GABA storage and release in the medial globus pallidus in L-DOPA-induced dyskinesia priming

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ABSTRACT

Levo-dihydroxyphenylalanine (L-DOPA) is the most effective treatment for Parkinson's disease; however, most patients develop uncontrollable abnormal involuntary movements known as L-DOPA-induced dyskinesia. L-DOPA-induced dyskinesia can be reduced by pallidotomy of the medial globus pallidus or pallidal deep brain stimulation, suggesting that the medial globus pallidus plays a significant role in the development of L-DOPA-induced dyskinesia. In the present study, the pathological changes of the medial globus pallidus in L-DOPA-induced dyskinesia were studied in rat models of Parkinson's disease (unilateral 6-hydroxydopamine lesioning) and L-DOPA-induced dyskinesia (L-DOPA injection in Parkinson's disease-model rats twice daily for 2 weeks, confirmed by display of dyskinesia-like abnormal involuntary movements). L-DOPA-induced dyskinesia-model rats displayed medial globus pallidus hypertrophy, enlarged axon terminals surrounding the dendrites of medial globus pallidus neurons, and increased density of synaptic vesicles in enlarged axon terminals on the lesioned side. Synaptic terminal enlargement reversed after discontinuation of L-DOPA. Histological studies revealed the enlarged synaptic terminals were those of GABAergic striatal (direct pathway) neurons. A single injection of L-DOPA enhanced GABA release in the medial globus pallidus on the lesioned side in L-DOPA-induced dyskinesia-model rats compared to Parkinson's disease-model rats. In addition, microinjection of muscimol, a GABAA receptor agonist, into the medial globus pallidus on the lesioned side of Parkinson's disease-model rats induced dyskinesia-like abnormal involuntary movements. Microinjection of bicuculline, a GABAA receptor antagonist, into the medial globus pallidus on the lesioned side alleviated L-DOPA-induced dyskinesia in Parkinson's disease-model rats that had received L-DOPA prior to the microinjection. These results indicate that priming for L-DOPA-induced dyskinesia comprises excessive GABA storage in axon terminals of the direct pathway and that expression of L-DOPA-induced dyskinesia is associated with enhanced GABA release into the medial globus pallidus after L-DOPA dosing and the resultant excessive stimulation of GABAA receptors.

Notes

Abbreviations: 5-HT, 5-hydroxytryptamine; 6-OHDA, 6-hydroxydopamine; 8-OH-DPAT, 8-hydroxy-2-(di-n-propylamino)tetralin; ABC, avidin-biotin-peroxidase complex; AIM, abnormal involuntary movement; AUC, area under the curve; BDA, biotin dextran amine; DAB, 3,3′-diaminobenzidine tetrachloride; DBS, deep brain stimulation; GABA, gamma-aminobutyric acid; GAD, glutamic acid decarboxylase; GFAP, glial fibrillary acidic protein; GP, globus pallidus; L-DOPA, levo-dihydroxyphenylalanine; LGP, lateral globus pallidus; LID, L-DOPA-induced dyskinesia; MGP, medial globus pallidus; NIH, National Institutes of Health; PB, phosphate buffer; SEM, standard error of the mean; VGAT, vesicular GABA transporter; VGluT1, vesicular glutamate transporter 1

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1. Introduction

Parkinson’s disease is caused by degeneration of nigrostriatal dopaminergic neurons and a subsequent loss of the neurotransmitter dopamine from the forebrain motor centers of the basal ganglia (Marsden, 1982). Treatment with a precursor of dopamine, levo-dihydroxyphenylalanine (L-DOPA), alleviates the main motor symptoms of the disease. Although L-DOPA is considered the most effective therapeutic option (Balestrino and Schapira, 2020), up to 80% of patients develop uncontrollable abnormal involuntary movements (AIMs) known as L-DOPA-induced dyskinesia (LID) within 5 years (Quinn, 1995; Rascol et al., 2000; Tran et al., 2018). Because of the gradual development and persistent character of LID, this motor complication has been suggested to be associated with maladaptive neuronal plasticity (Calabresi et al., 2000; Graybiel et al., 2000; Linazasoro, 2005) induced by intermittent unphysiological stimulation of dopamine receptors in the dopamine-denervated striatum following L-DOPA treatment (de la Fuente-Fernandez et al., 2004; Olanow and Obeso, 2000; Pavese et al., 2006). Despite the clinical importance of LID, the underlying priming process remains poorly understood (Brochtein et al., 2005). The threshold of L-DOPA dose for LID expression is reduced immediately after dopaminergic denervation and again after repetitive L-DOPA treatment (Cenci and Lundblad, 2006). Dopaminergic denervation induces loss of negative feedback via gamma-aminobutyric acid (GABA) receptor in the axon terminal of direct pathway striatal spiny projection neurons, leading to hyperactivity of the neurons and LID occurrence (Borgkvist et al., 2015). However, to date, little is known about how repetitive L-DOPA treatment primes LID.

In the present study, to simplify discussions of similarities between primates and rodents, we use the same terminology for rodents that is used in primates for the two subdivisions of the globus pallidus (GP). The structure commonly called the entopeduncular nucleus in rodents is instead called the medial globus pallidus (MGP), i.e., the internal segment of GP; the structure commonly called the globus pallidus in rodents is instead called the lateral globus pallidus (LGP), i.e., the external segment of GP, as per the widely accepted homology (Paxinos and Watson, 1998).

LID can be dramatically reduced by pallidotomy of the MGP (Fine et al., 2000; Lang et al., 1997). Pallidal deep brain stimulation (DBS) is also effective for LID (Volkmann et al., 2004). In an “unresolved paradox” (Lozano et al., 2000; Obeso et al., 2000), pallidotomy could instead, according to the present understanding of basal ganglionic circuitry (Albin et al., 1989; DeLong, 1990), reduce inhibition of motor thalamic nuclei, leading to motor area overactivation and dyskinesia induction; however, pallidotomy has a lasting anti-dyskinetic effect and repriming rarely occurs (Lang et al., 1997). These observations suggest that the MGP plays a central role in LID development (Lozano et al., 2000). Electrophysiological evidence also supports MGP involvement in LID occurrence. The onset of LID has been correlated with both over-reduction of the firing frequency (Boraud et al., 2001; Papa et al., 1999) and altered firing patterns (Boraud et al., 2001) in MGP neurons. Thus, L-DOPA produces persistent plastic changes in the basal ganglia that lead to LID.

