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The blood–brain barrier is intact after levodopa-induced dyskinesias in parkinsonian primates—Evidence from in vivo neuroimaging studies

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ABSTRACT

It has been suggested, based on rodent studies, that levodopa (L-dopa) induced dyskinesia is associated with a disrupted blood–brain barrier (BBB). We have investigated BBB integrity with in vivo neuroimaging techniques in six 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) lesioned primates exhibiting L-dopa-induced dyskinesia. Magnetic resonance imaging (MRI) performed before and after injection of Gadolinium-diethylenetriamine pentaacetic acid (Gd-DTPA) revealed an intact BBB in the basal ganglia showing that L-dopa-induced dyskinesia is not associated with a disrupted BBB in this model.

Introduction

Levodopa (L-dopa) is currently the primary treatment of motor symptoms in Parkinson's disease (PD). However, a major limitation of chronic L-dopa treatment is the development of dyskinesias after years of treatment (Fahn, 2003; Olanow et al., 2004). The pathophysiological mechanisms of L-dopa-induced dyskinesia are poorly understood, though non-physiological release of synaptic dopamine is likely to play a major role in its development (Obeso et al., 2000; Olanow et al., 2004; Olanow and Obeso, 2000). Recently, it has been suggested, based on studies in rodents, that L-dopa-induced dyskinesia may be associated with a disrupted blood–brain barrier (BBB) (Westin et al., 2006) and that this may in turn contribute to its pathophysiology, by further exacerbating dyskinesia (Westin et al., 2006).

The purpose of the present study was to investigate the integrity of the BBB using in vivo neuroimaging techniques in 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) lesioned parkinsonian primates exhibiting L-dopa-induced dyskinesias.

Methods

Induction of parkinsonian and dyskinetic symptoms

Six adult male macaque monkeys (*Macaca fascicularis*), aged 6–8 years and weighing 6–7 kg, were included in this study. Animals were housed in individual home cages at the New England Primate Research Center (NEPRC). All studies were approved by the Harvard Medical School Institutional Animal Care and Use Committee (IACUC). Parkinsonism was induced by weekly intravenous administration of low doses of MPTP (Sigma-Aldrich®) diluted in normal saline. Doses were given initially at 0.30 mg/kg to all animals but in some instances subsequently reduced to 0.15 mg/kg, due to symptom severity and individual sensitivity. Parkinsonian motor symptoms were rated weekly during and after MPTP administration on a Parkinson's Rating Scale (PRS) as developed for macaques (Imbert et al., 2000) and modified from the motor subscale of the Unified Parkinson's Disease Rating Scale (UPDRS) (Fahn, 2003), ranging from 0 to 24, with 24 being most severe. Stable PRS scores were obtained off L-dopa at least 3 months after the last MPTP dose and were considered stable if standard deviation did not change more than ±2 over 6 weeks (Table 1). All animals displayed a stable parkinsonian syndrome, including tremor, rigidity, bradykinesia, hypokinesia and posture/balance disturbances (Jenkins et al., 2004). Dopamine transporter loss

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Table 1
Animal characteristics, dosing and symptoms.

	MF 1	MF 2	MF 3	MF 4	MF 5	MF 6
Age (years)	8	8	8	6	6	7
Weight (kg)	6	6.1	7	5.9	6.1	6.1
MPTP (total mg)	39.5	16.4	10.3	7.2	13.4	13.2
MPTP (weeks)	34	10	7	4	8	10
PRS score	17	19	16	18	22	14
Putaminal ^{11}C-CFT binding % reduction	−55%	−67%	−60%	−69%	−46%	−59%
Daily L-dopa dose (mg/kg)	60–120	60–120	60–120	60–120	30–60	60–120
Duration of L-dopa treatment (weeks)	23	15	28	18	36	25
Maximal effective L-dopa dose (mg/kg)	60	60	60	60	30	60
Maximal effective time point after L-dopa (min)	90	90	90	90	90	90
Dyskinesia peak score	8	10	15	17	19	22
Dyskinesia severity score	2	2	3	3.4	2.4	2.4

MF = *Macaca fascicularis*; ^{11}C-CFT = 11C-(2β-carbomethoxy-3β-(4-fluorophenyl) tropane).

