inhibition of GP neurons was increased in PD, dyskinesia-off and dyskinesia-on states compared to control state, and cortically evoked late excitation in GP neurons was increased in PD and dyskinesia-off states. (3) Cortically evoked inhibition and late excitation of SNr neurons were reduced in PD and dyskinesia-off states. (4) During dyskinesia-on, cortically evoked inhibition was prolonged, and late excitation was reduced in the SNr. (5) Repeated L-dopa injection to sham-lesioned mice had little effects on GP and SNr activity. Inhibition and late excitation in the SNr are supposed to release movements and to stop movements, respectively. Thus, LID can be explained by increased facilitatory signal and loss of termination signal for movements.

Introduction

The basal ganglia (BG) are a group of nuclei located at the base of forebrain and receive inputs from the cerebral cortex and send outputs to the cerebral cortex via the thalamus. The striatum and subthalamic nucleus (STN) are two major input stations of the BG and receive cortical inputs. The output stations of the BG are the internal segment of the globus pallidus (GPi or entopeduncular nucleus in rodents) and substantia nigra pars reticulata (SNr). The external segment of the globus pallidus (GPe or GP in rodents) plays a role connecting all BG nuclei (Nambu, 2009; Steiner and Tseng, 2010). Three BG pathways connect input and output stations: the 'direct', 'indirect' and 'hyperdirect' pathways (Alexander and Crutcher, 1990, Nambu et al., 2002). The direct pathway arises from GABAergic striatal neurons and projects monosynaptically to the GPi or SNr. The indirect pathway arises from GABAergic striatal neurons and projects polysynaptically to the GPi/SNr through sequential connections of the GPe and STN. The hyperdirect pathway arises from glutamatergic STN neurons that receive direct cortical inputs and projects to the GPi/SNr (Nambu, 2008).

The BG play an important role in the execution of voluntary movement and motor learning. Dysfunction of the BG results in inability to control movement that can be seen in Parkinson's disease (PD) and Huntington's disease (Albin et al., 1989; DeLong, 1990; Nambu, 2008). PD is a neurological disorder that is caused by the loss of dopaminergic neurons in the substantia nigra pars compacta (SNc), which project to the striatum. Reduced dopamine signaling in the striatum leads to the appearance of its cardinal symptoms, including bradykinesia/akinesia, tremor at rest, rigidity, and loss of postural reflexes. These PD symptoms can be successfully treated by dopamine supplement therapy: Dopamine precursor L-dopa (levodopa) is administrated and is converted to dopamine in the brain (Cotzias et al., 1969; Fahn and the Parkinson study group, 2005).

Although L-dopa remains as gold standard for PD treatment, the progression of the disease and long-term L-dopa treatment induce motor complications. Most PD patients experience shorter motor response to each medication dose (wearing-off fluctuation), often accompanied with dyskinesia that appear during high plasma and brain level of L-dopa (Cenci, 2014). L-dopa-induced dyskinesia (LID) denotes an idiosyncratic mixture of choreiform (repetitive, jerky, and rapid involuntary movement) and dystonic (sustained muscle contraction) movements. LID is mostly severe 40-80 min after L-dopa administration (Cenci and Lundblad, 2006). About 30% of PD patients have LID after 4-6 years of L-dopa treatment, and most of them develop this

complication after 9 years of L-dopa treatment (Ahlskog and Mueneter, 2001; Cenci and Lundblad, 2006). The risks of developing LID include young age at PD onset, disease severity and duration, higher initial L-dopa dose, and duration of L-dopa treatment. Once LID appears, the same involuntary movements occur on every administration of L-dopa (Schrag and Quinn, 2000; Cenci and Lundblad, 2006; Jenner, 2008). Thus, prolonged L-dopa treatment should induce irreversible change in the BG.

The cellular mechanism underlying LID has been postulated, such as (1) abrupt dopamine increase in the striatum caused by unregulated dopamine efflux from serotonin neurons and defective dopamine clearance (Cenci and Lindgren, 2007; Lee et al., 2008; Rylander et al., 2010), (2) up-regulation of intracellular signaling of striatal direct pathway neurons (Cenci et al., 1998; Calon et al., 2000), (3) maladaptive plasticity of cortico-striatal synapses, and (4) increased GABAergic and opioidergic transmission in the GPi (Cenci, 2010; Lindgren et al., 2010). On the other hand, electrophysiological studies of LID are rather limited. A previous study reported that in the presence of dyskinesia, firing rate of GPi neurons declined profoundly in LID model monkey (Papa et al. 1999). However, loss of GPi activity by pallidotomy alleviates LID symptoms (Lozano et al., 1995). In the present study, we recorded neuronal activity of GP and SNr neurons from mice in control, PD, dyskinesia-off, and dyskinesia-on states under awake conditions and tried to elucidate the latent changes after long-term L-dopa treatment and electrophysiological mechanism underlying LID.

Materials and Methods

Animals

Sixteen young adult (8-11 week-old at the beginning of experiments) male ICR mice (SLC) were used in this study. They were kept in cages under 12-h light/dark cycle with free access to food and water. All experimental procedures were approved by the Institutional Animal Care and Use Committee of National Institutes of Natural Sciences.

Experimental design

The animals were separated into 3 groups. 6-hydroxydopamine (6-OHDA) was injected to the medial forebrain bundle (MFB) of the first group (6-OHDA lesioned) (n=10), the vehicle was injected to the MFB of the second group (sham lesioned) (n =3), and no lesion was made in the third group (no lesioned) (n = 3) (Fig. 1A). Fifteen days after 6-OHDA/vehicle injection, cylinder tests were carried out to evaluate PD symptoms. Operation for painless head fixation was performed in all 3 groups. After recovery from the operation, activity of GP and SNr neurons were recorded in 6-OHDA-lesioned mice (PD state) and in no-lesioned mice (control state). Then, 6-OHDA-lesioned and sham-lesioned mice were treated daily with L-dopa intraperitoneally (*i.p.*) for 11 d. Development of LID was evaluated every two days by abnormal involuntary movement tests (AIMs). Extracellular recordings from 6-OHDA lesioned mice under LID state were performed in 2 states: (1) dyskinesia-off sate, recordings at least 24 h after last L-dopa treatment, and (2) dyskinesia-on period, recordings during 20-120 min after L-dopa treatment when mice developed dyskinesia. Neuronal activity of sham-lesioned mice after 11 d L-dopa treatment was also examined in 2 state: (1) sham-lesioned L-dopa-off state, recordings at least 24 h after last L-dopa treatment, and (2) sham-lesioned L-dopa-on state, recordings during 20-120 min after L-dopa treatment.

6-OHDA injection

Thirty min prior to 6-OHDA or vehicle injection, selective noradrenaline reuptake inhibitor, desipramine hydrochloride (Sigma-Aldrich) was injected (25 mg/kg body weight, *i.p.*) to the mice. 6-OHDA hydrochloride (Sigma) dissolved (3 μg/μl) in sterile ice-cold saline containing 0.02% ascorbic acid or vehicle (sterile saline containing 0.02% ascorbic acid) was prepared within 2 h before the injection. Mice were anesthetized with ketamine hydrochloride (100 mg/kg, *i.p.*) and xylazine hydrochloride (5 mg/kg, *i.p.*), and fixed in the stereotaxic apparatus. The skull was exposed, and a hole was made over the right MFB. A glass micropipette (tip outer diameter, 100 μm)

connected to 1.0 µl Hamilton syringe was inserted into the right MFB (anterior -1.2 mm, lateral 1.1 mm, ventral 5.0 mm from the bregma). Then, 6-OHDA or vehicle was infused (1 µl, at 0.1 µl/min) with a microinfusion pump (WPI). After injection, a glass micropipette was left in a place for 5 min to allow diffusion away from the injection site, and was slowly retracted (Thiele et al., 2011). The scalp incision was then closed using surgical clips. Then 1 ml sterile saline was injected subcutaneously to prevent dehydration during the post-operative recovery period (Cenci and Lundblad, 2007). These procedures were performed under aseptic conditions.

Cylinder test

To evaluate PD symptoms after 6-OHDA lesion, behavioral test was done at day 15 following 6-OHDA injection. Mice were placed inside the transparent cylinder (inner diameter 10 cm, height 20 cm) and video-recorded. Two types of behaviors were assessed, rotational behavior and forelimb asymmetry. Mice that showed positive results with at least one of two tests were considered positive for PD symptoms and used for further experiments. *(1) Rotational behavior test.* The number of ipsilateral and contralateral turns to the 6-OHDA-lesion was counted for 5 min. Mice that exhibited at least 70% spontaneous ipsilateral rotational behavior during observation time were considered positive for rotational behavior test (Thiele et al., 2011). *(2) Forelimb asymmetry test.* Inside the cylinder, mice also exhibited exploratory behavior that included rearing up against the walls of the cylinder. Unilateral 6-OHDA-lesioned mice lose their ability to use contralateral paw to the lesion hemisphere during rearing up against cylinder walls. Use frame-by-frame viewing, the number of placement of contralateral paw (weight was fully supported only on the contralateral affected paw), ipsilateral paw (weight was fully supported on ipsilateral unaffected paw only) and both paw (simultaneously or when placement of one paw follows the other in quick succession) were counted (Cenci and Lundblad, 2007; Schallert et al., 2000; Thiele et al., 2011) until a minimum of 9 supporting wall contacts per testing session (Francardo et al., 2011). The percent usage of the contralateral paw was calculated with formula: [contralateral paw placements/ (ipsilateral + contralateral + both paw placements)] x 100. Mice that made less than 15% of the contralateral paw usage were considered positive for forelimb asymmetry test (Thiele et al., 2011).