Little pathological change has been reported to account for LID (Bastide et al., 2015; Jenner, 2002). However, we previously demonstrated in a rat LID-model that the MGP area is increased after repeated L-DOPA treatment (Tomiyama et al., 2004). Here, we aim to identify the processes underlying LID development by delineating the anatomic and neurochemical changes occurring in the MGP in a rat LID-model, including changes in GABA transmission in the MGP during dyskinesia.

2. Materials and methods

Male Wistar rats (Clea, Japan) weighing 280–320 g each were used in five experiments performed in accordance with the Guidelines for Animal Experimentation issued by Hirosaki University School of Medicine and the Guide for the Care and Use of Laboratory Animals (National Institutes of Health [NIH], USA). Animals were housed in individual cages and exposed to 12-h light-dark cycles. Food and water were provided ad libitum before and during the experiments. All efforts were made to minimize the number of animals and their suffering.

2.1. 6-hydroxydopamine lesions

Lesions in the dopaminergic system were generated on the right side of rats by 6-hydroxydopamine (6-OHDA) injection into the right medial forebrain bundle. Following anesthesia by intraperitoneal injection of pentobarbital (50 mg/kg body weight), the rat head was fixed in a stereotactic apparatus (David Kopf, USA) with the incisor bar set 3.3 mm below the horizontal. Thirty minutes before 6-OHDA injection, the rat was intraperitoneally injected with desipramine (25 mg/kg) to prevent denervation of noradrenergic neurons. 6-OHDA was then injected through a stainless steel needle (0.4 mm outer diameter) that was inserted through a small burr hole on the right side of the skull. The needle tip was placed 4.5 mm posterior to the bregma, 1.2 mm lateral to the sagittal suture, and 9 mm ventral to the skull surface, according to the atlas of Paxinos and Watson (Paxinos and Watson, 1998). 6-OHDA (8 μg/4 μl in saline with 0.01% ascorbic acid) was injected over 4 min, after which the needle was left in place for another 4 min to prevent backflow leakage. The same procedure was used to generate a sham lesion with the injection of saline only.

To evaluate the extent of 6-OHDA lesioning, after 2 weeks rats underwent rotational behavior testing. Apomorphine (0.05 mg/kg) was subcutaneously administered and 10 min later rats were placed in a stainless-steel bowl. After a 5-min accommodation period, the number of turns-to-the left (the side contralateral to the lesion) made by the rat was counted for 5 min. More than 20 contralateral turns indicated the loss of more than 99% of dopamine function in the striatum (Tanaka et al., 1999). Altogether, 73 rats were identified as successfully 6-OHDA-lesioned and divided into the following five experimental groups.

2.1.1. Experiment 1. Evaluation of behavior and MGP volume

Five weeks postoperatively, 18 6-OHDA-lesioned rats were randomly allocated to receive saline injections (Parkinson’s disease-model rats, n = 6; injections of 50 mg/kg L-DOPA methyl ester combined with 12.5 mg/kg benserazide (LID-model rats, n = 6); or injections of 50 mg/kg L-DOPA methyl ester with 12.5 mg/kg benserazide and 1 mg/kg 8-hydroxy-2-(di-n-propylamino)tetralin (8-OH-DPAT) (n = 6). The three groups received intraperitoneal injections twice daily for 2 weeks and all drugs (purchased from Sigma [Japan], except 8-OH-DPAT, which was obtained from Research Biochemicals International [USA]) were dissolved in saline. The high dose of L-DOPA was chosen based on our previous study in which we confirmed that it induced unphysiological fluctuation of extracellular dopamine in the 6-OHDA-lesioned striatum (Tanaka et al., 1999). 8-OH-DPAT is a 5-hydroxytryptamine (5-HT)1A receptor agonist, which has been shown to improve the fluctuations of the striatal dopamine level following L-DOPA treatment (Kannari et al., 2001) and LID-like behaviors (Lindenthal et al., 2015) in a rat model of Parkinson’s disease. Therefore we used 8-OH-DPAT to examine whether the drug affects the MGP volume alteration associated with repeated L-DOPA treatment.

The behavioral effect of administering L-DOPA to dopamine-denervated (6-OHPA-lesioned) rats was examined by recording AIMs on day 1, 7, and 14 according to the method of Cenci et al. (Cenci et al., 1998). Briefly, rats were observed individually for a 1-min monitoring period every 20 min for 3 h. Abnormal repetitive movements that affected the side of the body contralateral to the lesion were classified into axial dystonia, i.e., contralateral twisted posturing of the neck and upper body; orolingual dyskinesia, i.e., jaw movements and contralateral tongue protrusion; or forelimb dyskinesia, i.e., repetitive jerks of the contralateral forelimb. Each rat was scored on a severity scale
from 0 to 4 by two independent examiners blinded to the animal treatment conditions. The sum of the axial, orolingual, and limb scores was compared between 6-OHDA-lesioned rats treated with L-DOPA and those treated with L-DOPA plus B-OH-DPAT.

To measure the MGP volume, each rat was decapitated 12 h after the last treatment and their brain was immediately removed, frozen on powdered dry ice, and stored at −80 °C. Frozen brains were sectioned on a cryostat (Microm, Carl Zeiss, Germany) at 14-μm in the coronal plane that passes through the MGP, as shown in the atlas by Paxinos and Watson (Paxinos and Watson, 1998). The MGP volume was estimated as previously described (Gundersen et al., 1988). Briefly, every 4th section (10 sections in total) was stained according to the Klüver–Barrera method and viewed using an Olympus model SHZ-ILLB microscope (Olympus, Japan). MacSCOPE computer software (Mitani, Japan) was used in conjunction with a PowerMac G4 computer system (Apple Computer, Cupertino, California, USA) to digitize images. The MGP volume was calculated using the MGP area visible in each of the 10 sections and the distance between sections. The ratio of the MGP volume on the lesioned side to that on the intact side was calculated and compared among the treatment groups.

2.1.2. Experiment 2. Neuron-level morphology

Twenty-five unilaterally 6-OHDA-lesioned rats and one sham-operated rat were used to determine the neuron-level changes involved in MGP hypertrophy. Seven 6-OHDA-lesioned rats received twice-daily saline injections (Parkinson's disease-model) and 18 lesioned rats received twice-daily injections of L-DOPA with benserazide (LID-model), as described in experiment 1.

