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Dyskinesias Do Not Develop after Chronic Intermittent Levodopa Therapy in Clinically Hemiparkinsonian Rhesus Monkeys

Christopher A. Lieu^{a,b}, Milind Deogaonkar^c, Roy A. E. Bakay^d, and Thyagarajan Subramanian^{a,b}

^a Department of Neurology, The Pennsylvania State University College of Medicine, Hershey, PA, USA

^b Department of Neural & Behavioral Sciences, The Pennsylvania State University College of Medicine, Hershey, PA, USA

^c Center for Neurological Restoration, The Cleveland Clinic Foundation, Cleveland, OH, USA

^d Department of Neurosurgery, Rush University Medical Center, Chicago, IL, USA

Abstract

The stable 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)-induced hemiparkinsonian (HP) rhesus monkey model of Parkinson's disease (PD) has been frequently used to test preclinical experimental therapeutics targeted to treat patients with advanced PD who suffer from motor fluctuations and drug-induced dyskinesias. We retrospectively analyzed data from 17 stable HP rhesus monkeys treated long-term with chronic intermittent dosing of levodopa (LD) in an attempt to induce choreoathetoid and dystonic dyskinesias. Rhesus monkeys in stable HP state for greater than 6 months as confirmed by multiple blinded behavioral ratings and ¹⁸F-dopa Positron Emission Tomography (PET) were treated with optimal doses of LD to provide maximal amelioration of unilateral clinical parkinsonism without any adverse effects. Thereafter, each animal was given chronic intermittent daily challenge with doses of LD up to 700 mg/day orally or with 300 mg/kg/day parenteral injections. LD treatments failed to induce choreoathetoid and dystonic dyskinesias in these animals despite chronic intermittent high dose administration. These results suggest that the stable strictly unilateral HP rhesus monkey model of PD may not be a suitable animal model to test experimental therapeutics targeted against dyskinesias, and that bilateral parkinsonian rhesus models that readily demonstrate drug-induced dyskinesias and clinically relevant motor fluctuations are more appropriate for preclinical experimental testing of therapies designed to treat patients with advanced PD.

Keywords

Movement disorders; *Macaca mulatta*; basal ganglia; nigrostriatal degeneration; dopamine replacement therapy

Correspondence to: Thyagarajan Subramanian, MD, H109, Neurology and Neural & Behavioral Sciences, Penn State University Hershey Medical Center, 500 University Drive, Hershey, PA 17033, Phone: (717) 531-0003 x283767, Fax: (717) 531-0996, tsubram@yahoo.com.

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INTRODUCTION

PD is characterized by loss of dopaminergic nigrostriatal neurons leading to bradykinesia, tremor, and rigidity. Dopamine replacement by oral LD remains a highly effective therapy for PD, however, undesirable choreoathetoid and dystonic dyskinesias, motor fluctuations, and drug-induced hallucinations are well known side effects of long-term LD therapy. Epidemiological studies show that drug-induced dyskinesias are particularly frequent in young onset PD patients (onset of disease before the age of 40) and in early onset PD patients (disease onset between the age of 40 and 55) [1]. LD-induced dyskinesias (LID) are separately classified as peak dose dyskinesias, diphasic dyskinesias, and off-period dystonia [esupp ref1]. Furthermore, off period dyskinesias in PD patients treated with embryonic mesencephalic tissue transplants into the striatum have been identified [2,esupp ref2]. Nigrostriatal degeneration and pulsatile stimulation due to intermittent oral dosing of LD has been postulated to cause denervation supersensitivity of striatal dopamine receptors which “prime” PD patients to develop LID [3, esupp ref3].

Rhesus monkeys rendered hemiparkinsonian (HP) by MPTP have been a commonly used model of PD to test preclinical experimental therapeutics. Many investigators utilize the HP rhesus monkey because of the convenience of animal husbandry, built-in control that can be used for side-to-side behavioral comparisons, and the availability of considerable neuroscientific literature [4–10, esupp ref4–ref6]. We retrospectively reviewed 10 year data from stable HP rhesus monkeys treated with chronic intermittent doses of LD using a standardized protocol to determine the occurrence of choreoathetoid or dystonic LID in such animals.