Surgery

To painlessly fix the head of the awake mouse to the stereotaxic apparatus for extracellular recording, a small U-shape head holder was mounted on mouse's head as

described previously (Chiken et al., 2008; Sano et al., 2013). Briefly, each mouse was anesthetized with ketamine hydrochloride (100 mg/kg, *i.p.*) and xylazine hydrochloride (5 mg/kg, *i.p.*), and fixed in the stereotaxic apparatus. The skull was widely exposed, and the periosteum and blood on the skull were completely removed. The exposed skull was then covered with bone adhesive resin (Bistite II; Tokuyama Dental) and acrylic resin (Unifast II; GC), and U-shape head holder made of acetal resin was mounted and fixed with acrylic resin on the mouse head. All surgical procedures were performed under aseptic conditions.

After recovery from the surgery (2 or 3 d later), the mouse was lightly anesthetized with ketamine hydrochloride (50-100 mg/kg, *i.p.*) and then put into the stereotaxic apparatus with its head restrained using the U-shape head holder. A part of the skull in the right hemisphere, ipsilateral to 6-OHDA/vehicle injection side, was removed in order to access the motor cortex, GP and SNr. Two pairs of bipolar stimulating electrodes (tip distance 300-400 µm, made of Teflon coated 50 µm-diameter tungsten wires) were implanted chronically into the primary motor cortex, one into the orofacial region and the other into the forelimb region (caudal forelimb region), and fixed using acrylic resin (Chiken et al., 2008; Sano et al., 2013). These regions were confirmed by intracortical microstimulation (train of 10 pulses at 333Hz, 200µs duration, 30-35 µA).

Recording neural activity

After full recovery from the operation, a mouse was placed in the stereotaxic apparatus using the U-frame head holder in awake conditions (Fig. 1B, C). A glass-coated elgiloy-microelectrode (1.2-1.5MΩ at 1 kHz) for single-unit recording was inserted vertically into the brain through the dura mater using a hydraulic microdrive (Narishige Scientific Instrument). The target area for GP was 0.3-0.8 mm posterior and 1.4-2.2 mm lateral to bregma, and for SNr was 2.5-3.2 mm posterior and 1.5 -2.0 mm lateral to bregma (Franklin and Paxinos, 2008; Chiken et al., 2008; Sano et al., 2013). Unit activity of GP and SNr neurons recorded from the microelectrode was amplified and filtered (0.1 - 2 kHz), converted to digital data with a homemade window discriminator, and sampled at 2.0 kHz using a computer for online data analysis. Spontaneous discharges and the responses to cortical stimulation (200 µs single pulse at 0.7 Hz, 20-50 µA strength) through the stimulating electrode implanted in the motor cortex were recorded (Chiken et al., 2008; Sano et al., 2013).

L-dopa treatment

L-dopa (Dopaston, 25 mg/ml, Sankyo) and the peripheral DOPA decarboxylase inhibitor, benserazide hydrochloride (Sigma-Aldrich) were dissolved in sterile saline (L-dopa 0.9 mg/ml and benserazide 1.5 mg/ml). The mice received *i.p.* injection of this solution (10 ml/kg), corresponding to injection of L-dopa (9 mg/kg) and benserazide (15 mg/kg), once a day for 11 consecutive days.

Abnormal involuntary movement tests (AIMs)

During chronic L-dopa treatment, abnormal involuntary movements or dyskinesia in mice were evaluated using AIMs scale based on resembled scale used in human dyskinesia (Cenci and Lundblad, 2007; Thiele et al., 2011). Once mice were injected with L-dopa (9 mg/kg with 15 mg/kg benserazide, *i.p.*), mice were placed in a separate cage to allow them to be observed individually. Each mouse was monitored for 2 min every 20 min starting 20 min after L-dopa injection. AIMs in 3 different body parts were observed: axial dyskinesia, forelimb dyskinesia and orofacial dyskinesia. In axial dyskinesia, the neck and upper trunk are twisted toward contralateral side of the lesion. Forelimb dyskinesia is characterized by small circular movements of the contralateral forelimb to and from the snout. Orofacial dyskinesia is identified by repetitive and purposeless chewing movements of the jaw sometimes with or without tongue protrusion to the contralateral side of the lesion, twitching of facial muscles (Cenci and Lundblad, 2007; Francardo et al., 2011; Thiele et al., 2011). AIMs in each body part were scored on a severity scale from 0 to 4: 0, absent; 1, present during less than half of the observation time; 2, present more than half of the observation time; 3, present all the time but interrupted by sensory distraction, such as pencil placed in front of the mouse; and 4, present all the time and not interrupted with sensory distraction (Francardo et al., 2011; Thiele et al., 2011). AIMs at each time point were the sum of AIMs in 3 different body parts. As dyskinesia symptoms lasted 120 min after L-dopa injection in this study, AIMs were observed till 120 min, i.e., 6 times. AIMs of the day were the sum of AIMs at each time point. Thus, the maximum AIMs score of the day was 72.

Recording neural activity under dyskinesia-off and dyskinesia-on states

For recording during dyskinesia-off state, at least 24 h after last L-dopa administration, mice were put in the stereotaxic apparatus in awake conditions. Meanwhile for recording during dyskinesia-on state, an intravenous catheter (26 gauge) connected to a syringe containing L-dopa was inserted intraperitoneally into the mice (Fig. 1B). During recording, L-dopa was injected (9 mg/kg with 15 mg/kg benserazide) through

the catheter. Mice usually started to develop dyskinesia symptoms 10 min after L-dopa injection. Recording under dyskinesia-on state was started after 20 min.

Data analysis

The spontaneous firing rate was calculated from continuous digitized recording for 50 s. Several parameters for firing patterns were also calculated from the first 30 s of the same recordings, which include the coefficient of variance (CV) of interspike intervals (ISIs), number of bursts, and number of pauses (Sano et al., 2013; Chiken et al., 2008). Bursts were detected using Poisson surprise method. The spike train judged to be a burst if at least three spikes can be found during 30 s spontaneous recording and if its surprise value was >2.0 (Legendy and Salcman, 1985; Chiken et al., 2008). Meanwhile, pauses during 30 s of spontaneous recording were detected using the method of Elias et al. (2007) that basically used similar algorithm for burst detection. The spike train judged to be train that contains pauses if at least one pause was observed in 30 s of spontaneous recording (Chiken et al., 2008). The spontaneous firing pattern was also analyzed by constructing autocorrelograms (bin width of 0.5 ms) from continuous digitized recordings for 50 s.

The responses to cortical stimulation were analyzed by making peri-stimulus time histogram (PSTHs) (bin width of 1 ms) for 100 stimulation trials. The mean value and standard deviation (SD) of the discharge rate during 100 ms preceding onset of stimulation were considered as the baseline of discharge rate. Changes in neuronal activity in response to cortical stimulation were judged significant if the discharge rate during at least two consecutive bins reached a significance level of $p < 0.05$ (one-tailed t test) (Chiken et al., 2008; Sano et al., 2013). The latency of each response was defined as the time at which the discharge rate first exceeded this level. The responses were judged to end when four consecutive bins fell below the significance level. The amplitude of each response was defined as the number of spikes during significant changes minus the number of spikes of the baseline discharge in the 100-trials PSTH (the area of the response; positive and negative values indicate excitatory and inhibitory responses, respectively). For population PSTHs, the PSTH of each neuron with significant response to motor cortical stimulation was smoothed with Savitzky-Golay filter (OriginPro 2015; polynomial order, 2; points of window, 10) and averaged.

The spontaneous firing rates and patterns and the neuronal responses to cortical stimulation of GP and SNr neurons were compared between normal, PD, dyskinesia-off, and dyskinesia-on states.

Histology

After final recording, several GP and SNr recording sites were marked by passing cathodal DC current (20 µA for 20 s) through the recording electrode. Then mice were deeply anesthetized with sodium pentobarbital (100 mg/kg, *i.p.*) and perfused transcardially with 0.01M PBS followed by 10% formalin in 0.01M PBS. The brains were removed, postfixed in 10% formalin at 4°C overnight then cryoprotected in graded sucrose (10% sucrose in 0.01M PBS overnight, 20% sucrose in 0.01M PBS, 30% sucrose in 0.01 M PBS) at 4°C.

Frontal sections (40 µm) were cut with freezing microtome and collected in 0.01 M PBS (Sano et. al., 2013). Brain sections containing the motor cortex, GP or SNr were mounted on MAS-coated slides (Matsunami Glass), air dried, and stained with Neutral Red. The cortical stimulating sites and the recording sites in the GP and SNr were confirmed according to the lesions made by cathodal DC current and traces of the electrode tracks. Other sections containing the SNc were used for immunohistochemistry to evaluate tyrosine hydroxylase (TH) positive cells in lesion hemisphere. Free-floating sections were incubated with primary antibodies against mouse TH (1:1000, Sigma-Aldrich) at 4°C overnight, then visualized with biotinylated secondary antibody (1:500, Vector laboratories) and the ABC method (Vectastain Elite ABC kit; Vector laboratories). The sections were mounted, air dried, cover-slipped and examined under a light microscope.

Results

Cylinder test

Assessment of PD symptoms using cylinder test was done in 10 6-OHDA-lesioned and 3 sham-lesioned mice (Fig. 2A). Majority of 6-OHDA-lesioned mice (8 out of 10 mice) exhibited ipsilateral rotational behavior more than 70%, positive for rotational behavior test. Only positive mice were used for further experiments. No abnormal rotational behavior observed in sham-lesioned mice. Mice were also tested for forelimb asymmetry use. In present study, all mice that were positive for rotational behavior test also showed less than 15% contralateral paw placement, positive for forelimb asymmetry test.