2.1.2.1. Light microscopy evaluations. Twelve hours after the last injection, five of the saline-treated and six of the L-DOPA-treated rats were anesthetized with pentobarbital (50 mg/kg) and transectally perfused with 4% PFA in 0.1 M phosphate buffer (PB), pH 7.4. After perfusion, the brains were immediately removed and immersed in the same fixative for 5 h at 4 °C. The brains were then embedded in paraffin and sectioned at 4 μm in the coronal plane through the MGP in five consecutive sections that were subsequently stained using the Klüver–Barrera method.

To measure MGP areas and the gray matter in the MGP, the stained sections were viewed using an Olympus model SHZ-ILLB microscope and MacSCOPE was used in conjunction with a PowerMac G3 to digitize images. An Olympus model BH-2 microscope in conjunction with MacSCOPE was used in conjunction with a PowerMac G3 to digitize images and measure the cross-sectional area of the MGP. The cross-sectional areas were compared among the treatment groups.

2.1.2.2. Immunohistochemistry evaluations. To measure immunoreactivity for vesicular GABA transporter (VGAT) and vesicular glutamate transporter 1 (VGLUT1) with light microscopy, a vibratome (Vibratome, St. Louis, Missouri, USA) was used to cut 50-μm-thick sections through the MGP of one 6-OHDA-lesioned rat treated with L-DOPA. To assess immunohistochemistry for glial fibrillary acidic protein (GFAP) with light microscopy, 4-μm-thick sections were prepared using a sliding microtome (HM430, Thermo Fisher Scientific, Waltham, Massachusetts, USA). The sections were immunostained with a VECTASTAIN® avidin-biotin-peroxidase complex (ABC) kit (Vector Laboratories, Burlingame, California, USA) and incubated with rabbit anti-VGAT antibody (1:1000) (Miyazaki et al., 2003), rabbit anti-VGLUT1 antibody (1:1000) (Miyazaki et al., 2003), and rabbit anti-GFAP antibody (ready-to-use kit, Dako, Glostrup, Denmark). The reaction was visualized by incubation with 0.02% 3,3′-diaminobenzidine tetrachloride (DAB) and 0.005% H2O2 in 0.05 M Tris-HCl buffer, pH 7.6, for 10 min at 21 °C.

For electron microscopy, the brains of one rat from the saline-treated group and one from the L-DOPA-treated group were fixed with 4% paraformaldehyde and 0.1% glutaraldehyde in 0.1 M PB. A vibratome was used to cut 50-μm-thick sections through the MGP. The sections were incubated in 5% normal goat serum, followed by overnight incubation at 4 °C with rabbit anti-VGAT antibody (1:1000) (Miyazaki et al., 2003), rabbit anti-VGLUT1 antibody (1:1000) (Miyazaki et al., 2003), rabbit anti-dynorphin antibody (1:1000; Phoenix Pharmaceuticals, Inc., Belmont, CA, USA), rabbit anti-substance-P antibody (1:1; Nichirei, Tokyo, Japan), or rabbit anti-parvalbumin antibody (1:1000; Swant, Bellinzona, Switzerland). The sections were incubated in biotinylated secondary antibody (1:200) and ABC (1:200). The reaction was developed with DAB (0.1 mg/ml) containing 0.0015% hydrogen peroxide. The immunolabeled sections were post-fixed in 1% glutaraldehyde and 1% osmium tetroxide, dehydrated in ethanol, embedded in epoxy resin, and stained with lead citrate, and then viewed using a Hitachi H-300 electron microscope (Hitachi, Tokyo, Japan).

2.1.2.3. Electron microscopy evaluation of synaptic areas. MGP synaptic areas were evaluated by electron microscopy in one sham-operated rat, one lesioned saline-treated rat, and 10 lesioned L-DOPA-treated rats. Twelve hours after the last saline or L-DOPA treatment, one brain from a sham-operated rat, one brain from a rat in the saline-treated group, and two brains from rats in the L-DOPA-treated group were fixed with 2.5% glutaraldehyde in 0.1 M PB, pH 7.4 and sectioned at 50 μm using a vibratome. The other eight lesioned rats were sacrificed at 5 weeks (3 rats), 10 weeks (3 rats), or 30 weeks (2 rats) after stopping L-DOPA, with their brains processed for electron-microscopic examinations.

Ultra-thin sections were obtained from the MGP area using an ultramicrotome (MT2, Sorvall, Norwalk, Conn., USA) and viewed using a Hitachi H-300 electron microscope. For quantitative evaluations, photomicrographs (×22,500) were taken of randomly selected electron microscope fields and the area of the axon terminals making synaptic contacts with dendrites and the number of synaptic vesicles in the terminals were measured using NIH image software version 1.61.

2.1.3. Experiment 3. Tracer study of synaptic terminals of direct and indirect pathways

Eight uninjuredly 6-OHDA-lesioned rats were injected twice daily for 2 weeks with L-DOPA with benserazide (LID-model), as described in experiment 1. Three days after the last injection, rats were anesthetized by intraperitoneal injection of 50 mg/kg pentobarbital and placed in a stereotactic head holder. A 10% solution of biotin dextran amine (BDA) (Molecular Probes, Eugene, Ore., USA) in 0.01 M PB (pH 7.2) was iontophoretically injected on each side into the striatum and LGP in five and three animals, respectively. The stereotactic coordinates (Paxinos and Watson, 1998) for injections into the dorsolateral part of the striatum were 0.5 mm anterior to the bregma, 3.0 mm lateral to the sagittal suture, and 5.0 mm ventral to the dura mater. For injections into the globus pallidus, the coordinates were 1.0 mm posterior to the bregma, 3.0 mm lateral to the sagittal suture, and 6.0 mm ventral to the dura mater. To inject the BDA solution, a glass micropipette (30 μm-tip diameter) was used with a pulse (7 s on/off) positive current (5 to 7 A) for 20–30 min.