in the posterior putamen was measured by positron emission tomography (PET) studies and binding of the dopamine transporter tracer ^{11}C-(2β-carbomethoxy-3β-(4-fluorophenyl) tropane) (CFT) at the stable stage, at least 3 months after last MPTP administration, as previously described (Brownell et al., 1998). Animals then received daily intramuscular (i.m.) injections of L-dopa methylester (Sigma-Aldrich®) in combination with the peripheral decarboxylase inhibitor benserazide (Sigma-Aldrich®), diluted in normal saline and injected at 1 ml, for the induction of dyskinesia. L-dopa was administered according to individual animal response and tolerance at 30, 60 or 120 mg/kg daily for 15–36 weeks. Benserazide was co-administered at 10–15 mg/kg per dose. Dyskinesia severity was rated weekly by two independent observers at 30, 60 and 90 min after a single i.m. administration of L-dopa (30 or 60 mg/kg) in combination with benserazide (10–15 mg/kg). Abnormal movements were classified as chorea (rapid, random flicking movements), athetosis (sinuous, writhing distal limb movements) dystonia (sustained twisting movements resulting in abnormal posturing), myoclonus (jerky) or stereotypy (repetitive purposeless behavior). Severity was rated according to the Dyskinesia Disability Severity scale as described (Bezard et al., 2003; Pearce et al., 1995), ranging from 1 to 4, based on frequency and interference with normal behavior by 0 = absent; 1 = mild, fleeting and dyskinetic movements and postures (<5 in 10 min); 2 = moderate, more prominent and abnormal dyskinesia but not interfering with normal behavior (~5–20 in 10 min); 3 = marked, frequent dyskinesia, intruding on normal behavior (21–50 in 10 min); 4 = severe, virtually continuous dyskinesia, disabling the animal. Sum of dyskinesia scores (peak scores) at the maximally effective dose and time point were obtained and severity (disability) scores were calculated by dividing the total score by the number of affected regions, as previously described (Sanchez-Pernaute et al., 2007).

MRI studies with Gadolinium-diethylenetriamine pentaacetic acid (Gd-DTPA) contrast enhancement

After developing reproducible dyskinesias, animals underwent neuroimaging studies. L-dopa was administered until the morning of the study. Animals were anesthetized with a Ketamine (10 mg/kg)/Xylazine (1.5 mg/kg) combination i.m. Atropine was administered at 0.04 mg/kg i.m. Anesthesia was maintained with halothane (1–1.5%) while the animal was intubated but free breathing. The animal was placed in an MRI compatible head frame (Kopf Instruments®) on a water heating blanket to maintain body temperature. Respiratory rate, heart rate, SpO_2 and body temperature were constantly monitored throughout the procedure. MRI studies were performed on a 3 T Allegra system (Siemens®, Erlangen, Germany) using a transmit–receive 3 inch surface coil. The animal's head was placed in the center of the surface coil such that the coil fit over the skull, above the eyes. After collection of baseline images, Gd-DTPA was administered intravenously at 0.3 mmol/kg and