AIMs

AIMs were observed in 6-OHDA-lesioned mice. On day 1, L-dopa injection (9 mg/kg with 15 mg/kg benserazide, *i.p.*) induced AIMs whose score was 17.9 ±8.8 (n=8) (Fig. 2B, left). On day 5, AIMs score increased significantly to 25.6 ±6.5. After 5 days, AIMs score reached a plateau. On the other hand, AIMs were not observed in sham-lesioned mice.

AIMs appeared 20 min after L-dopa injection and reached their peak around 60 mins after L-dopa injection. AIMs score gradually decreased after 80 min and totally diminished around 120 min after L-dopa injection (Fig. 2B, right).

TH immunostaining

Dopamine depletion in LID mice was confirmed by counting TH positive neurons in the SNc. In LID mice, the number of TH positive neurons in the lesioned side was significantly decreased compared to the intact side (Fig. 2C). On the other hand, TH positive neurons were comparable between ipsilateral and contralateral sides in sham-lesioned mice.

Spontaneous activity of GP and SNr neurons in PD and dyskinesia states

Spontaneous activity of 116 GP and 69 SNr neurons were recorded from normal mice (n = 3) as control. Spontaneous activity from 6-OHDA-lesioned mice was also recorded: 105 GP and 61 SNr neurons in PD state (n = 4), 114 GP and 55 SNr neurons in dyskinesia-off state (n = 4), 59 GP and 29 SNr neurons in dyskinesia-on state (n = 4). One mouse died in the course of dyskinesia-on recording.

The firing rates and patterns of GP and SNr neurons were compared between

these states. GP and SNr neurons fired continuously and irregularly at high discharge rate > 50 Hz as observed in digital spike traces and autocorrelograms (Fig. 3A, B). Firing rate of GP neurons was reduced in PD and dyskinesia-off states and increased in dyskinesia-on state compared to control state (Table 1). Irregularity and the number of bursts increased in PD and dyskinesia-off states, but they returned to normal in dyskinesia-on state (Fig. 3A; Table 1, $p < 0.05$, one way ANOVA). In SNr neurons, there were no significant changes in firing rates between control, PD, dyskinesia-off, and dyskinesia-on states. Irregularity increased only in dyskinesia-on state, and the number of bursts increased in PD, dyskinesia-off, and dyskinesia-on states (Fig. 3B; Table 1, $p < 0.05$, one way ANOVA).

Responses of GP neurons to cortical stimulation in PD and dyskinesia states

Motor cortical stimulation can induce responses in BG nuclei that mimic information processing during voluntary movements (Nambu et al., 2002; Tachibana et al., 2008; Chiken et al., 2008). Cortically evoked responses in the BG are altered in hyperkinetic and hypokinetic movement disorders (Chiken et al., 2008; Kita and Kita, 2011; Nishibayashi et al., 2011). Hence, cortically evoked responses of GP and SNr neurons were recorded in control, PD, dyskinesia-off, and dyskinesia-on states. Response patterns to cortical stimulation were quantified by constructing PSTHs.

Motor cortical stimulation induced responses in 109 out of 116 GP neurons in control state, 98 out of 105 GP neurons in PD state, 91 out of 114 GP neurons in dyskinesia-off state, and 49 out of 59 GP neurons in dyskinesia-on state. In PD state, cortical stimulation induced triphasic response composed of early excitation, inhibition and late excitation as seen in control state, but the duration and amplitude of inhibition and late excitation were significantly increased compared to control state (Fig. 4; Table 2, $p < 0.05$, one way ANOVA). In dyskinesia-off state, cortical stimulation induced triphasic response with increased inhibition and late excitation as seen in PD state. In dyskinesia-on state, cortical stimulation induced triphasic response, but the duration and amplitude of inhibition were significantly increased compared to control state, meanwhile the duration and amplitude of late excitation was decreased and returned to control level (Fig. 4 and Table 2, $p < 0.05$, one way ANOVA). Besides major triphasic response, other responses including biphasic response, such as excitation-inhibition, inhibition-excitation, and excitation-excitation, and monophasic response such as early excitation, inhibition and late excitation also were found (Fig. 4B).

Changes in cortically evoked response of GP neurons were also evident in

population PSTHs (Fig. 5A). The amplitude and duration of inhibition were increased in PD, dyskinesia-off and dyskinesia-on states as compared to control state. The amplitude and duration of late excitation were increased in PD and dyskinesia-off states and decreased in dyskinesia-on state, whereas early excitation remained unchanged in all states.

Responses of SNr neurons to cortical stimulation in PD and dyskinesia states

Motor cortical stimulation induced response in 63 out of 69 SNr neurons in control state, 41 out of 61 SNr neurons in PD state, 42 out of 55 SNr neurons in dyskinesia-off state, and 22 out of 24 SNr neurons in dyskinesia-on state.

In control state, cortical stimulation induced triphasic response composed of early excitation, inhibition and late excitation in SNr neurons (Fig. 6A). But other response patterns including biphasic response, such as excitation-inhibition, excitation-excitation and inhibition-excitation and monophasic response, such as early excitation, inhibition and late excitation, were also observed (Fig. 6B). In PD state, cortical stimulation typically induced monophasic early excitation, and over 90% of neurons exhibited response without inhibition, such as early excitation, excitation-excitation and late excitation. In dyskinesia-off state, the percentage of neurons exhibiting response without inhibition was smaller than that in PD state, but it was still much larger than that in control state. However, in dyskinesia-on state, response with inhibition, such as excitation-inhibition-excitation, excitation-inhibition, inhibition-excitation and inhibition, became major in SNr neurons.

Changes in cortically evoked response of SNr neurons were also observed in population PSTHs (Fig. 5B) and quantitatively analyzed (Table 2). Cortically evoked inhibition disappeared in PD and dyskinesia-off states and recovered in dyskinesia-on states (Fig. 5B). The amplitude and/or duration of cortically evoked inhibition was significantly reduced in PD and dyskinesia-off states compared to control state (Table 2, $p < 0.05$, one way ANOVA) and increased in dyskinesia-on state. The amplitude and duration of late excitation were reduced in all disease states as compare to control state (Table 2, $p < 0.05$, one way ANOVA). The late excitation reduced in dyskinesia-off state and was almost absent in dyskinesia-on state (Fig. 5B).

Activity of GP and SNr neurons in sham-lesioned mice

Spontaneous activity of GP and SNr neurons was also recorded in sham-lesioned mice after 11-days L-dopa treatment, 109 GP and 48 SNr neurons in L-dopa-off-state, and 90

GP and 40 SNr neurons in L-dopa-on-state. Firing rates and patters were not significantly different from those in control state (Table 3). Cortically evoked responses of GP and SNr neurons were also observed, 90 GP and 38 SNr neurons in L-dopa-off state, and 86 GP and 39 SNr neurons in L-dopa-on state (Table 4 and Fig. 7). Amplitude and/or duration of early excitation, inhibition and late excitation of GP neurons were increased in L-dopa-off and L-dopa-on states. However, responses of SNr neurons showed similar patterns in control, L-dopa-off and L-dopa-on states. These results indicate that prolonged L-dopa treatment to normal mice induces neither dyskinesia nor abnormal neuronal activity.

Discussion

The present study showed following results: (1) Spontaneous activity of GP and SNr neurons showed a little changes in term of its firing rate, irregularity and number of bursts in PD, dyskinesia-off and dyskinesia-on states compared to normal state. (2) Cortically evoked inhibition of GP neurons was increased in PD, dyskinesia-off and dyskinesia-on states compare to control state, and late excitation of GP neurons was increased in PD and dyskinesia-off states, (3) Cortically evoked inhibition and late excitation of SNr neurons were decreased in PD and dyskinesia-off states. (4) In dyskinesia-on state, cortically evoked inhibition of SNr neurons was prolonged compared to control, PD and dyskinesia-off states, and late excitation was decreased compared to control state. (5) Repeated L-dopa injection to sham-lesioned mice had little effects on GP and SNr activity. Thus, the changes of phasic signals thorough the direct and indirect pathway, rather than the changes in the spontaneous firing rates and patterns, seems to be responsible for PD, dyskinesia-off and dyskinesia-on states (Fig. 8).

Changes in firing rates and patterns of GP and SNr neurons

Based on the classical model of the BG functions, hypokinetic and hyperkinetic movement disorders are supposed to be induced by activity imbalance between direct and indirect pathways. For example, in PD increased mean firing rates of GPi/SNr neurons and subsequent decreased activity in thalamic and cortical neurons are considered to induce akinesia in PD (DeLong, 1990; Nambu, 2008). On the other hand, in hyperkinetic disorder, such as ballism and dystonia, decreased firing rates of GPi/SNr neurons have been suggested. Actually, recording neuronal activity in the GPe and GPi in PD monkeys and PD patients exhibiting LIDs showed increased GPe and decreased GPi activity (Papa et al., 1999; Merello et al., 1999; Boraud et al., 2001). Apart from mean firing rate changes, recent studies emphasize firing pattern changes (Nambu et al., 2015). PD patients with LID exhibited low-frequency (6-8 Hz) oscillatory activity in the STN and GPi.

In this study, firing rates of GP neurons were decreased in PD and dyskinesia-off states and were increased in dyskinesia-on state, agreeing with the firing rate model described above. Firing pattern changes of GP and SNr neurons in the present study also agree with the firing pattern model. On the other hand, firing rates of SNr neurons showed little change in the present study, disagreeing with the firing rate model.