On day 7 after BDA injection, rats were re-anesthetized and perfused transcardially with 100 ml of saline followed by 500 ml of 4% paraformaldehyde in 0.1 M PB (pH 7.4). The brain was removed and post-fixed for 1 h in the same fixative, then placed in 30% sucrose in 0.1 M PB, and stored at 4 °C overnight. The next morning, the brain was cut in 50-μm-thick sections in the coronal plane. The sections were incubated in ABC solution (ABC Elite kits; Vector) for 2 h at 21 °C followed by DAB incubation with 0.03% nickel ammonium sulfate. We used an Olympus model BH-2 microscope with MacSCOPE on a PowerMac G3 to digitize images and measure the cross-sectional area of 100 BDA-labeled synaptic boutons in the MGP on the intact and lesioned sides.
2.1.4. Experiment 4. Microdialysis evaluation of GABA release

Five unilaterally 6-OHDA-lesioned rats were treated with saline (Parkinson’s disease-model) and five rats with L-DOPA (LID-model), as described in experiment 1. Five sham-operated rats that received twice-daily saline injections for 14 days were also used. Twelve hours after the last injection, with rats under 50 mg/kg pentobarbital anesthesia, a stainless steel guide cannula (Eicom Co., Kyoto, Japan) was stereotactically implanted in the MGP on the lesioned side aimed at a site 1.5 mm dorsal to the MGP (2.8 mm posterior to the bregma, 2.8 mm lateral to the sagittal suture, and 7.4 mm ventral to the skull surface) according to the atlas of Paxinos and Watson (Paxinos and Watson, 1998). Guide cannulas were fixed to the skull by dental cement and screws. Two days later, rats were placed under anesthesia and a microdialysis probe with a 1.5-mm active membrane (Eicom) was inserted through the guide cannula. The following morning, we started continuous dialysis through the probe at a constant flow rate of 1 μl/min of an artificial cerebrospinal fluid solution (138 mM NaCl, 2.7 mM KCl, 1.2 mM CaCl2, 1.0 mM MgCl2/6H2O, and 1.5 mM Na2HPO4/12H2O).

We started to collect dialysates 6 h after the beginning of the perfusion. Baseline samples were collected every 20 min over the next 120 min. Subsequently, the rats received a single intraperitoneal injection of 50 mg/kg L-DOPA with 12.5 mg/kg benserazide and samples were collected again every 20 min for 120 min. Each sample was immediately mixed with 20% methanol and stored at −80 °C.

The extracellular GABA level was determined by high-performance liquid chromatography with o-phthalaldehyde-derived fluorescence detection (Zhu et al., 2006). The analytical column (100 mm × 3.0 mm internal diameter; Maxis Inc., Hiroshima, Japan) was packed with Mightysil RP-18 (particle size, 3 μm; Kanto Chemicals, Tokyo, Japan) and maintained at 30 °C. The excitation and emission wavelengths of the fluorescence detector were 340 and 445 nm, respectively. A linear gradient elution was performed over 30 min at a flow rate of 400 μl/min for both the “mobile” (0.05 M PB containing 25% methanol, pH 6.0) and “static” phases (0.05 M PB containing 40% methanol, pH 3.5).

The rats were then sacrificed by decapitation under deep anesthesia, and their brains were quickly removed and frozen on powdered dry ice. The brains were sectioned on a cryostat at the level of the MGP. The location of microdialysis probes was determined on consecutive coronal sections, and only rats in which the microinjection cannula was determined on consecutive coronal sections, and only rats in which the probe had been placed in the MGP without excessive tissue damage were included in the analyses.

The mean baseline level of GABA was determined using the six baseline fluid collections for each rat. L-DOPA-induced GABA release at each collection time was calculated as the difference between the mean baseline value and the extracellular GABA level in that sample. The cumulative L-DOPA-induced increase in extracellular GABA levels was determined by subtracting the area under the curve (AUC) of the baseline fractions from the AUC after L-DOPA challenge.

2.1.5. Experiment 5. Dyskinesia and microinjection of GABA_A receptor agonist and antagonist

Under pentobarbital anesthesia (50 mg/kg), a stainless steel guide cannula was stereotactically implanted in the MGP of 12 unilaterally 6-OHDA-lesioned hemiparkinsonian rats, as described in experiment 4. Two days later, a microinjection stainless steel cannula (35-g) (Eicom) protruding 0.5-mm from the guide cannula was placed under light ether anesthesia. After dissolution in saline (60 ng/μl), 1 μl of muscimol, a GABA_A receptor agonist, was microinjected into the lesioned side of the MGP for 2 min in four rats. Four other rats displaying L-DOPA-induced AIMs received intraperitoneal injection of L-DOPA (50 mg/kg) with benserazide (12.5 mg/kg) 20 min prior to the microinjection of bicuculline methiodide, a GABA_A receptor antagonist (dissolved in saline [0.5 μg/μl] with injection of 1 μl for 5 min). The remaining 6-OHDA-lesioned rats received microinjection of saline (1 μl) into the MGP on the lesioned side for 2 min. The rats were video-recorded for 10 min following injection, and AIM scores (Cenci et al., 1998) were independently recorded every minute by two blinded examiners. The rats were decapitated under deep anesthesia the next day, and their brains were quickly removed and frozen on powdered dry ice. The brains were sectioned on a cryostat at the level of the MGP. The location of the microinjection cannula was determined on consecutive coronal sections stained with hematoxylin and eosin, and only rats without excessive tissue damage were included in the analyses.

2.2. Statistical analysis

Statistical analyses were performed using the Statview computer software program, version 5.0 (Abacus Concepts, California, USA). Differences were considered to be significant at a level of P < .05.

2.2.1. Experiment 1

Differences in the AIM scores between the 6-OHDA + L-DOPA and the 6-OHDA + L-DOPA + 8-OH-DPAT groups were evaluated using two-factor repeated measures ANOVA. Differences in the volume ratios of the MGP among the three groups were assessed using one-factor ANOVA followed by post-hoc comparisons with the Tukey–Kramer test.

2.2.2. Experiment 2

Differences in the total MGP areas and MGP gray matter areas were evaluated using one-factor ANOVA followed by post-hoc comparisons with the Tukey–Kramer test. The number of MGP neurons was evaluated using one-factor ANOVA followed by post-hoc comparisons with the Tukey–Kramer test. Differences between the areas of nerve cell bodies in the intact and lesioned sides of each rat were compared using unpaired Student’s t-tests. Differences in the areas of axon terminals having synaptic contacts with dendrites and densities of vesicles in the axon terminals were compared between the intact and lesioned sides using Student’s t-test.