MATERIALS AND METHODS

Animals

Seventeen adult female rhesus monkeys (*Macaca mulatta*, 3–6 kg) used as “negative controls” (no treatment or placebo) over the period from 1997–2007 were evaluated in this study. Behavioral observations reported in this retrospective study were done prior to enrollment into any experimental therapeutics protocol. In all cases, randomization into the “negative control” arm had been completed with no consideration of the hypothesis presented in this paper. Procedures were carried out in compliance with the NIH Guide for the Care and Use of Laboratory Animals (NIH Publications No. 80-23, revised 1978), and approved by the institutional animal care and use committee. All efforts were made to minimize pain and discomfort to the animals.

Behavioral Evaluations

The timeline for the experimental design is shown in Fig. 1. Through operant conditioning, the movement time of a fine motor task (FMT) was evaluated using a modified food picking test done twice weekly utilizing two sets of 3×2 matrix of food wells embedded in a Plexiglass board [4]. Each set of food wells can be accessed by the monkey only using the arm that is being tested. Six raisins were placed on the board for each trial. The time required to retrieve all six raisins was taken for each arm over 10 trials.

A clinical rating scale based on the motor portion of the Unified Parkinson’s Disease Rating Scale (mUPDRS) [8] was performed by a physician-scientist (T.S.) in a blinded fashion twice weekly and videotaped. The rater was blinded to the hypothesis presented in this paper at the time the ratings were performed. The mUPDRS consists of subjective rater-dependent but validated assessment of vocalization/hooting, facial expression, tremor, muscle tone/rigidity, hypokinesia, finger dexterity, foot agility, balance/postural instability, spontaneous gait, dystonia and choreiform limb dyskinesia, separately scored from 0 (none) to 4 (severe).

Total scores of 1–5 denote very mild, 6–10 indicate mild, 11–15 indicate moderate, 16–19 indicate moderate to severe and 20–25 indicate severe hemiparkinsonism. Scores over 30 generally indicate bilateral parkinsonism and are unexpected after unilateral intracarotid MPTP injections that cause a stable HP state.

Intracarotid Injection of MPTP

Slow retrograde left intracarotid infusion of MPTP solution (0.5 mg/kg body weight) at 1 mg/ml was done manually over 15 minutes as previously described in detail [7,8]. After the initial injection, animals were observed for the genesis of stable unilateral right hemiparkinsonism. If they did not achieve this state, additional intracarotid MPTP injections were performed up to 4 times at the same dose separated by 2 weeks each time. Cumulative dose of MPTP ranged from 0.5–2.5 mg/kg.

Levodopa Responsiveness in Stable HP Animals

The stability of the right HP state was confirmed using FMT and mUPDRS for a minimum of 6 months before testing the animal for responsiveness to LD therapy and for the genesis of LID. Animals were treated for 3–12 months with LD/dopa-decarboxylase inhibitor. Oral LD treatments were initiated at 100 mg LD/25 mg carbidopa (CD) b.i.d. and increased by 100 mg LD every 72 hours until no further improvement in mUPDRS and FMT (each taken 1.5 hours after LD) to determine the optimal dose. Animals were operant conditioned to accept medications, visually monitored and videotaped for 12 hours thus ensuring complete drug consumption [esupp ref6]. During a drug washout period for 4 weeks, behavioral evaluations continued. Plasma dopamine levels were measured using gas chromatography at pre-LD and post-optimal LD [11].

To test the effects of intermittent high dosing of LD to cause dyskinesias, animals received single daily doses of LD/CD at 700/175 mg/day orally or escalating single injections of LD/benserazide starting at 50/12.5 mg/kg/day and up to 300/12.5 mg/kg/day for 3–4 weeks. Animals were carefully observed and videotaped for a minimum of 12 hours to further evaluate the beneficial effects of LD and determine onset of LID. Thereafter, each 12 hour video was de-identified, scrambled such that they were in random order and carefully evaluated by a blinded rater (T.S.) using a standard dyskinesia rating scale. This dyskinesia rating scale ranged from 0 (no dyskinesias) to 4 (severe dyskinesias) and took into account only unequivocal limb dyskinesias in the form of chorea, ballismus, flinging of arms, violent jerks or athetosis and dystonias modeled after the scale described by Kurlan et al. [12].