Changes of cortically evoked responses of GP and SNr neurons

An important issue on the mechanisms underlying LID is how activity in motor cortical neurons is transmitted to the BG. By modifying the firing rate model of BG functions, the dynamic activity changes in the GPi/SNr could explain the pathophysiological mechanism of hypokinetic and hyperkinetic movement disorders (Nambu, 2008). Cortical stimulation evokes a triphasic response consisting of early excitation, inhibition, and late excitation in the GP and GPi/SNr (Ryan and Clark, 1991; Fujimoto and Kita, 1992; Kita, 1992; Yoshida et al., 1993; Kitano et al., 1998; Maurice et al., 1999). Previous study in 6-OHDA-lesioned rat showed a clear increase of cortically evoked late excitation in the GP and early excitation in the GPi (Kita and Kita, 2011). In addition, L-dopa treatment in 6-OHDA-lesioned rats decreased cortically evoked late excitation in the GP (Kita and Kita, 2011).

In the present study, it is shown that cortically evoked late excitation of GP neurons was significantly increased in dyskinesia-off sate similar to PD state compared to normal state (Figs 4, 5A and Table 2), and returned to normal level in dyskinesia-on state. It was also observed that cortically evoked inhibition of GP neurons was increased in PD, dyskinesia-off and dyskinesia-on states. The early excitation, inhibition and late excitation evoked in the GP by cortical stimulation are considered to be mediated by the cortico-STN-GP, cortico-striato-GP and cortico-striato-GP-STN-GP pathways, respectively (Ryan and Clark, 1991; Maurice et al., 1999; Nambu et al., 2000; Kita et al., 2004; Tachibana et al., 2008) (Fig. 8). Thus, activity of the cortico-striato-GP pathways should be increased in PD, dyskinesia-off and dyskinesia-on states. Activity of the cortico-striato-GP-STN-GP pathway should be increased in PD and dyskinesia-off states, but should be decreased in dyskinesia-on state.

This study also showed decreased inhibition of SNr neurons in PD and dyskinesia-off states and decreased late excitation in dyskinesia-off and dyskinesia-on states (Figs. 6, 5B and Table 4). The early excitation, inhibition and late excitation evoked in the SNr by cortical stimulation are considered to be mediated by the cortico-STN-SNr hyperdirect, cortico-striato-SNr direct and cortico-striato-GP-STN-SNr indirect pathways, respectively (Ryan and Clark, 1991; Maurice et al., 1999; Nambu et al., 2000; Kita et al., 2004; Tachibana et al., 2008) (Fig. 7). Thus, activity of the cortico-striato-SNr direct pathway should be decreased in PD and dyskinesia-off states and recovered in dyskinesia-on state. On the other hand, activity of the cortico-striato-GP-STN-SNr indirect pathway should be decreased in dyskinesia-off and dyskinesia-on states. Decreased late excitation of SNr neurons in dyskinesia-on state should be explained by the decreased inhibition in the GP through

the GP-STN-SNr pathway.

Pathophysiological mechanisms underlying PD, dyskinesia-off and dyskinesia-on states

Based on the dynamic model of the BG functions (Mink and Thach, 1993; Hikosaka et al., 2000; Nambu et al., 2002; Sano et al., 2013), signals through the cortico-STN-SNr hyperdirect pathway excite the SNr and reset all cortical activity (Fig. 8, Normal). Then, signals through the cortico-striato-SNr direct pathway inhibit the SNr and release appropriate movements, and finally, signals through the cortico-striato-GP-STN-SNr indirect pathway excite the SNr again and stop the movements released by the signals through the direct pathway. This model can be used to explain the results of the present study. In PD state, inhibition through the direct pathway is significantly decreased, as a result, signals through the direct pathway cannot release movements, resulting in akinesia (Fig. 8 PD). In dyskinesia off state, inhibition through the direct pathway remains decreased and excitation through the indirect pathway is decreased. The decrease of late excitation in the SNr is considered to be the latent change after long-term L-dopa treatment As a result, signals through the direct pathway is still weak to release movements (Fig. 8 Dyskinesia-off). In dyskinesia-on state, inhibition through the direct pathway is restored to normal level, and excitation through the indirect pathway is significantly decreased, as a result, release signals are restored, stop signals are reduced, and dyskinesia is induced (Fig. 8 Dyskinesia-on). Up-regulation of intracellular signaling of striatal direct pathway neurons (Cenci, 2010; Lindgren et al., 2010) and increased GABAergic (Cenci et al., 1998) and opioidergic transmission in the GPi (Cenci et al., 1998) may explain the increased inhibition through the direct pathway (Cenci, 2010; Lindgren et al., 2010). On the other hand, cellular mechanism underlying activity changes in the indirect pathway remains to be elucidated.

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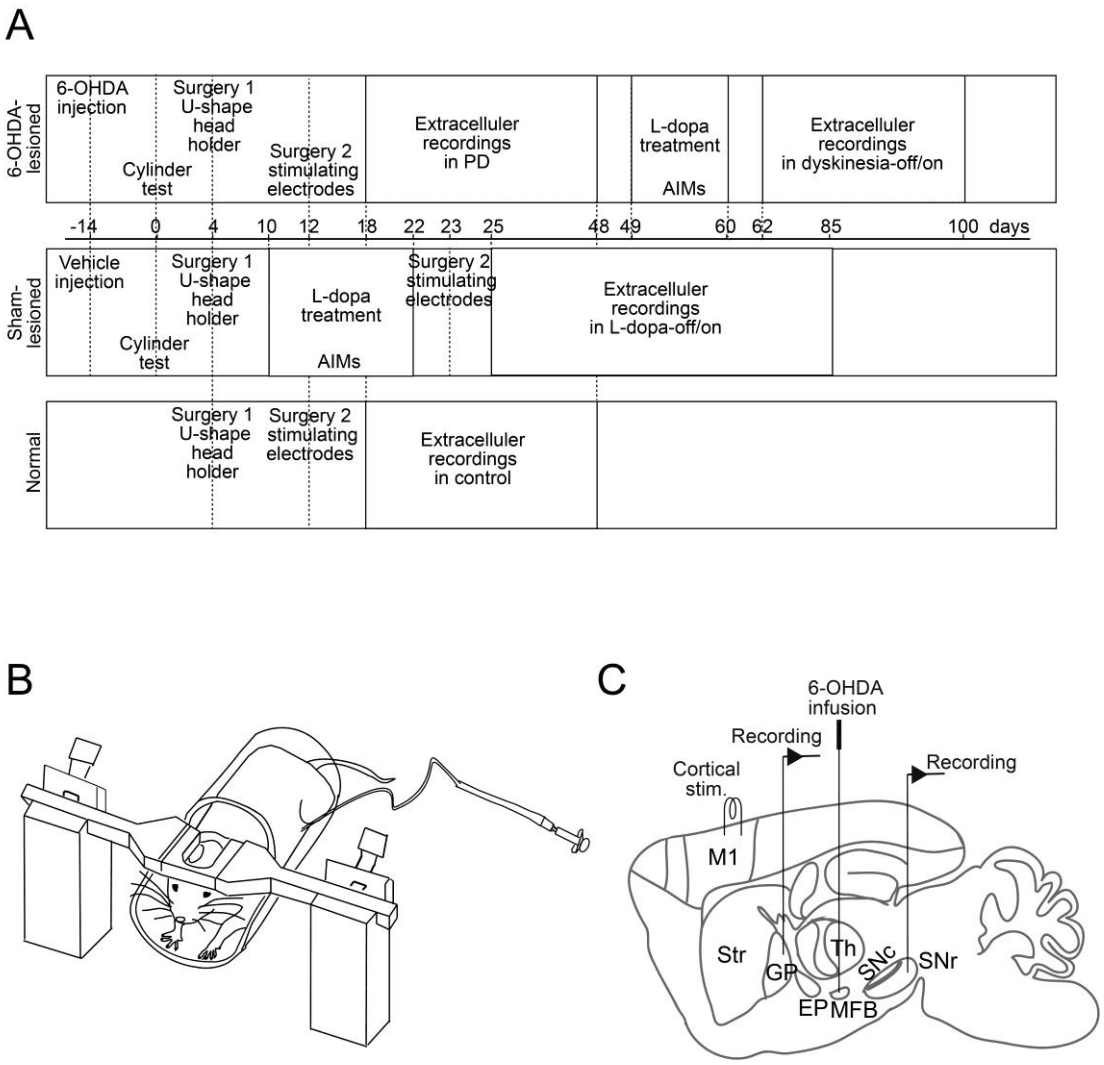

Figure 1. Experimental setup. A, Schedule of 6-OHDA/vehicle injection into the medial forebrain bundle (MFB), behavioral tests, surgery, L-dopa-treatments and extracellular recordings in 6-OHDA-lesioned and sham-lesioned mice along with normal mice. **B,** A mouse was positioned with its head restrained during extracellular recording, and an intravenous catheter connected to a syringe containing L-dopa was inserted intraperitoneally into a mice. **C,** Schematic diagram showing the location of cortical stimulating sites, recording sites, and 6-OHDA injection target. EP, entopeduncular nucleus; GP, globus pallidus; M1, primary motor cortex; SNc and SNr, substantia nigra pars compacta and reticulata; Str, striatum; Th, thalamus.