2.2.3. Experiment 3

Differences in the cross-sectional areas of synaptic boutons on the intact and lesioned sides of the MGP in each rat were explored using unpaired Student’s t-test.

2.2.4. Experiment 4

Baseline levels of GABA release in the MGP were compared using the Kruskal–Wallis test followed by post-hoc Mann–Whitney U test. Statistical analysis was performed for L-DOPA-induced changes in GABA release from the basal levels by two-factor repeated measures ANOVA. The mean cumulative increases in GABA induced by L-DOPA injection were evaluated using the Kruskal–Wallis test followed by post-hoc Mann–Whitney U test.

2.2.5. Experiment 5

The AIM score at the time examined was compared to the basal value of each rat using paired Student’s t-test.

2.2.6. Data availability

All the raw data are available from the corresponding author upon request.

3. Results

3.1. Behavior and MGP volume

Experiment 1 confirmed that L-DOPA induced AIMs in hemiparkinsonian rats and showed that these LID-model rats had hyperrophy of the MGP on the lesioned side (Fig. 1).

6-OHDA-lesioned rats treated with saline (Parkinson’s disease-model) did not show any AIMs (Fig. 1A). At day 1, the total AIM scores
were not different between hemiparkinsonian rats treated with L-DOPA alone and L-DOPA plus 8-OH-DPAT \((P = .19)\) (Fig. 1A). Fourteen days of treatment with L-DOPA alone (LID-model) led to a significant, progressive increase in AIM scores. However, 8-OH-DPAT significantly improved AIMs over 14 days (Fig. 1A; treatment effect: \(F = 15.39, P < .01\); time effect: \(F = 19.94, P < .0001\); treatment-time interaction: \(F = 8.96, P < .01\)). The score of each AIM subtype (axial dyskinesia, orolingual dyskinesia, and forelimb dyskinesia) on day 14 was also significantly lower in the L-DOPA plus 8-OH-DPAT group compared to the L-DOPA alone group (Supplementary Fig. 1).

In the Parkinson’s disease-model, the MGP volume was similar on the lesioned and intact sides (Fig. 1B and C). In contrast, in the LID-model, the MGP had a significantly greater volume on the lesioned side (Fig. 1B and C). However, treatment with L-DOPA with 8-OH-DPAT resulted in less hypertrophy compared to treatment with L-DOPA alone (Fig. 1B and C).

### 3.2. Neuron-level changes

Experiment 2 showed enlargement of GABAergic synaptic terminals in the MGP of the lesioned side in LID-model rats (Fig. 2).

The MGP hypertrophy on the lesioned side of rats treated with L-DOPA was similar to that observed in experiment 1. Furthermore, experiment 2 showed that the increase in the MGP area on the lesioned side resulted mainly from an increase in the gray matter area (Fig. 2A and B). The area of fiber bundles in the MGP darkly stained by the Klüver–Barrera method was similar for rats in the Parkinson’s disease-model and LID-model (data not shown). This suggested that MGP hypertrophy was due to an increase in the number of neurons or size of nerve cell bodies, glial proliferation, or changes in neuropils. To
Fig. 2. Morphological and immunohistochemical evaluations in the medial globus pallidus (MGP) in unilaterally 6-hydroxydopamine (OHDA)-lesioned (dopamine-denervated) rats treated with saline and levo-dihydroxyphenylalanine (L-DOPA).

(A) The gray matter area in the MGP on the lesioned side was larger in an L-DOPA-treated rat (image on the right) compared to that in a saline-treated rat (image on the left). Scale bar, 200 μm.

(B) Compared to saline-treated rats (n = 5), rats treated with L-DOPA (n = 6) had a larger mean MGP area on the lesioned side (“Total MGP area”) (*P < .05). The larger MGP was primarily due to a larger mean area of the gray matter (“Gray matter area”) (*P < .05). Error bars represent standard error of the mean (SEM).

(C) In a 6-OHDA-lesioned rat repeatedly treated with L-DOPA, Klüver–Barrera staining showed larger nerve cell bodies in the MGP on the lesioned side (right) compared to the intact side (left). Scale bar, 20 μm.

(D) In the five saline-treated rats (left), nerve bodies in the MGP had similar areas in the intact and 6-OHDA-lesioned sides, but in the six L-DOPA-treated rats (right), the mean area of nerve bodies was significantly larger on the lesioned side (**P < .01). Error bars represent SEM.

(E) Immunoreactivity for vesicular GABA transporter in the gray matter of the MGP was more widely distributed on the lesioned side compared to the intact side of an L-DOPA-treated 6-OHDA-lesioned rat. Scale bar, 200 μm.
elucidate which of these mechanisms might account for the gray matter enlargement, we counted the number of MGP neurons with nucleoli and measured the area covered by MGP cell bodies on consecutive sections through the MGP. Dopaminergic denervation following L-DOPA treatment did not change the number of MGP neurons. On each section, approximately 20 neurons with nucleoli were seen on both sides in the LID-model (mean 20.6 ± 0.9 [standard error of the mean; SEM] and 18.8 ± 1.5 cells per section on the intact and lesioned sides, respectively) and in the Parkinson’s disease-model (mean 20.3 ± 2.7 and 18.3 ± 1.0 cells per section on the intact and the lesioned sides, respectively).

Subsequently, we found a significant increase in the neuronal cell body area in the MGP on the lesioned side of LID-model rats (Fig. 2C and D). Specifically, the mean area of nerve cell bodies on the lesioned side in LID-model rats was 193.5 ± 11.7 μm², compared to 123.2 ± 4.5 μm² in Parkinson’s disease-model rats (P < .001). The mean areas of nerve cell bodies in the MGP on the intact side of LID-model and Parkinson’s disease-model were not significantly different (133 ± 6.0 μm² in the LID-model and 125.0 ± 3.6 μm² in the Parkinson’s disease-model rats) (Fig. 2D). However, the increased area of nerve cell bodies in the MGP on the lesioned side of LID-model rats (Fig. 2D) was much smaller than that of the gray matter (Fig. 2B).

Immunohistochemical staining revealed no significant difference in GFAP immunoreactivity in the MGP on the lesioned side of Parkinson’s disease-model or LID-model rats (Supplementary Fig. 2), suggesting that glial proliferation did not account for MGP hypertrophy.