Evaluation of dopaminergic nigrostriatal loss

Dihydroxy-6-¹⁸F-fluorophenylalanine (¹⁸F-dopa) PET imaging was done after attaining a stable HP state in randomly chosen animals and Ki values (sec⁻¹) obtained to confirm unilateral nigrostriatal deficit (N=8). Post-mortem dopamine transporter immunoautoradiography (DAT-IAR) was also used to quantitatively assess the loss of dopaminergic fibers in the striatum in randomly chosen animals (N=12, anonymous numbers were assigned for each brain and randomization table was used). Tyrosine hydroxylase (TH) immunohistochemistry was used to assess the loss of dopaminergic substantia nigra (SN) neurons in all monkeys. Details for these techniques have been previously described [7].

Quantitative estimates of SN TH-positive (TH+) neurons were performed using the optical fractionator probe (Stereo Investigator, MBF Bioscience) using systematic random sampling as determined by unbiased stereological counting procedures (N=3). Every 12th section through the SN was evaluated. Briefly, both the left (ipsilateral to intracarotid MPTP) and right (contralateral to intracarotid MPTP) SN was first outlined at low magnification (4×). A

sampling site grid was then computer-generated with unbiased counting frames ($125 \times 125 \mu\text{m}$) and superimposed onto the SN outline. Top and bottom guard zones were applied to each site, and a dissector height of $25 \mu\text{m}$ was used. Section thickness was measured at each site and counts for SN TH+ neurons were performed at high magnification. Total SN TH+ neurons for each hemisphere were then calculated using the Stereo Investigator software.

Statistics

Fisher's exact test and Student's t-test were used to measure the change in mUPDRS and FMT. One-way analysis of variance with Bonferroni's multiple comparison post-test was used to compare stereological counts. Data are expressed as mean $\pm$ SEM. Significance was set at $p < 0.05$.

RESULTS

Confirmation of Stable HP State and LD Responsiveness

FMT and mUPDRS behaviorally confirmed HP status. Right FMT significantly increased from 6.54 ± 0.17 seconds pre-MPTP to 23.0 ± 2.4 seconds post-MPTP ($p < 0.001$). Left FMT remained unaffected, 6.68 ± 0.25 seconds pre-MPTP and 6.19 ± 0.18 seconds post-MPTP ($p > 0.05$) (Fig. 2A). The mean dose of LD that produced optimal resolution of parkinsonism as measured by the mUPDRS was 200 mg b.i.d. The mean mUPDRS score post-MPTP was 12.6 ± 1.3 which improved to 3.6 ± 0.39 ($p = 0.02$) after optimal LD. At washout, the mUPDRS returned to 11.7 ± 1.0 ($p = 0.02$) (Fig. 2B). Additionally, FMT performance post-LD improved on the right side following optimal dose and returned to baseline when LD was washed-out. The mean post-MPTP FMT during this trial was 29.9 ± 1.7 seconds, which improved to 9.1 ± 1.2 after LD, worsening back to 21.4 ± 1.6 after LD washout ($p = 0.045$) (Fig. 2C). Median plasma dopamine levels pre-LD were 180 pg/ml and increased to 22,220 pg/ml on optimal LD dose.

Levodopa-Induced Dyskinesia Assessment

No unequivocal limb dyskinesias in the form of chorea, ballismus, flinging of arms, violent jerks or athetosis and no dystonias were observed in any of the animals for up to 12 months of LD treatments. Furthermore, deliberate chronic over-dosing up to 700 mg/day (approximately 140 mg/kg/day) of oral LD or 300 mg/kg/day of injectable LD caused no apparent limb dyskinesias. Some animals did show ill-sustained contraversive circling, occasional motor tics or minor stereotypy on both sides of the body for less than 1 minute/hour of video after this high dose LD treatment (700mg/day). We did not include circling, rare simple motor tics or minor stereotypies in our study as indicators of dyskinesias. Tics and stereotypies were defined by comparison against 12 hour videos obtained from these stable HP animals before treatments with any anti-PD medication.