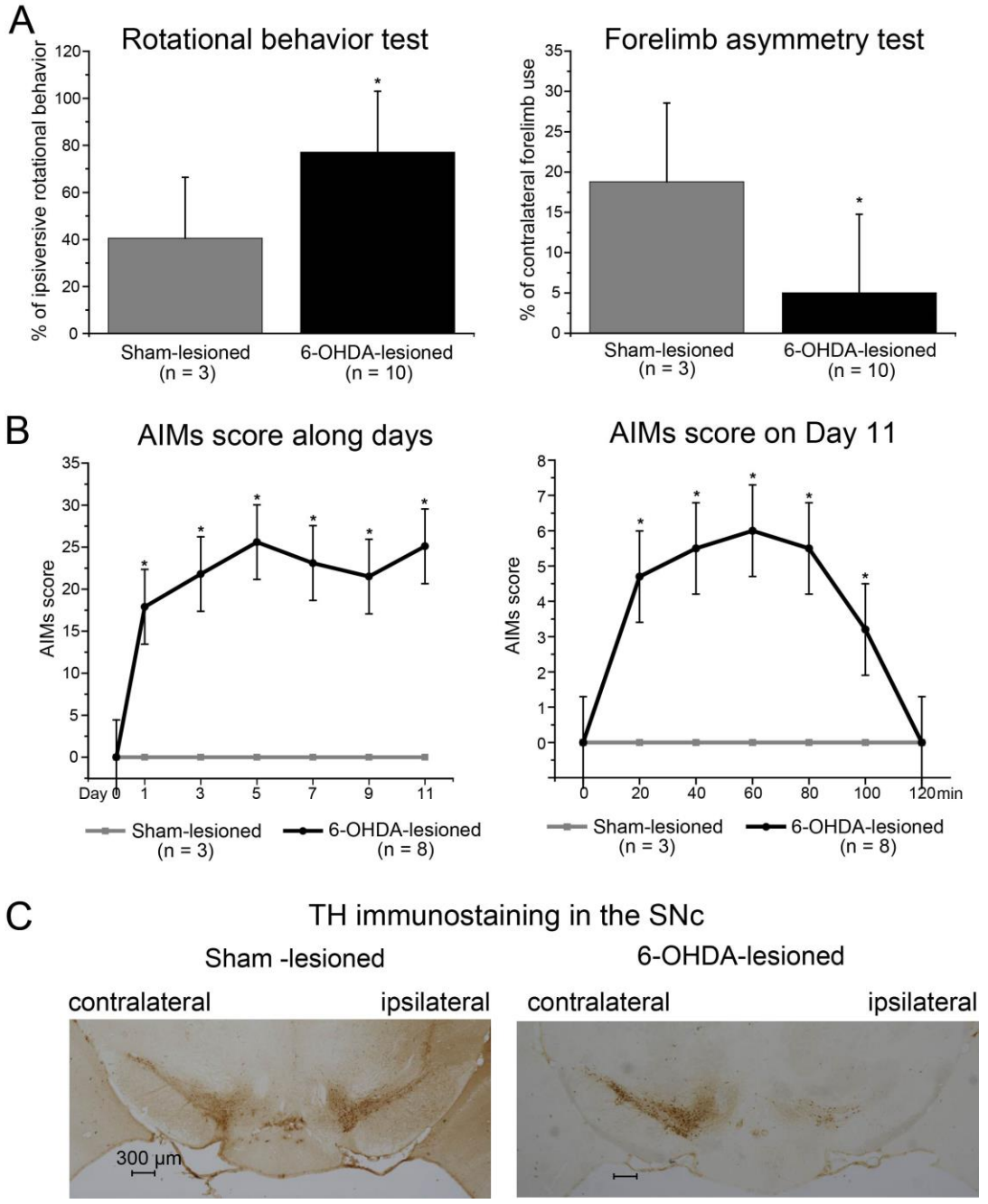

Figure 2. A, Rotational behavior (left) and forelimb asymmetry (right) tests in cylinder test of sham-lesioned and 6-OHDA-lesioned mice (* indicates $p<0.05$ compared to sham-lesioned mice, one-way ANOVA). **B,** Abnormal involuntary movements (AIMs) scores along 11 days of chronic L-dopa treatment to sham-lesioned and 6-OHDA-lesioned mice (left) and their time course of on Day 11 after one single injection of L-dopa (right) (* indicates $p<0.05$ compared to sham-lesioned mice, one-way ANOVA). **C,** Tyrosine hydroxylase immunostaining of the ipsilateral and contralateral SNc of sham-lesioned and 6-OHDA-lesioned mice.

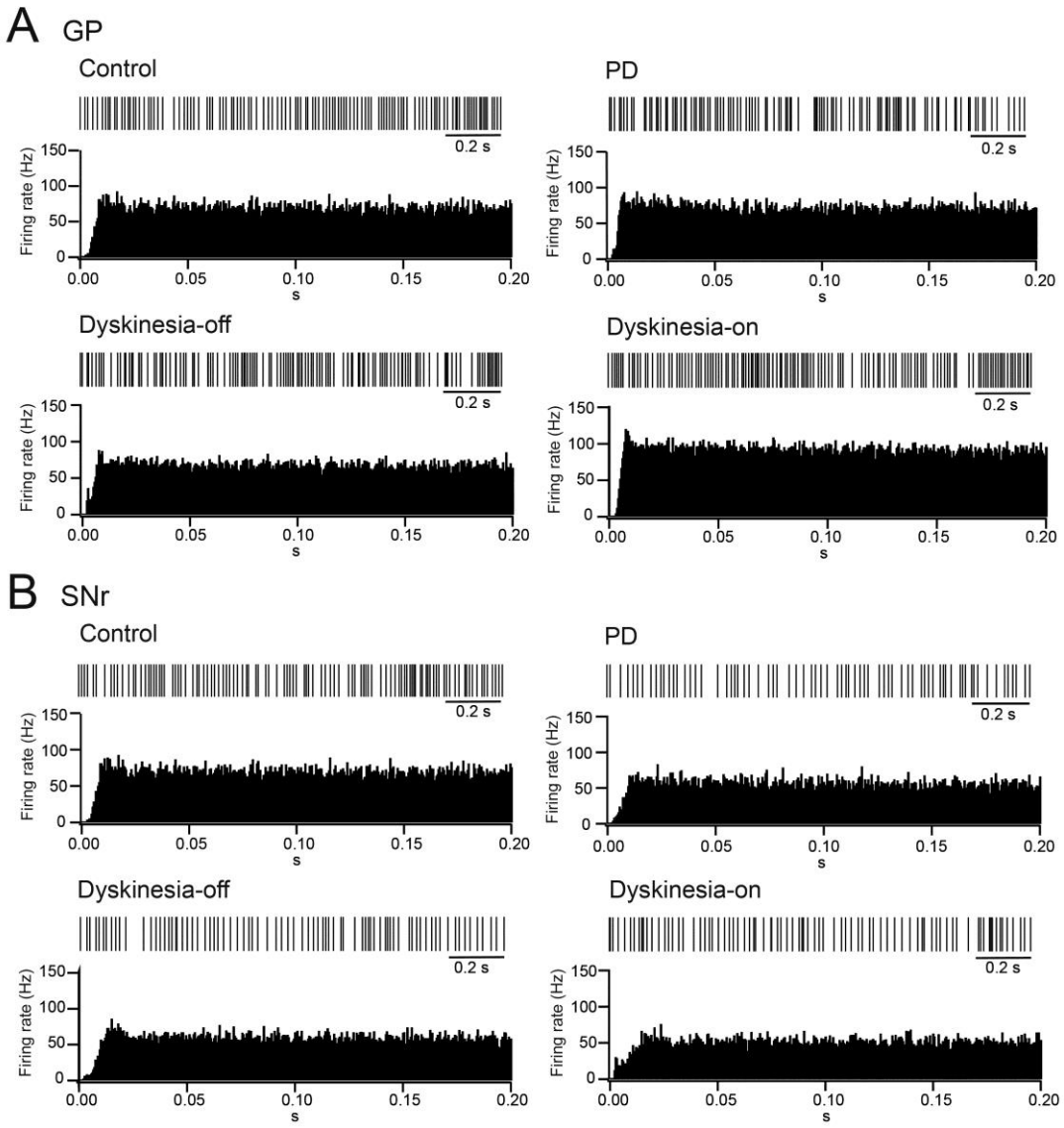

Figure 3. Spontaneous activity of GP (A) and SNr (B) neurons in control, PD, dyskinesia-off and dyskinesia-on states. Digitized spikes and autocorrelograms are shown in each state.

