Behavioural impact of a double dopaminergic and serotonergic lesion in the non-human primate

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Serotonergic (5-HT) neurons degenerate in Parkinson’s disease. To determine the role of this 5-HT injury—besides the dopaminergic one in the parkinsonian symptomatology—we developed a new monkey model exhibiting a double dopaminergic/serotonergic lesion by sequentially using 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) and 3,4-methylenedioxy-N-methamphetamine (MDMA, better known as ecstasy). By positron emission tomography imaging and immunohistochemistry, we demonstrated that MDMA injured 5-HT nerve terminals in the brain of MPTP monkeys. Unexpectedly, this injury had no impact on tremor or on bradykinesia, but altered rigidity. It abolished the L-DOPA-induced dyskinesia and neuropsychiatric-like behaviours, without altering the anti-parkinsonian response. These data demonstrate that 5-HT fibres play a critical role in the expression of both motor and non-motor symptoms in Parkinson’s disease, and highlight that an imbalance between the 5-HT and dopaminergic innervating systems is involved in specific basal ganglia territories for different symptoms.

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Abbreviations: 5-HT = serotonin; BPND = non-displaceable binding potential; DASB = N,N-dimethyl-2-(2-amino-4-cyanophenylthio)benzylamine; MDMA = 3,4-methylenedioxy-N-methylamphetamine; MPTP = 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine; PE2I = N-(3-iodoprop-2E-enyl)-2beta-carbomethoxy-3beta-(4-methylphenyl)nortropane; SERT = serotonergic transporter
Introduction

Idiopathic Parkinson’s disease is traditionally considered as a neurological pathology characterized by a progressive, irreversible and ultimately disabling motor deficit. It is related to dopamine depletion in the basal ganglia, which is a consequence of preferential degeneration of dopaminergic neurons localized in the substantia nigra pars compacta. This neuropathology is characterized clinically by cardinal motor symptoms (akinesia/bradykinesia, rigidity, resting tremor) and L-DOPA-induced motor complications (including motor fluctuations and dyskinesias) as an adverse effect of long-term dopamine replacement (Obeso et al., 2000). Parkinsonian patients may also display non-motor symptoms, including frequent and disabling neuropsychiatric symptoms (Chaudhuri and Schapira, 2009). Dopamine replacement therapy (L-DOPA or dopamine agonists), used for the treatment of motor symptoms, improves some neuropsychiatric symptoms (apathy, depression, anxiety) but promotes others (euphoria, psychosis, impulse control disorders) (Chaudhuri and Schapira, 2009).

It is accepted that a lesion of the dopamine nigrostriatal pathway is involved in the pathophysiology of bradykinesia and rigidity, motor fluctuations and L-DOPA-induced dyskinesia in parkinsonian patients (Politis, 2014). However, an indirect link has been shown between the degeneration of serotonergic neurons and (i) the stage of the disease; and (ii) the severity of tremor in parkinsonian patients (Huot et al., 2011a; Politis, 2014; Politis and Niccolini, 2015). Serotonin (5-HT) dysfunction is also associated with motor complications such as dyskinesia (Rylander et al., 2010; Politis et al., 2014). Indeed, striatal serotonergic terminals may contribute to L-DOPA-induced dyskinesia in parkinsonian patients via aberrant processing of exogenous L-DOPA and release of dopamine as false neurotransmitter (Politis et al., 2014). Regarding neuropsychiatric symptoms, their physiopathological substrates remain complex and incompletely understood, although there is a significant dopamine contribution. Indeed, the lesion of the dopamine mesolimbic pathway is related to the expression of hypodopaminergic symptoms such as apathy and depression, in parkinsonian patients (Thboois et al., 2010; Politis, 2014). Serotonin dysfunction is also linked to the physiopathology of non-motor symptoms, such as fatigue and depression (Huot et al., 2011a; Politis, 2014; Politis and Niccolini, 2015). It is also involved in the physiopathology of psychosis (Zahodne and Fernandez, 2008).

The alteration of the 5-HT innervating system occurs with the disease progression and its associated treatment, as seen for L-DOPA-induced dyskinesias, but its causal role in the expression of the wide range of parkinsonian motor or non-motor symptoms still remains to be explored. Is this 5-HT alteration involved in compensatory mechanisms or in the expression of specific symptoms, induced by the disease or the symptomatic treatment? To answer these questions, we developed for the first time a monkey model of Parkinson’s disease presenting a double dopamine/5-HT lesion as a result of sequential use of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) and 3,4-methylenedioxy-methamphetamine (MDMA, better known as the recreational drug ecstasy) in the macaque Macaca fascicularis. Indeed, the MPTP monkey model is the gold standard for toxin-based animal models as it reproduces most of the clinical and pathological hallmarks of Parkinson’s disease (Porras et al., 2012). And the specific neurotoxic effects of MDMA towards the 5-HT innervating system have been well characterized in the normal monkey (Ricaurte et al., 2000). We used two groups of MPTP-intoxicated monkeys, those that recovered from their motor symptoms (MPTP recovered monkeys), and those that stayed symptomatic (MPTP symptomatic monkeys) before MDMA. We combined PET imaging, behavioural assessments and neuroanatomy to determine: (i) the efficiency of MDMA in inducing a lesion of the 5-HT innervating system in previously dopamine-depleted monkeys; and (ii) the causal link between the serotonergic fibre alteration driven by MDMA and the parkinsonian symptoms including cardinal motor symptoms (akinesia, rigidity and tremor) and responses to L-DOPA therapy (dyskinesia and neuropsychiatric-like behaviours).