Since MGP neurons receive GABAergic and glutamatergic projections (DeLong, 1990), we also tested for immunoreactivity to their respective markers, VGAT and VGluT1. The immunoreactive area for VGAT in the gray matter of the MGP was larger on the lesioned side compared to the intact side in the LID-model (Fig. 2E). However, immunoreactivity to VGluT1 was low on both MGP sides of the LID-model (data not shown). Thus, an increase in axons and/or axon terminals of GABAergic neurons contributes to MGP hypertrophy in the LID-model.

Our examination of the MGP ultrastructure in sham-operated, Parkinson’s disease-model, and LID-model rats (Fig. 3A) showed that axon terminals surrounding dendrites in the MGP appeared swollen on the lesioned side in the LID-model (Fig. 3A) compared to the Parkinson’s disease-model (Fig. 3A). Measurements confirmed that the axon terminal area surrounding the dendrites of MGP neurons was significantly greater on the lesioned side compared to the intact side of the LID-model rats (Fig. 3B), but not in the sham-operated (Fig. 3B) or Parkinson’s disease-model rats (Fig. 3B).

The synaptic vesicle density was greater on the lesioned compared to the intact side of the LID-model rats (Fig. 3C), but not of the Parkinson’s disease-model rat (Fig. 3A-2, 3A-3, 3C). Thus, the enlargement of the synaptic terminals and increase in synaptic vesicle density are associated with enlargement of the gray matter in the MGP on the lesioned side of LID-model rats. Furthermore, all three LID-model rats showed significant swelling of synaptic terminals 5 weeks after L-DOPA discontinuation (Figs. 3A-4 and 3B). However, none of the LID-model rats sacrificed 30 weeks after L-DOPA discontinuation showed synaptic terminal swelling (Figs. 3A-6 and 3B). The reversibility of synaptic terminal enlargement after L-DOPA discontinuation confirmed that L-DOPA treatment induced the enlargement.

Immunoelectron microscopy revealed that all the synaptic terminals that were swollen (area greater than 1 μm²) on the lesioned side of LID-model rats showed VGAT immunoreactivity (e.g., Fig. 3D), whereas enlarged axon terminals did not show VGluT1 immunoreactivity (data not shown). Thus, the GABAergic terminals, not the glutamatergic terminals, were enlarged in the MGP on the lesioned side of the LID-model. The dendrites surrounded by enlarged axon terminals were immunoreactive for parvalbumin (Fig. 3D), a marker for projection neurons to the motor thalamus (Rajakumar et al., 1994), suggesting the involvement of the MGP neurons in motor circuitry.

There are two main GABAergic projections to the MGP; one from the striatum (the direct pathway) and the other from the LGP (Parent and Hazrati, 1995). The direct pathway involves dynorphin and substance P with GABA (Gerfen et al., 1990). In our rat LID-model, swollen synaptic terminals in the MGP on the lesioned side showed immunoreactivity for dynorphin and substance P (Fig. 3D), indicating that these were terminals of the striatum-MGP direct pathway. However, these results do not exclude the possibility that axon terminals of the LGP-MGP pathway were also enlarged.

In experiment 2, only one sham-operated rat was used in the ultrastructural examination of the MGP with L-DOPA treatment. In experiment 4, five sham-operated rats were used in the microdialysis evaluation of GABA release. We did not examine sham-operated rats in other experiments. We considered that the intact side of the striatum and MGP of 6-OHDA-lesioned Parkinson’s disease-model rats was almost normal, although hemidopaminergic denervation may slightly affect the pathology of contralateral side. Since we did not find any apparent pathological changes in the intact side of the MGP, the effect of L-DOPA likely requires a 6-OHDA-lesion to trigger globus pallidus abnormality.

3.3. Tracer study of synaptic terminals in the MGP

In experiment 3, injection of the anterograde tracer BDA into the striatum or the LGP on both sides of LID-model rats showed enlargement of the cross-sectional area of BDA-labeled synaptic terminals on the lesioned side in the striatum but not the LGP (Fig. 4A and B). This confirmed that axon terminals of the direct pathway became enlarged, but those of LGP-MGP did not. Additionally, axons of projection neurons of the striatum to the MGP appeared to be thickened on the lesioned side (Fig. 4A, top right).

3.4. Microdialysis evaluation of GABA release in the MGP

We hypothesized that abnormal GABA transmission in the MGP was involved in the emergence of LID, which was confirmed by our study involving in vivo microdialysis challenge with L-DOPA (experiment 4).

Baseline GABA levels in the MGP of LID-model, Parkinson’s disease-model, and sham-operated rats were 37.0 ± 4.8 pmol/sample, 19.9 ± 3.1 pmol/sample, and 28.9 ± 2.6, respectively, with a significant difference between the LID-model and the Parkinson’s disease-model. In the LID-model, systemic L-DOPA challenge resulted in increased extracellular GABA levels in the MGP measured through a probe histologically confirmed to have been placed in the MGP (Fig. 5A) that were higher than those in the Parkinson’s disease-model (Fig. 5B, main effect of repeated L-DOPA treatment: F = 6.55, P < .05). The changes in GABA levels induced by L-DOPA challenge were significantly different between the Parkinson’s disease-model and sham-operated controls (Fig. 5B, main effect of dopaminergic denervation: F = 26.73, P < .01). The mean cumulative level of GABA release in response to L-DOPA challenge was significantly different between the sham-operated control, Parkinson’s disease-model, and LID-model rats (Fig. 5C; P < .01). The cumulative increase of GABA release was higher on the lesioned side of LID-model than Parkinson’s disease-model rats (Fig. 5C; P < .05). The mean cumulative level of GABA release in the Parkinson’s disease-model was higher than that of the sham-operated control (Fig. 5C; P < .01). Thus, experiment 4 showed that L-DOPA challenge increased GABA release in the MGP on the lesioned side in both LID-model and Parkinson’s disease-model rats, but significantly more in the LID-model rats.