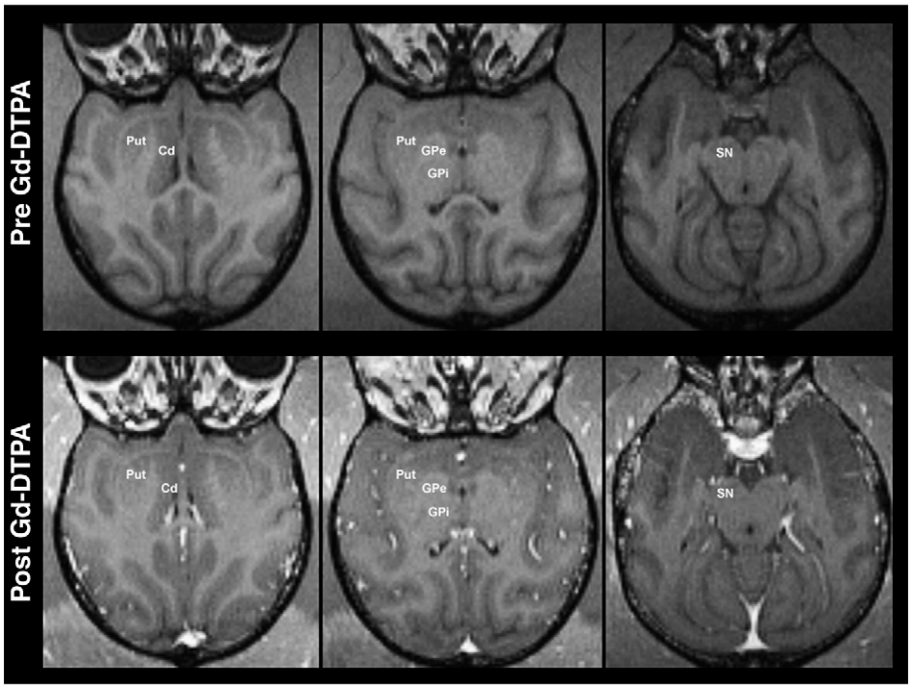

Fig. 1. The BBB of the basal ganglia is intact as shown by Gadolinium-DTPA (Gd-DTPA) MRI studies in dyskinetic monkeys. T1 weighted axial brain MRI images before (upper panel) and after (lower panel) peripheral injection of Gadolinium-DTPA, 0.3 mmol/kg to a dyskinetic macaque. Post Gd-DTPA there is marked signal enhancement of the hypothalamic/pituitary region, structures lacking a BBB, and the sagittal sinus, but no signal enhancement of the basal ganglia, including the putamen, caudate, globus pallidus and substantia nigra is observed. Put = putamen; Cd = caudate nucleus; GPe = globus pallidus externa, GPi = globus pallidus interna SN = substantia nigra, Pit/Hyp = pituitary/hypothalamic region. L = left; R = right. Bright spots near SN are contrast filled blood vessels.

serial gradient echo imaging was continued with a flip angle alpha of 25° and short TE (TR/TE = 235/4.5 ms) with 30 s temporal resolution, and high resolution (0.65 mm isotropic) T1-weighted sequence (TR/TI/TE = 1910/1100/3.1 ms) were collected. MRI data acquisition occurred over a total of 20 min (Fig. 2). At the conclusion of the study, the animals were extubated and placed in a warmed cage until fully recovered. Regions of interest (ROIs) were hand drawn of the SN, the putamen, the caudate, the pituitary–hypothalamic region, the sagittal sinus and jaw muscle on the various MRI images, and the average image intensity was used for the quantitative analysis using the serial gradient echo images as a function of time after injection of Gd-DTPA and delayed enhancement was analyzed using the high resolution T1 weighted images. Statistical analyses were performed with the GraphPad Prism® program, version 5.01.

Results

Animals received a weekly low dose of the neurotoxin MPTP for a total of 4–34 weeks, with a total cumulative MPTP dose of 7.2–39.5 mg. This resulted in moderate to severe parkinsonian symptoms in all six animals, with an average PRS score of 18 ± 2.7 (range 14–22), that remained stable at least 3 months after the last MPTP dose (Table 1). All animals displayed a significant loss of dopamine transporter binding in the putamen with an average reduction of 59 ± 7.4 % (*t*-test; $p<0.001$), as measured by PET and the dopamine transporter tracer ^{11}C-CFT. After 15–36 weeks of daily L-dopa treatment, all six animals developed dyskinesias as defined by the presence of abnormal involuntary movements, mainly choreiform, dystonic and stereotypic movements affecting limbs, axial body, tail, and orolingual muscles (Table 1).

Animals then underwent an MRI brain scan with Gd-DTPA. Visual inspection of high resolution T1 weighted images, revealed no increase in signal intensity post Gd-DTPA in the basal ganglia, including the substantia nigra, in any animal. Signal enhancement was observed in structures lacking a BBB, namely the pituitary/hypothalamus region (pit/hyp), in addition to the sagittal sinus and jaw muscles, thus serving as an internal control of Gd-DTPA delivery (Fig. 1). A region of interest (ROI) quantitative analysis (see Methods), confirmed an intact BBB in the basal ganglia in all six animals. One way ANOVA across brain regions showed that there were no significant differences between images of the caudate nucleus (Cd), putamen (Put), SN or occipital cortex (OccCx) with either the serial gradient echo sequences ($F23,3 = 1.27$; $p>0.3$) or the high resolution (0.65 mm isotropic) T1-weighted sequence ($F23,3 = 1.40$; $p>0.25$) whereas there were highly significant differences between the Cd, Put, SN or OccCx and either jaw muscle, pit/hyp or sagittal sinus, as expected (Figs. 2A and B).