Evaluation of Dopaminergic Nigrostriatal Loss in Stable HP Monkeys

^{18}F -dopa PET imaging showed a significant reduction of ^{18}F -dopa uptake and metabolism in the striatum ipsilateral to the side of MPTP administration and preservation of signal on the contralateral side (Fig. 3A). Animals demonstrated an 87% reduction in uptake in the ipsilateral caudate and a 79% reduction in uptake in the ipsilateral putamen. For example, K_i values (sec^{-1}) were 1.32×10^{-5} and 2.025×10^{-5} in the ipsilateral caudate and putamen, respectively. The K_i values (sec^{-1}) were 10.0×10^{-5} and 9.53×10^{-5} in the contralateral caudate and putamen, respectively. The average DAT-IAR values of the lesioned side ipsilateral to MPTP injection in the caudate were 603 dpm/mg and 486 dpm/mg in the putamen. The contralateral side was 4936 dpm/mg and 3951 dpm/mg in caudate and putamen, respectively. The dopaminergic fiber loss was less marked in the ventromedial

striatum and the tail of caudate (Fig. 3B). TH immunohistochemistry showed extensive loss of TH staining in the striatum and nigra on the lesioned side as compared to the contralateral side (Fig. 3C). Unbiased stereological counts of SN TH⁺ neurons from previous studies using normal rhesus monkeys aged 3–10 years old report an average of $178,198 \pm 51,802$ SN TH⁺ neurons unilaterally [esupp ref7–8]. TH⁺ counts from our animals were $18,418 \pm 4,425$ in the left SN (ipsilateral to intracarotid MPTP) ($p < 0.05$) and $99,667 \pm 11,711$ in the contralateral hemisphere (CE = 0.14) (Fig. 3D). There was no anatomical or histological evidence of placebo-induced changes either in the striatum or nigra in these animals.

DISCUSSION

Our results confirm that strictly unilateral HP rhesus monkeys do not develop LID. The experiments described here closely resemble the clinical dosing regimen of dopaminergic medications in PD patients. Animals were exposed to chronic intermittent optimal oral doses of LD then very high doses of LD both orally and parenterally in sufficient amounts that should have lead to the genesis of choreoathetoid and dystonic LID. However, despite careful assessments for LID, none were observed for up to 12 months of testing. Several other investigators using the strictly HP rhesus monkey have anecdotally observed the lack of choreoathetoid and dystonic LID, but to the best of our knowledge not reported in the literature though systematic investigation. It was previously reported that high doses of LD produced dyskinesias in “HP” rhesus monkeys [12], but careful read of this previous literature indicate that these animals in fact had clinical and pathological evidence of bilateral parkinsonism. This was acknowledged in that article by the authors themselves (see page 116–117 of [12]). Our systematic blinded retrospective review is the first of its kind to report the lack of LID in HP rhesus monkeys despite exposure to large intermittent doses of LD.

LID has been attributed to nigrostriatal degeneration and nonphysiological pulsatile stimulation of striatal dopamine receptors from chronic intermittent LD [3, esupp ref3]. This theory would predict that HP rhesus monkeys should develop unilateral (on the contralateral limbs) dyskinesias when given chronic intermittent high dose LD treatments. However, this did not occur. It is unclear what mechanisms are involved in the lack of LID in these animals. We discuss here the potential mechanisms that may explain our findings. Future experiments are warranted to validate these theories.

One possibility is that there is an interhemispheric inhibitory influence that prevents the genesis of unilateral dyskinesias from the less affected hemisphere in the HP rhesus monkey. The nature of this inhibitory influence is unclear but could be mediated via the small percentage of interhemispheric fibers of the nigrostriatal pathway that immediately crossover at the mesencephalon from the opposite hemisphere [13–15]. Such crossover fibers represent an estimated 5% of SN dopaminergic fibers in the rat and up to 13% in monkeys. This theory would suggest that a threshold of bilateral nigrostriatal denervation is essential for the genesis of dyskinesias. This theory is supported in part by the finding that HP rhesus monkeys when overlesioned (with additional doses of MPTP), to cause bilateral but asymmetric parkinsonism, readily develop dyskinesias that are identical to LID seen in PD. These animals have bilateral but markedly asymmetric nigrostriatal denervation [16]. Similarly, systemic MPTP causing bilateral parkinsonism in rhesus monkeys also readily develop LID [17–19]. Thus, one possibility is that HP monkeys that we report here have interhemispheric inhibition of LID. As a corollary, rats with less than 95% unilateral loss of nigrostriatal innervation as a result of striatal 6-hydroxydopamine (6-OHDA) lesioning (e.g., Sauer and Oertel model) and monkeys with <85% unilateral nigrostriatal denervation from intracarotid injections of MPTP are likely to have intact (on both sides) interhemispheric dopaminergic fibers. Moreover, the Ungerstedt 6-OHDA lesioned rat model of PD that