3.5. Microinjection of GABA_A receptor agonist and antagonist into the MGP

To confirm that GABA receptor stimulation in the MGP induces dyskinesia-like AIMs, and that inhibition of GABA transmission in the MGP improves L-DOPA-induced AIMs, we performed microinjection of a GABA_A receptor agonist (muscimol) and antagonist (bicuculline) into
**A** 1. sham 2. vehicle 3. L-DOPA 12 hrs 4. L-DOPA 5 wks 5. L-DOPA 10 wks 6. L-DOPA 30 wks

**B** Area of axon terminal

**C** Synaptic vesicle density

**D** VGAT Parvalbumin Substance-P Dynorphin

(caption on next page)
the MGP on the lesioned side of Parkinson's disease-model rats (experiment 5). Muscimol injection induced AIMS similar to L-DOPA-induced AIMS (Fig. 6A-6D, Video 1). Additionally, bicuculline significantly alleviated L-DOPA-induced AIMS, which were revived after discontinuation of bicuculline microinjection (Fig. 6A-6D, Video 2). Saline injection into the MGP on the lesioned side of Parkinson's disease-model rats had no behavioral effects (Fig. 6A-6D), suggesting that tissue damage by microinjection did not cause any behavioral changes. Thus, experiment 5 showed that activation of GABA_A receptors in the MGP of Parkinson's disease-model rats induced dyskinesia-like AIMS, and inhibition of GABA signaling improved L-DOPA-induced AIMS.

4. Discussion

In this study, we found pathologic abnormalities in rats rendered dyskinetic on the lesioned side, including MGP hypertrophy, axon terminal enlargement of GABAergic neurons projecting from the striatum to the MGP (direct pathway), and an increased synaptic vesicle density in the enlarged terminals. These results provide evidence that LID priming involves plastic morphologic changes resulting from excessive GABA storage in axon terminals of the direct pathway from the striatum to the MGP. We showed that enhanced GABA release occurred in the MGP of LID-model rats following L-DOPA injection. Furthermore, disruption of GABA transmission in the MGP improved LID-like involuntary movements. These findings prove the hypothesis that GABA-mediated excessive signaling into the output nucleus of the basal ganglia is associated with LID (Cenci, 2007).

4.1. L-DOPA treatment and MGP hypertrophy in the LID-model

Our finding that administration of the 5-HT_1As receptor agonist 8-OH-DPAT in conjunction with L-DOPA has a beneficial effect on the level of AIMS is consistent with previous studies in which co-administration of 8-OH-DPAT with L-DOPA inhibited LID in parkinsonian monkeys (Iravani et al., 2006) and rats (Lindenbach et al., 2015). Specifically, we found a lower AIM incidence and less MGP hypertrophy in 6-OHDA-lesioned rats treated with 8-OH-DPAT additional to L-DOPA versus L-DOPA alone. It has been previously established that 5-HT_1As receptor agonists can reduce LID in rodent models of Parkinson's disease (Lanza and Bishop, 2018). Thus, our findings suggest that MGP hypertrophy is directly associated with AIM development.

Other studies have linked LID development to supra-physiologic swings in striatal dopamine levels following L-DOPA treatment (Pavese et al., 2006). We have previously found that serotonin neurons are the primary site for the storage and release of L-DOPA-derived dopamine into the dopamine-denervated striatum of 6-OHDA-lesioned rats (Tanaka et al., 1999), and that pretreatment with 8-OH-DPAT alleviates the marked fluctuations in the dopamine level following L-DOPA treatment (Kannari et al., 2001). Accordingly, 8-OH-DPAT co-administration would be expected to improve AIMS by attenuating marked swings in striatal dopamine levels following every L-DOPA administration, further supporting the 5-HT_1As receptor as a promising target in LID treatment (Lanza and Bishop, 2018). The dose of L-DOPA in the present study was high, and may be irrelevant to clinical situations; indeed, a lower L-DOPA dose can induce AIMS (Nishijima et al., 2018b). Future studies using lower L-DOPA doses are warranted to support our findings and interpretations.

4.2. Involvement of direct pathway in development of MGP hypertrophy

Dopamine acts in the striatum principally through the D1 and D2 dopamine receptors, which segregate to the direct and indirect striatal projection neurons, respectively (Albin et al., 1989). Accordingly, the enlargement of axon terminals of the direct pathway striatal neurons in L-DOPA-treated rats observed in the present study strongly supports the concept that dyskinesia develops because of repetitive abnormal stimulation of D1 receptors (Bezard et al., 2001; Obeso et al., 2000). Consistently, two studies have found persistent D1 receptor supersensitivity in dopamine-denervated striatum (Corvol et al., 2004; Gerfen et al., 2002). Another study has found increased D1 receptor signaling in a LID-model (Aubert et al., 2005). Furthermore, in 6-OHDA-lesioned rats treated with L-DOPA, D1 receptor hypersensitivity is associated with synaptic plasticity alterations at corticostriatal synapses (Picconi et al., 2003) and morphologic changes in dendritic spines of striatal direct pathway neurons (Nishijima et al., 2018b). Additionally, via D1 receptor stimulation, intermittent L-DOPA treatment contributes to the overexpression of D3 dopamine receptors co-expressed with D1 receptors in neurons of the direct pathway and to the development of LID or behavioral sensitization to L-DOPA (Bordet et al., 1997; Bordet et al., 2000).

In one gene expression study, chronic L-DOPA treatment of 6-OHDA-lesioned rats resulted in increased levels of dynorphin and glutamic acid decarboxylase (GAD_67) (an isofrom of the enzyme that synthesizes GABA) mRNA in the striatum of the lesioned side, and the increase in GAD_67 mRNA correlated with increased AIMS (Cenci et al., 1998). Neurons of the direct pathway express dynorphin with GABA...
Gerfen et al., 1990) and over-express GAD67 mRNA in the rat LID-model (Nielsen and Soghomonian, 2004). These studies suggest that chronic L-DOPA administration induces abnormal GABAergic transmission from the direct pathway.

It has been suggested that neurons of the direct pathway are underactive when nigrostriatal dopamine neurons are destroyed, because the excitatory effects of D1 dopamine receptor activity are lost (Albin et al., 1989). A transient marked increase in dopamine levels in the

Fig. 4. Effects of levo-dihydroxyphenylalanine treatment on axon terminals of neurons projecting to the medial globus pallidus (MGP) from the striatum (Str) (n = 5) or lateral globus pallidus (LGP) (n = 3) in unilaterally 6-hydroxydopamine-lesioned (dopamine-denervated) rats.

(A) Results of biotinylated dextran amine labeling of axons (arrows) and axon terminals (arrow heads) in representative sections on the intact and lesioned sides. Synaptic boutons (axon terminals) in the direct (Str-MGP) pathway appeared enlarged on the lesioned side but those in the LGP-MGP pathway appeared similar in size on both sides. Scale bar, 40 μm.