Discussion

This is the first in vivo demonstration of the integrity of the BBB in parkinsonian primates exhibiting L-dopa-induced dyskinesia. The induction of dyskinesia by the administration of daily high dose L-dopa over several months to MPTP lesioned, parkinsonian primates, did not lead to a leaking BBB. It is conceivable that in the case of a disrupted BBB, this could lead to high and uncontrolled levels of L-dopa entering the brain following systemic L-dopa therapy, further exacerbating non-physiological synaptic release of dopamine (Olanow et al., 2004; Westin et al., 2006). Also, the BBB is usually impermeable to carbidopa, a peripheral L-dopa decarboxylase inhibitor, and if disrupted and rendered permeable, this could compromise physiological L-dopa decarboxylation in the brain (Carvey et al., 2005). Finally, in gene therapy, a dysfunctional BBB could possibly result in a different distribution of secreted gene products (Isacson and Kordower, 2008) or in the case of cell transplantation, exposure to immune factors and rejection (Isacson and Kordower,

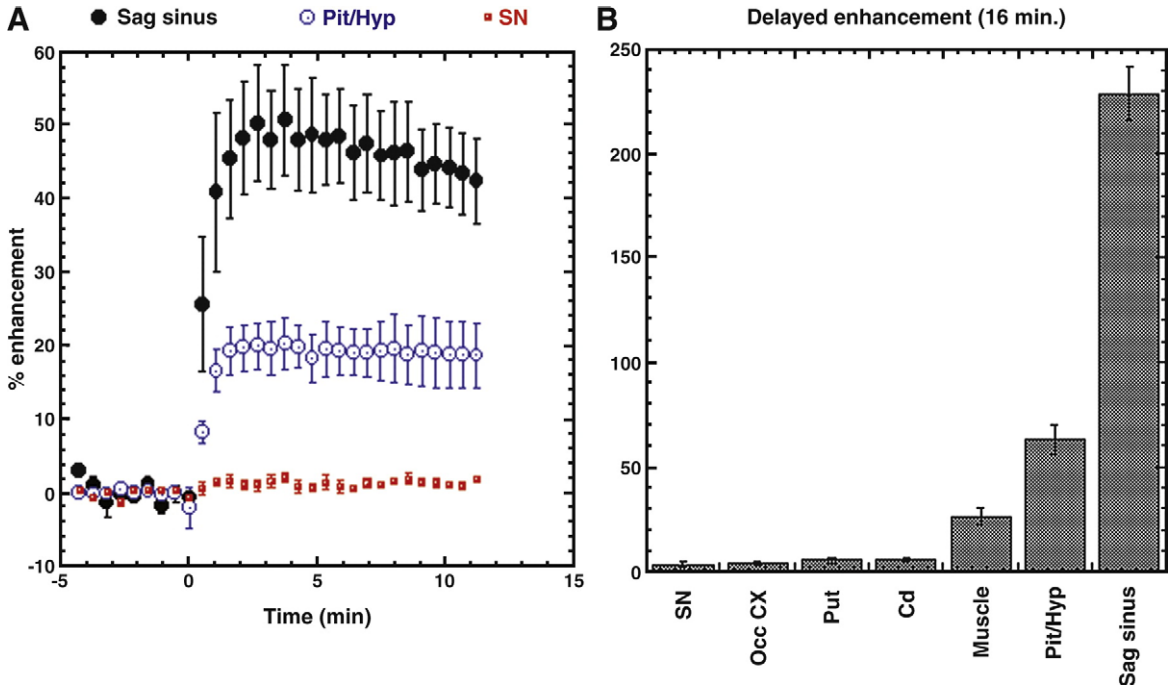

Fig. 2. Quantitative results following injection of Gadolinium-DTPA in dyskinetic monkeys show an intact BBB of the basal ganglia. (A) Averaged plot across all animals showing the effects of 0.3 mmol/kg Gd-DTPA as a function of time using serial gradient echo imaging with a flip angle alpha of 25° and short TE (TR/TE = 235/4.5 ms) with 30 s temporal resolution. Injections were made during serial imaging for comparison effects and are shown in the sagittal sinus vein (Sag sinus), the pituitary/hypothalamic region (Pit/Hyp) and the substantia nigra (SN). There is no increase in the SN aside from a small contribution attributable to the intrinsic blood volume. (B) Bar plot showing the averages across all animals for signal enhancement at an average of 16 min after GD-DTPA injection using a high resolution (0.65 mm isotropic) T1-weighted sequence (TR/TI/TE = 1910/1100/3.1 ms). The regions shown are the same as in (A) but also include putamen (Put), caudate (Cd), jaw muscle (muscle), and occipital cortex (OccCx) as a control gray matter region. One way ANOVA across brain regions showed that there were no significant differences between images of the Cd, Put, SN and OccCx with either the gradient echo data in (A) ($F23,3 = 1.27$; $p>0.3$) or in (B) ($F23,3 = 1.40$; $p>0.25$). As expected, there were highly significant differences between the latter four regions and either muscle, pit/hyp or sagittal sinus.