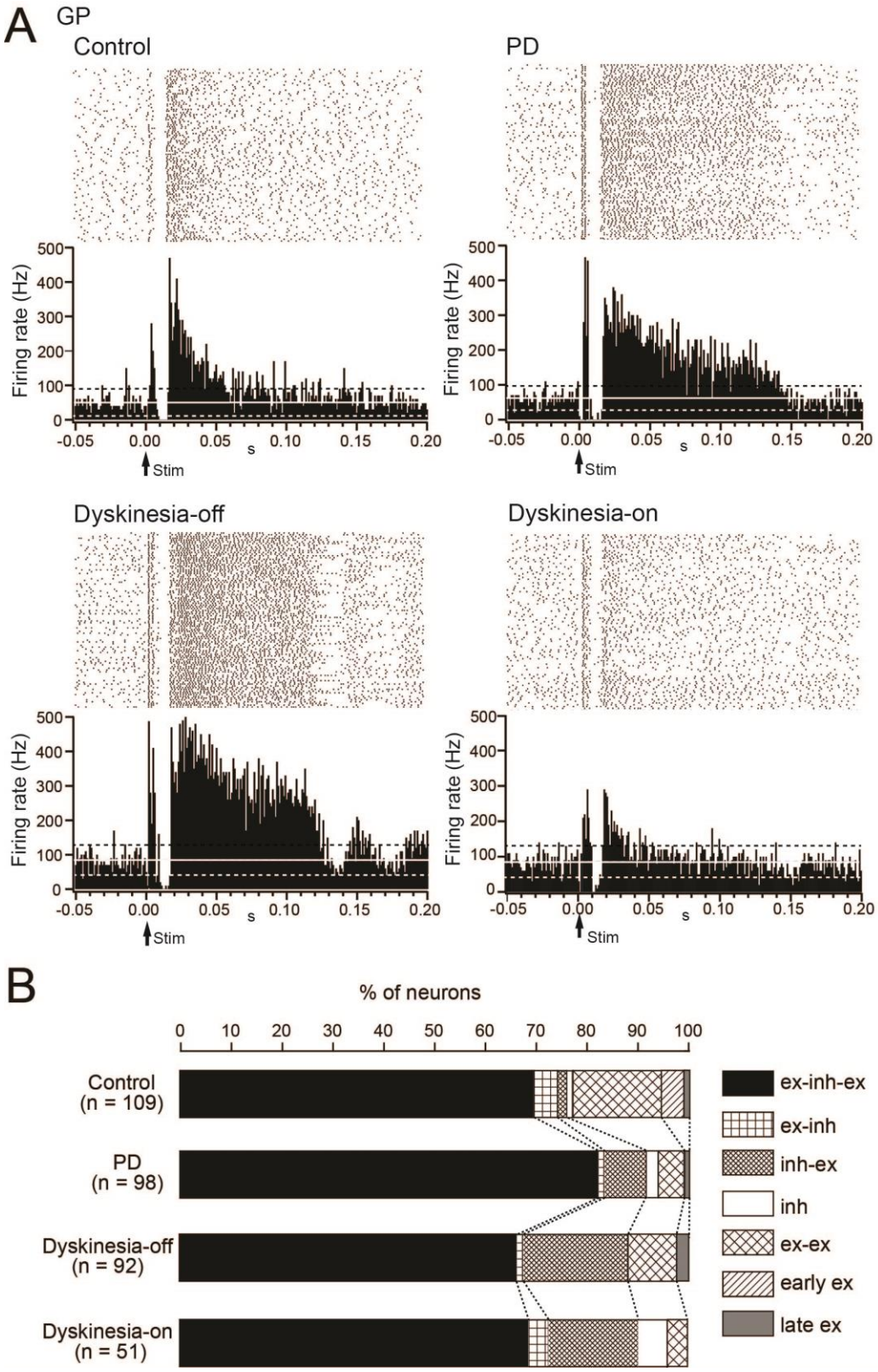
A
GP
Control
PD
Dyskinesia-off
Dyskinesia-on
Firing rate (Hz)
500
400
300
200
100
0
-0.05
0.00
0.05
0.10
0.15
0.20
s
Stim
B
% of neurons
0
10
20
30
40
50
60
70
80
90
100
Control
(n = 109)
PD
(n = 98)
Dyskinesia-off
(n = 92)
Dyskinesia-on
(n = 51)
ex-inh-ex
ex-inh
inh-ex
inh
ex-ex
early ex
late ex

Figure 4. Responses of GP neurons to cortical stimulation in control, PD, dyskinesia-off and dyskinesia-on states. A, Raster and peristimulus time histograms (PSTHs) of typical response pattern in each state. Cortical stimulation (200 μs duration single pulse and 50 μA strength) was delivered for 100 stimulus trials on the time 0. The mean firing frequency and statistical levels of $p < 0.05$ (one-tailed *t* test) calculated from 100 ms prior to the stimulation onset are indicated by a white solid line, a black dotted line (upper limit) and white (lower limit) dotted line, respectively. **B,** Proportions of GP neurons classified based on response patterns evoked by motor cortical stimulation in in each state.

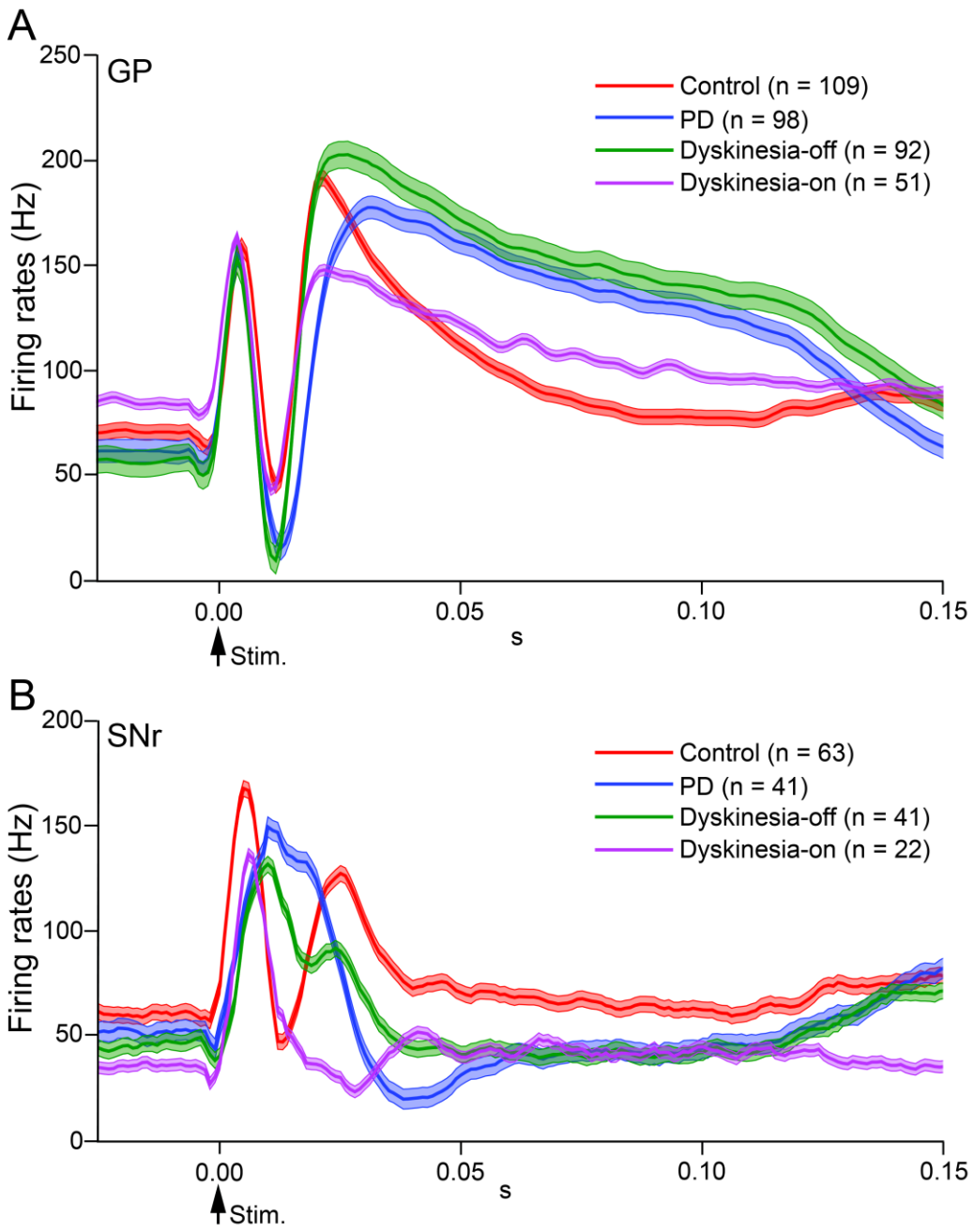

Figure 5. Population PSTHs of GP (A) and SNr (B) neurons in control, PD, dyskinesia-off and dyskinesia-on states. PSTH in each sate is shown by different color. Cortical stimulation was delivered in time 0. The light-colored areas represent ± SEM. The numbers of neurons used are indicated by n.

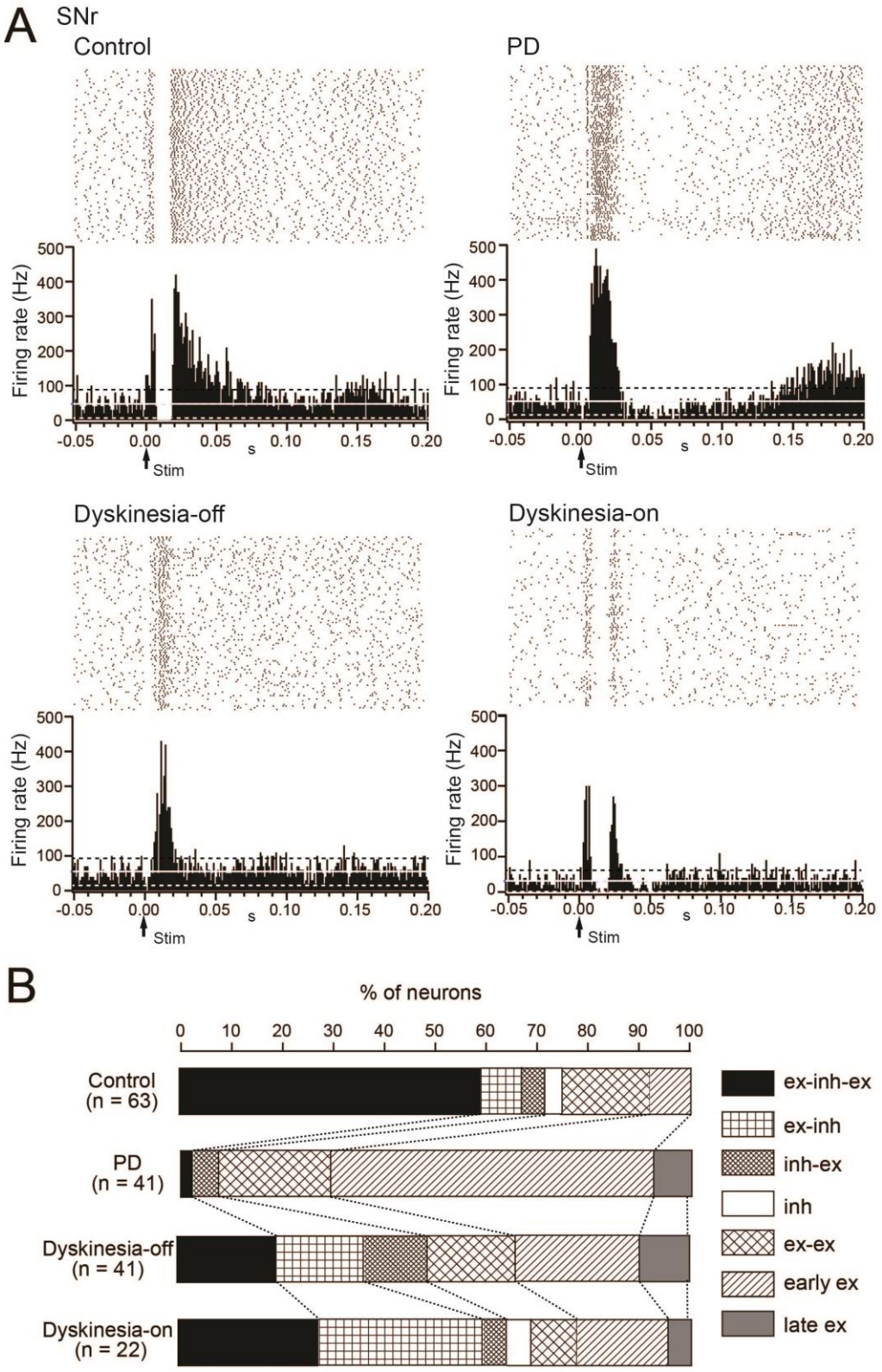
A
SNr
Control
PD
Dyskinesia-off
Dyskinesia-on
Firing rate (Hz)
500
400
300
200
100
0
-0.05
0.00
0.05
0.10
0.15
0.20
s
Stim
B
% of neurons
0
10
20
30
40
50
60
70
80
90
100
Control
(n = 63)
PD
(n = 41)
Dyskinesia-off
(n = 41)
Dyskinesia-on
(n = 22)
ex-inh-ex
ex-inh
inh-ex
inh
ex-ex
early ex
late ex