causes >95% unilateral loss of striatal dopamine innervation do develop involuntary movements that are similar to some degree to LID [20], whereas rats that receive a partial lesion that cause only 50% striatal denervation do not develop LID [21]. Similarly, as discussed earlier, the overlesioned HP rhesus and bilateral parkinsonian rhesus monkeys readily develop LID. We are not aware of similar reports on the status of interhemispheric nigrostriatal fibers in humans, but we would speculate that humans have at least as many dopaminergic nigrostriatal crossover fibers as in monkeys. Another possible pathway that may modulate dyskinesias is the interhemispheric and intrahemispheric corticostriatal connections [esupp ref9–10]. It is well known that the cortical synaptic connections to the striatum are altered in response to dopamine depletion that pathophysiologically alters the output of medium spiny neurons and subsequent basal ganglia nuclei [esupp ref11–12]. Future studies are necessary to evaluate if changes to these corticostriatal connections influence the genesis of LID.

The second possibility is that the protection against LID in our HP animals is simply a lack of sufficient unilateral nigral denervation and do not involve the crossover fibers or interhemispheric inhibition. This theory would suggest that extensive unilateral nigrostriatal denervation is sufficient for the genesis of contralateral LID. However, this theory appears to be unlikely in the animals we report here as they had near complete loss of all ipsilateral nigrostriatal fibers as demonstrated by DAT-IAR (>80%, mean DAT-IAR drop from 4500 to 540), ¹⁸F-dopa PET imaging showing near complete loss of ¹⁸F-dopa uptake in the lesioned left hemisphere (>80% reduction demonstrated by striatal Ki values) and histology (SN TH+ stereology 178,000 to 18,000-proof of 90% reduction). This degree of denervation represents almost complete denervation of the ipsilateral nigrostriatal pathway (the remaining 10% representing fibers that crossover to the opposite hemisphere). Thus, the lack of sufficient ipsilateral nigrostriatal denervation in the 17 monkeys we report here is unlikely to explain their resistance to develop LID despite chronic 12 month intermittent exposure to LD. As reported in the literature, any additional intracarotid injections in these HP animals either fail to cause additional clinical parkinsonism (so-called MPTP resistance) or cause parkinsonism in the previously asymptomatic side such that these animals are now clinically bilaterally parkinsonian [12].

As a clinical correlate to our observations in the HP monkey, idiopathic PD patients with strictly unilateral symptoms (stage I Hoehn and Yahr) do not develop LID until they progress to stage II (bilateral parkinsonism) despite clearly demonstrating neurodegeneration (on PET imaging and in autopsy studies) in the SN bilaterally even in stage I disease. This clinical observation is true even among young onset PD patients who have the highest (>80%) risk of developing LID [1]. Moreover, it is now clear from numerous early PD clinical trials including those that exclusively used LD as a treatment, that PD patients in stage I disease do not develop LID despite imaging studies in these early PD patients confirming the asymmetric bilateral dopaminergic denervation [22]. Although many clinicians are aware of this phenomenon in idiopathic PD patients, we are unaware of any systematic study that has examined the lack of LID in stage I patients (requiring LD withdrawal and examination in the “on” and “off” states to confirm their clinical stage over an extended period of longitudinal follow-up). Rare case reports of patients with secondary parkinsonism (non-idiopathic PD patients) or with hemiatrophy-HP syndromes who developed LID had severe near complete destruction of the SN and the adjacent mesencephalon where crossover fibers reside [23,24], suggesting that crossover fibers were destroyed in these patients. Future anatomical studies in post-mortem brains of patients who had developed LID or hemi-LID are needed to validate this hypothesis in humans.

The third, but much less likely possibility to explain the lack of LID in our animals is that this is a species-specific finding that somehow protects HP rhesus monkeys from LID.

Investigators report the genesis of “dyskinesias” in normal and partially lesioned monkeys other than rhesus macaques [25–29, esupp ref13–15]. The discrepancies with these reports and the present study most likely stems from how these investigators chose to define LID (e.g., circling counted as dyskinesias), variations in the amount of LD administered (e.g., LD dose 10 times of what is required for optimal anti-parkinsonian benefits) and routes of administration (e.g., intraperitoneal). In the present study, we strictly defined LID to include only choreoathetoid and dystonic movements that are readily seen in the severely bilateral parkinsonian rhesus monkey and are identical to what is seen in humans with advanced PD on LD therapy. We did not include circling, simple motor tics or minor stereotypies in our study. Circling was rarely (<1 event/hour of video) noted in our study, and only with very high doses of LD.