(B) Measurements of mean cross-sectional areas of synaptic boutons confirm that boutons of the Str-MGP pathway were larger on the lesioned sides but areas were not different for boutons of the LGP-MGP pathway (**P < .01). Error bars represent standard errors of the mean.
suggested that an increase in GABA synthesis occurs in the neurons of the direct pathway in the LID-model. However, the half-life of L-DOPA is short, and although L-DOPA administration is followed by a rapid increase in the level of dopamine in the dopamine-denervated striatum, it rapidly declines (Kannari et al., 2001). Consequently, the excitatory effects of activation of D1 receptors on neurons of the direct pathway (Albin et al., 1989) may also be rapidly lost, in turn decreasing the activity of neurons synthesizing excessive GABA. This sudden switch in the activity of neurons of the direct pathway may be responsible for the increase in GAD67 mRNA levels in the neurons (Nielsen and Soghomonian, 2004) and the increase in the number of synaptic vesicles, which presumably contain GABA in their axon terminals. Repeated abnormal swings in neuronal activity due to pharmacologic dosing with L-DOPA may cause axon terminals to enlarge, leading to MPG hypertrophy.

Our finding that L-DOPA discontinuation alleviated the enlargement of axon terminals also indicates that their enlargement results from L-DOPA treatment and is reversible as the priming for LID in patients with Parkinson’s disease (Bejjani et al., 2000).

It should be noted that we do not provide direct quantitative evidence of GABA storage in the axon terminals in the MPG of the LID-model rats. However, since we observed MPG hypertrophy with enlarged axon terminals of direct pathway striatal projection neurons; an increased density of synaptic vesicles; positive VGAT immunoreactivity of the axon terminals in the MPG on the 6-OHDA-lesioned side; and enhanced GABA release from the MPG after acute L-DOPA treatment, we speculate that additional synaptic vesicles in the MPG of LID-model rats contain excessive GABA.

4.3. Relationship between MPG hypertrophy, GABA transmission, and expression of dyskinesia

Our results support the hypothesis that increased GABAergic transmission in the MGP is involved in LID genesis (Cenci, 2007). Acute L-DOPA administration can elicit dyskinesia provided the dose is sufficiently high and dopaminergic denervation is severe enough (Cenci and Lundblad, 2006). The reduction of LID threshold by dopaminergic denervation can be explained by a loss of negative feedback via GABA_A receptor in the axon terminal of the direct pathway neurons (Borgkvist et al., 2015). The finding that L-DOPA induces more GABA release into the MPG in Parkinson’s disease-model rats than in controls probably reflects the loss of negative feedback. However, repeated L-DOPA administration further reduces the threshold dose for LID (Lundblad et al., 2002). Thus, LID development is determined by two factors, dopaminergic denervation (lesion-induced plasticity) and priming (L-DOPA-induced plasticity) (Borgkvist et al., 2018; Cenci and Lundblad, 2006).

In our study, differences in behavioral and pathologic manifestations between the Parkinson’s disease-model and LID-model reflect L-DOPA-induced plasticity. The excessive GABA storage, ready to be released into the MGP, in axon terminals of the direct pathway observed in LID-model rats represents the priming process of dyskinesia. This priming step in LID development provides a rationale for the dramatic efficacy of surgical manipulations such as pallidotomy and pallidal DBS for LID in patients with Parkinson’s disease (Fine et al., 2000).

However, these pathologic changes were found in rats sacrificed when not displaying AIMs (12 h after L-DOPA treatment). Accordingly, rather than MPG hypertrophy, the direct cause of dyskinesia may be an increase in GABA release into the MPG triggered by L-DOPA treatment. Consistently, microinjection of a GABA_A receptor agonist into the MPG on the lesioned side induced dyskinetic movements in Parkinson’s disease-model rats while injection of an antagonist alleviated L-DOPA-induced dyskinetic movements. Thus, these results suggest that excessive GABA transmission in the MPG is directly related to dyskinesia occurrence. It is conceivable that such enhanced GABA release causes electrophysiological abnormalities in MGP neurons via GABA_A receptors.
including decreased firing frequency and alteration of firing pattern, which are thought to be directly related to the emergence of dyskinesia (Boraud et al., 2001; Papa et al., 1999). Experiments in which both a GABAA receptor agonist and antagonist (muscimol and bicuculline, respectively) are microinjected into the MGP would be valuable for further understanding the role of GABAA receptor stimulation in LID expression, and should be conducted in future studies.

Administering an L-DOPA challenge to LID-model (“primed”) rats resulted in enhanced GABA release in the MGP, maybe from enlarged axon terminals of the direct pathway, and more severe AIMs compared to non-primed Parkinson’s disease-model rats. Accordingly, the GABA release level probably determines the severity of dyskinesia. This result is compatible with previous findings that L-DOPA-induced GABA release is increased in the substantia nigra reticulata, another output nucleus of the basal ganglia, in LID-models (Bido et al., 2011; Mela et al., 2012; Mela et al., 2007; Paolone et al., 2015; Rangel-Barajas et al., 2008; Rangel-Barajas et al., 2011; Yamamoto et al., 2006). We speculate the following mechanism for how repeated L-DOPA treatment triggers increased GABA release in the MGP. In Parkinson’s disease-model rats, the striatal dopamine level is relatively low. Repeated L-DOPA treatment causes marked fluctuation of extracellular dopamine levels in the striatum and excessive GABA release in MGP for only a short period after L-DOPA injection. During “OFF time”, i.e. the interval of L-DOPA treatment when dyskinesia-like behavior disappears, synaptic vesicles containing GABA may gradually increase in the primed MGP. Subsequently, intermittent L-DOPA injection may trigger excessive GABA release.

Interestingly, we found that L-DOPA injection appeared to cause a decrease in GABA release in the MGP of sham-operated rats. This paradoxical result may be explained by endogenous dopamine release into the striatum before L-DOPA injection. The microdialysis procedure is likely to be a stressor for rats, and may induce dopamine release in the non-dopamine denervated striatum of the sham-operated rats, resulting in GABA release in the MGP. Thus, when L-DOPA was injected, the axon terminals in the MGP of sham-operated rats might have already lost their stored GABA, causing the GABA release to be lower than the baseline values.

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Declaration of Competing Interest

The authors report no competing interests in relation to the research covered in the submitted manuscript.

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