Figure 6. Response of SNr neurons to cortical stimulation in control, PD, dyskinesia-off and dyskinesia-on states. A, Raster and PSTHs of typical response pattern in each state. **B,** Proportions of SNr neurons classified based on response patterns evoked by motor cortical stimulation in each state.

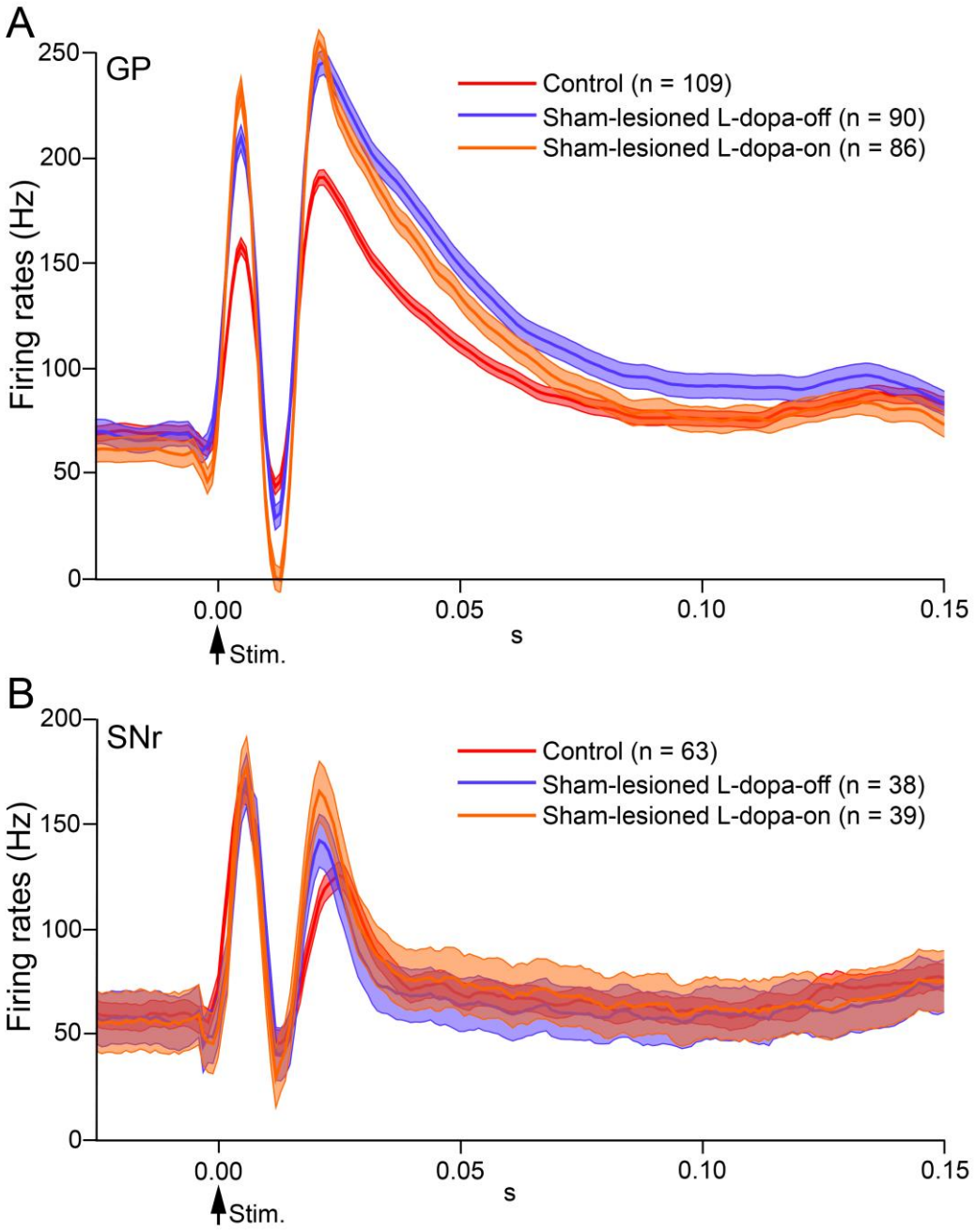

Figure 7. Population PSTHs of GP (A) and SNr (B) neurons in control, sham-lesioned L-dopa-off and sham-lesioned L-dopa-on states. PSTH in each sate is shown by different color. Cortical stimulation was delivered in time 0. The light-colored areas represent ± SEM. The numbers of neurons used are indicated by n.

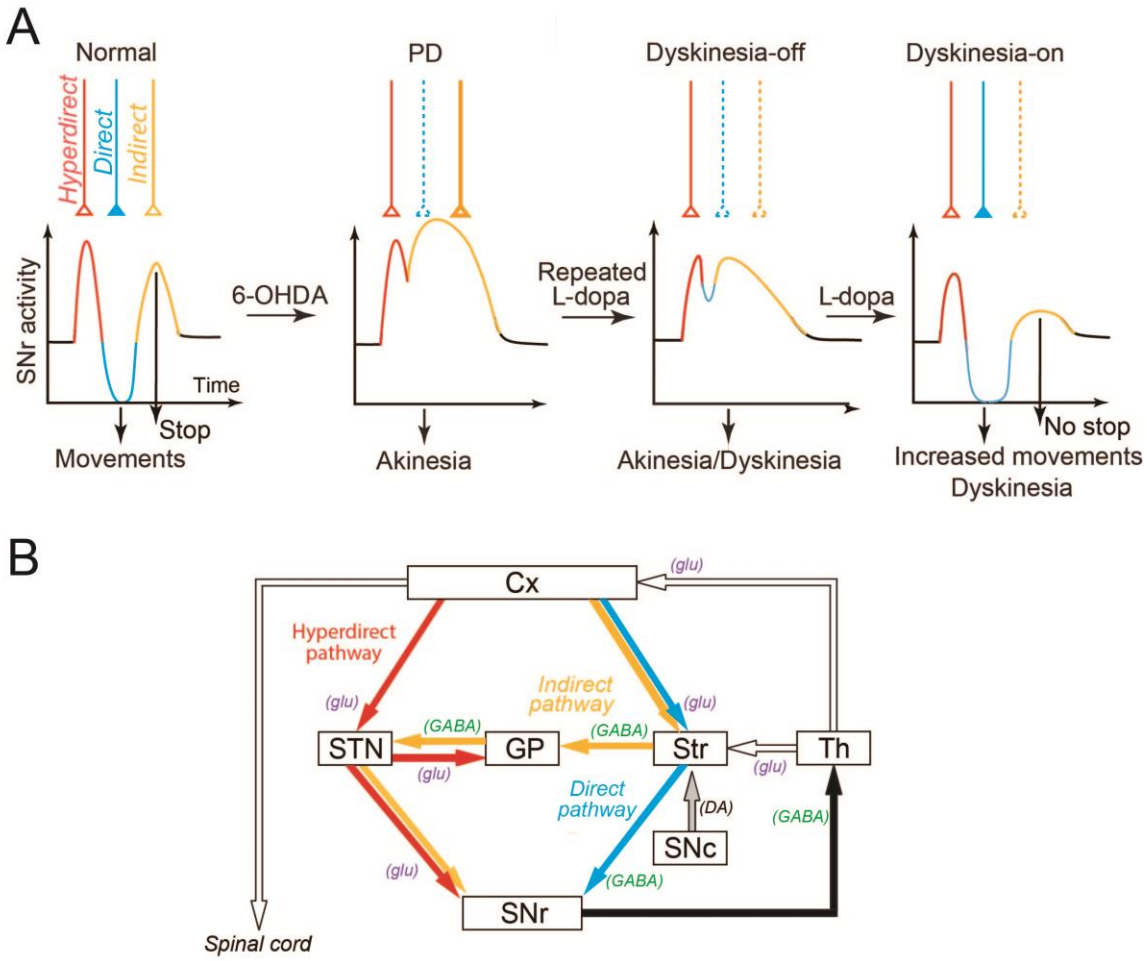

Figure 8. Pathophysiological mechanism of LID. A, Cortically evoked responses of SNr neurons in normal, PD, dyskinesia-off and dyskinesia-on states are shown with supposed activity of the hyperdirect, direct and indirect pathways. Dotted lines indicate decreased activity. **B,** Circuit diagram showing the hyperdirect, direct and indirect pathways of the BG.