2008). The findings of an intact BBB in the present study may therefore have implications for existing and new therapies for PD.

BBB integrity has also been studied in clinical and experimental models of Parkinson's disease. For example, a PET study of ^{11}C-verapamil uptake in the brain demonstrated a decreased function of the P-glycoprotein (P-gp) transporter in the BBB of PD patients (Kortekaas et al., 2005). Findings from a rodent study have suggested that L-dopa-induced dyskinesia may be associated with a compromised BBB (Westin et al., 2006). Postmortem analysis of 6-OHDA lesioned rats rendered dyskinetic after a 2 week course of L-dopa, revealed a BBB with long-term structural changes in the basal ganglia, particularly in its output regions; the entopeduncular nucleus and the substantia nigra pars reticulata, as demonstrated by increased immunostaining for albumin and a reduction in endothelial barrier antigen (EBA) expression (Westin et al., 2006). However, no external tracer such as horseradish-peroxide (HRP) was administered (Westin et al., 2006). HRP is a glycoprotein with a small molecular weight that produces a fluorimetric or luminescent derivative of the labeled molecule, and can be administered intravenously, subsequently allowing it to be histologically detected and quantified and has been widely used as a histological marker of BBB integrity (Harris et al., 2002). EBA is rodent specific and may not be applicable to the clinical setting (Sternberger and Sternberger, 1987). Finally, Westin et al. found a high rate of cell proliferation in the basal ganglia and newly born microvessels (Westin et al., 2006). These observations were specifically associated with the development of dyskinesia and not L-dopa treatment alone (Westin et al., 2006).

We have developed a slow, progressive model of L-dopa-induced dyskinesia, by the administration of L-dopa over several months, to chronically MPTP lesioned non-human primates (Jenkins et al., 2004; Sanchez-Pernaute et al., 2007). Whereas Parkinson's disease patients usually develop dyskinesias only after several years of L-dopa treatment, we have used substantially higher doses of L-dopa than clinically applied, for the induction of dyskinesias in primates, in order to shorten the length of the induction phase (Sanchez-Pernaute et al., 2007). Nevertheless, this model may more realistically simulate the progressive pathogenesis of dyskinesia in clinical PD, than current rodent models of L-dopa-induced dyskinesias do.

MRI studies with Gd-DTPA enhancement are widely used to detect BBB changes in a variety of neurological conditions, such as multiple sclerosis (Kermode et al., 1990; Soon et al., 2007), including subtle BBB changes associated with non-enhancing lesions (Soon et al., 2007), as well as stroke (Wardlaw et al., 2008), intracerebral neoplasm (Ludemann et al., 2002) and head injury (Beaumont et al., 2000). We have chosen to use Gd-DTPA MRI to detect BBB integrity in our in vivo model of L-dopa-induced dyskinesia of primates, as it is a well established, clinically useful marker to evaluate BBB integrity. It has the advantage over HRP and albumin, that it can be readily used in vivo, whereas the analysis of HRP and albumin leakage is suitable for postmortem studies. Furthermore, Gd-DTPA is a much smaller molecule than both albumin and HRP and therefore should be more sensitive to subtle BBB permeability changes (Harris et al., 2002; Schmiedl et al., 1991). Notably, if a molecule as large as albumin can leak across the BBB it must indicate a very high permeability surface area product (Westin et al., 2006). Given that we could not see leakage of a small molecule like Gd-DTPA in the present study, it must mean that there was minimal opening of the BBB in our model.

While we found no evidence of BBB damage after chronic L-dopa administration in our study, it cannot be excluded that other microvascular effects of L-dopa treatment might have occurred in this model. For example, the possibility of L-dopa-induced microvascular proliferation and increased cerebral blood volume cannot be excluded (Westin et al., 2006). Furthermore, it cannot be excluded, as was recently demonstrated, that L-dopa treatment is associated with increased cerebral blood flow and dissociation of cerebral blood flow and metabolism in the striatum (Hirano et al., 2008).

In conclusion, in primates rendered parkinsonian with MPTP, repeated L-dopa treatment or dyskinesia did not disrupt the BBB in the basal ganglia, as detected with MRI neuroimaging using Gd-DTPA. These findings contrast with studies of the BBB in rodent models of L-dopa-induced dyskinesia.

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