The risk of developing LID has been attributed to the degree of lesion in both SN (and potentially the locus coeruleus), dosing regimen of LD, and sensitivity of detecting dyskinesias [12,22, esupp ref16–17]. We demonstrate that HP monkeys reported here had stable unilateral nigrostriatal degeneration through blinded clinical ratings and fine motor performance testing, quantitative analysis of histological sections, DAT-IAR and ¹⁸F-dopa PET imaging.

Previous studies have demonstrated an 86% decrease in SN TH+ neurons ipsilaterally and 25% decrease in SN TH+ neurons contralaterally in HP young rhesus monkeys in comparison to historical data in normal rhesus monkeys using unbiased stereological counts with the optical fractionator method [esupp ref7–8, ref18]. Our stereological SN TH+ counts demonstrate a 90% decrease in the ipsilateral SN and 44% decrease in the contralateral SN compared to previous reports in normal rhesus monkeys. Taken together, these studies demonstrate a near complete unilateral loss of nigrostriatal neurons (except for the 10% of fibers that cross-over to the opposite striatum) was present in the HP rhesus monkeys reported in our study.

The extent of SN lesioning in this animal model is at its maximum unilaterally. Any additional lesioning with MPTP causes bilateral symptoms. The regimen of LD dosing we used simulated the clinical experience in PD patients with chronic, high intermittent doses. Identical dosing regimen readily induces LID in bilateral parkinsonian monkeys. These animals were carefully observed by an experienced clinician scientist for a minimum of 12 hours each day for several months using randomized blinded video rating to detect LID. Thus, lack of sufficient unilateral lesioning, inadequate LD dosing or the lack of careful clinical observation could not explain the lack of LID in these strictly unilateral HP monkeys. In the present study, we did not collect biochemical data because each animal was perfused and brains fixed for histological analysis. We did not obtain stereological counts of neurons in the locus coeruleus that are known to play a role in PD [esupp ref16–17], although its role in the genesis of LID in HP rhesus monkeys is unknown. Future studies are warranted utilizing dopamine biochemical analysis techniques and stereological counts of the locus coeruleus in strictly unilateral HP rhesus monkeys that are chronically exposed to LD to further strengthen our findings.

The strictly unilateral stable HP rhesus monkey model of PD has been extensively used by researchers to test various preclinical therapies including cell transplantation and gene therapy [4,6,7,9,30]. Our results suggest this strictly unilateral rhesus monkey model of PD, which has a proven track record of excellent utility in pathophysiological and electrophysiological studies, is not a suitable model to test experimental therapeutics targeted to improve advanced PD. In particular, treatments that seek prophylactic and palliative treatments for LID or pathophysiology of LID cannot be meaningfully tested in this model. The severely parkinsonian bilateral rhesus monkey model or the overlesioned

HP monkey model that readily exhibit all the classic hallmarks of LID seen in PD patients may be a more appropriate model to test experimental therapeutics in advanced PD and to examine the pathophysiology of LID.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1. Experimental procedure for MPTP exposure, LD administration, mUPDRS, FMT, and observation of levodopa-induced dyskinesias (LID).

Fig. 2.
 (A) FMT before and after MPTP. Contralateral side remains unaffected post MPTP ($***p < 0.001$). Improvement in the mUPDRS (B) and FMT on the affected side (C) while on levodopa therapy which reverses back after levodopa washout ($*p < 0.05$).

Fig. 3. Confirmation of HP state. (A) Trans-axial ^{18}F -dopa PET scans from HP rhesus monkeys. (B) DAT-IAR to estimate quantitative dopaminergic fiber loss in the striatum. (C) View of TH staining in the striatum and nigra in the HP rhesus monkey. (D) Unbiased stereological counts of unilateral SN TH+ neurons. Right nigra is contralateral to intracarotid MPTP and left nigra is ipsilateral to intracarotid MPTP-expressed as percentage (* $p < 0.05$ vs. normal).