Table 1. Firing rates and patterns of GP and SNr neurons in control, PD, dyskinesia-off and dyskinesia-on states

	Control	PD	Dyskinesia-off	Dyskinesia-on
GP				
No. of neurons	116	105	114	59
Firing rates (Hz)	70.9 ± 22.2	59.1 ± 18.2[1]	54.7 ± 20.3[1]	89.3 ± 39.2[1,2,3]
ISI CV(ms)	9.7 ± 4.6	19.6 ± 9.0[1]	22.0 ± 11.3[1]	12.0 ± 8.4[2.3]
No. of bursts during 30 s	2.0 ± 5.1	9.0 ± 13.4[1]	12.2 ± 11.3[1]	5.2 ± 9.8[3]
No. of pauses during 30 s	0.03 ± 0.2	0.7 ± 1.6[1]	0.9 ± 1.7[1]	0.5 ± 1.5
SNr				
No. of neurons	69	61	55	24
Firing rates (Hz)	61.3 ± 20.3	54.8 ± 13.6	56.7 ± 15.4	51.6 ± 21.5
ISI CV(ms)	10.3 ± 5.7	12.0 ± 5.2	11.3 ± 4.7	25.8 ± 26.4[1,2,3]
No. of bursts during 30 s	1.9 ± 3.6	7.0 ± 7.6[1]	6.1 ± 7.4[1]	9.0 ± 11.4[1]
No. of pauses during 30 s	0.1 ± 0.5	0.02 ± 0.1	0.15 ± 0.4	1.4 ± 2.3[1,2,3]

Values are means ± SD.

[1] $p < 0.05$, significantly different from control (ANOVA and Tukey's post hoc test)

[2] $p < 0.05$, significantly different from PD (ANOVA and Tukey's post hoc test)

[3] $p < 0.05$, significantly different from dyskinesia-off (ANOVA and Tukey's post hoc test)

Table 2. Response parameters of GP and SNr neurons to cortical stimulation in control, PD, dyskinesia-off and dyskinesia-on state

	Control	PD	Dyskinesia-off	Dyskinesia-on
GP				
No. of neurons	109	98	91	49
Early excitation				
Latency (ms)	3.4 ± 1.4	3.4 ± 1.3	3.2 ± 1.3	3.0 ± 1.3
Duration (ms)	5.7 ± 6.0	4.2 ± 2.3	3.1 ± 2.1[1]	4.4 ± 8.0
Amplitude (spikes)	77.5 ± 60.9	68.7 ± 54.1	68.7 ± 58.2	65.7± 99.6
Inhibition				
Latency (ms)	9.6 ± 2.7	9.6 ± 2.4	7.8 ± 2.1[1,2]	8.8 ± 1.7
Duration (ms)	5.7 ± 4.4	9.1 ± 4.5[1]	8.5 ± 4.6[1]	8.1 ± 5.8[1]
Amplitude (spikes)	-38.7 ± 33.5	-52.6 ± 32.7[1]	-45.5 ± 28.9	-57.7 ± 37.1[1]
Late excitation				
Latency (ms)	17.3 ± 2.6	21.1 ± 4.2[1]	18.8 ± 4.5[1,2]	17.3 ± 3.5[2]
Duration (ms)	47.3 ± 44.8	92.9 ± 42.9[1]	113.0 ± 47.7[1,2]	47.4 ± 59.5[2.3]
Amplitude (spikes)	448.4. ± 471.8	919.0 ± 617.4[1]	1380.4 ± 823.7[1,2]	398.9 ± 570.3[2,3]
SNr				
No. of neurons	63	41	42	22
Early excitation				
Latency (ms)	3.8 ± 1.5	5.3 ± 2.4[1]	5.0 ± 2.4[1]	4.5 ± 2.4
Duration (ms)	6.0 ± 3.7	9.3 ± 6.9[1]	9.8 ± 9.3[1]	7.1 ± 4.6
Amplitude (spikes)	82.3 ± 55.1	124.0 ± 126.0	129.0 ± 143.6	104.4 ± 66.3
Inhibition				
Latency (ms)	11.5 ± 3.3	12.9 ± 1.6	16.5 ± 8.7[1]	15.0 ± 5.3
Duration (ms)	7.0 ± 5.4	3.1 ± 8.9[1]	4.0 ± 5.5	11.6 ± 12.8[1,2,3]
Amplitude (spikes)	-40.8 ± 38.9	-13.5 ± 38.0[1]	-14.4 ± 19.3[1]	-41.3 ± 46.9[2.3]
Late excitation				
Latency (ms)	20.6 ± 6.1	15.0 ± 3.9[1]	18.7 ± 7.7	30.6 ± 9.5[1,2,3]
Duration (ms)	19.5 ± 33.4	5.8 ± 6.7[1]	8.4± 11.4[1]	4.6 ± 6.5[1]
Amplitude (spikes)	218.8 ± 414.5	75.0 ± 109.3[1]	71.8 ± 108.2[1]	38.2 ± 62.3[1]

Values are means ± SD.

[1] $p < 0.05$, significantly different from control (ANOVA and Tukey's post hoc test)

[2] $p < 0.05$, significantly different from PD (ANOVA and Tukey's post hoc test)

[3] $p < 0.05$, significantly different from dyskinesia-off (ANOVA and Tukey's post hoc test)

Table 3. Firing rates and patterns of GP and SNr neurons in control and sham-lesioned states

		Sham-lesioned	
	Control	L-dopa-off	L-dopa-on
GP			
No. of neurons	116	109	90
Firing rates (Hz)	70.9 ± 22.2	67.0 ± 19.2	66.2 ± 18.4
ISI CV(ms)	9.7 ± 4.6	10.7 ± 5.4	10.7 ± 6.6
No. of bursts during 30 s	2.0 ± 5.1	2.1 ± 4.3	3.0 ± 7.4
No. of pauses during 30 s	0.03 ± 0.2	0.1 ± 0.4	0.1 ± 0.5
SNr			
No. of neurons	69	48	40
Firing rates (Hz)	61.3 ± 20.3	58.7 ± 17.7	64.9 ± 20.0
ISI CV(ms)	10.3 ± 5.7	11.0 ± 5.7	10.3 ± 6.2
No. of bursts during 30 s	1.9 ± 3.6	2.5 ± 3.7	2.5± 4.9
No. of pauses during 30 s	0.1 ± 0.5	0.1 ± 0.4	0.2 ± 1.0

Values are means ± SD.

Values are not significantly different (ANOVA test).

Table 4. Response parameters of GP and SNr neurons to cortical stimulation in control and sham-lesioned states

		Sham-lesioned	
	Control	L-dopa-off	L-dopa-on
GP			
No. of neurons	109	90	86
Early excitation			
Latency (ms)	3.4 ± 1.4	3.5 ± 1.2	3.2 ± 1.1
Duration (ms)	5.7 ± 6.0	4.2 ± 2.3[1]	5.2 ± 2.1
Amplitude (spikes)	77.5 ± 60.9	90.1± 60.2	105.8 ± 56.8[1]
Inhibition			
Latency (ms)	9.6 ± 2.7	8.7 ± 2.7	9.2 ± 1.7
Duration (ms)	5.7 ± 4.4	8.6 ± 5.1[1]	8.0 ± 2.7[1]
Amplitude (spikes)	-38.7 ± 33.5	-51.4 ± 32.7[1]	-48.1 ± 23.0
Late excitation			
Latency (ms)	17.3 ± 2.6	18.2 ± 2.1[1,]	18.5 ± 1.7[1]
Duration (ms)	47.3 ± 44.8	69.0 ± 47.2[1,]	67.0 ± 39.0[1]
Amplitude (spikes)	448.4. ± 471.8	657.6 ± 457.4[1,]	626.2 ± 387.9[1]
SNr			
No. of neurons	63	38	39
Early excitation			
Latency (ms)	3.8 ± 1.5	3.8 ± 2.0	4.0 ± 2.1
Duration (ms)	6.0 ± 3.7	8.9 ± 10.5	8.8 ± 10.1
Amplitude (spikes)	82.3 ± 55.1	124.6 ± 152.0	123.2 ± 123.5
Inhibition			
Latency (ms)	11.5 ± 3.3	11.0 ± 2.7	10.4 ± 2.8
Duration (ms)	7.0 ± 5.4	6.7 ± 9.2	6.2 ± 4.6
Amplitude (spikes)	-40.8 ± 38.9	-33.5 ± 40.35	-29.7 ± 22.3
Late excitation			
Latency (ms)	20.6 ± 6.1	19.6 ± 3.4	19.4 ± 3.4
Duration (ms)	19.5 ± 33.4	14.3 ± 24.1	21.3 ± 30.9
Amplitude (spikes)	218.8 ± 414.5	121.1 ± 193.6	185.4.2 ± 250.1

Values are means ± SD.

[1] $p < 0.05$, significantly different from control (ANOVA and Tukey's post hoc test)