Materials and methods

Ethical statement

All studies were carried out in accordance with European Communities Council Directive of 2010 (2010/63/UE) as well as the recommendations of the French National Committee (2013/113).

Animals

Twenty adult macaque monkeys (19 males, one female) were used in this study. Taking into consideration the three R’s (Reduction, Refinement, and Replacement) for animal experimentation, we used some of the behavioural and anatomical data obtained on three African green monkeys from a previous study (Mounayar et al., 2007). All monkeys weighed between 4 and 6 kg and were aged between 3 and 5 years. They were kept under standard conditions (12-h light cycles, 23°C and 50% humidity).

Dopaminergic lesion

Five of these monkeys (Cercopithecus aethiops sabaeus CA33, CA34, CA 37 and M. fascicularis MF1 and MF2) were recovered parkinsonian monkeys that had been involved in previous studies (Mounayar et al., 2007; Neumane et al., 2012). The monkeys were dopamine-depleted by receiving MPTP injections (0.3–0.5 mg/kg, intramuscular) under light anaesthesia (ketamine 0.5 mg/kg, atropine 0.05 mg/kg) on consecutive days (acute protocol; n = 8) or every 4 to 5 days (progressive protocol; n = 11) until the emergence of parkinsonian symptoms. MPTP intoxication was stopped once most of the motor complications (dyskinesia and neuropsychiatric-like behaviours) were observed. Twenty adult macaque monkeys (19 males, one female) were used in this study. Taking into consideration the three R’s (Reduction, Refinement, and Replacement) for animal experimentation, we used some of the behavioural and anatomical data obtained on three African green monkeys from a previous study (Mounayar et al., 2007). All monkeys weighed between 4 and 6 kg and were aged between 3 and 5 years. They were kept under standard conditions (12-h light cycles, 23°C and 50% humidity).
parkinsonian symptoms had appeared. The appearance of symptoms was assessed by the rating scale of Schneider and Kovelovski (1990), which includes several items rated with a total score of 29. The higher the score, the more symptomatic the monkey. Monkeys receiving progressive MPTP intoxication became symptomatic but recovered (MPTP recovered monkeys, n = 11), whereas monkeys receiving acute MPTP intoxication remained symptomatic (MPTP symptomatic monkeys; n = 8).

Serotonergic lesion

The lesion of serotonergic fibres (and not 5-HT somas) was specifically induced in 11 MPTP-intoxicated macaque monkeys following MDMA injections twice daily for four consecutive days (5 mg/kg, subcutaneously) according to a previous study in normal monkeys (Ricaurte et al., 2000).

Chronic l-DOPA treatment

Chronic l-DOPA treatment was applied to seven MPTP-intoxicated monkeys before and after MDMA. Briefly, 2 months after the MPTP intoxication, the monkeys received l-3,4-dihydroxyphenylalanine methyl ester hydrochloride (l-DOPA) combined with benzerazide hydrochloride (ratio 5:1) twice daily for 2 months, followed by a 2-month washout period preceding MDMA. Following stabilization of the MDMA-driven lesion (5 weeks), the monkeys underwent a second l-DOPA/benserazide treatment, identical to the first.

Behavioural assessments

Parkinsonism

The severity of parkinsonian symptoms was assessed longitudinally using the rating scale proposed by Schneider and Kovelovski (1990) as previously described (Mounayar et al., 2007; Neumane et al., 2012). The higher the score, the more symptomatic the monkey. Rigidity was clinically assessed by scoring its severity (0, absent; 1, mild; 2, severe) in the shoulders, elbows, wrists, hips, knees and ankles (total score on 24) (adapted from the Movement Disorder Society Unified Parkinson’s Disease Rating Scale III; Goetz et al., 2008).

Parkinson’s disease disability

The severity of Parkinson’s disease disability was scored similarly to Bezard and co-workers (Ko et al., 2014). Four items were assessed: the range of movement, bradykinesia, the postural abnormality and the tremor. The Parkinson’s disease disability score was rated by analysing video recordings in 5-min periods every 50 min for 350 min. The Parkinson’s disease score ranges from 0 to 10.

Dyskinesia

The severity of l-DOPA-induced dyskinesias was assessed using the Non-Human Primate Dyskinesia rating scale (Fox et al., 2012) by analysing video recordings (observation for 5 min, every 50 min for a total period of 350 min after injection of l-DOPA). The higher the score, the more dyskinetic the monkey.

Neuropsychiatric-like behaviour

The neuropsychiatric-like behaviour in response to l-DOPA was assessed with the scale defined by Fox et al. (2010), by analysing video recordings (observations every minute for 10 min at the peak dose of l-DOPA, i.e. 145–155 min after injection). Four categories of behaviour with different items were characterized: agitation (hyperkinesia, vocalization), hallucinatory-like response behaviours to non-apparent stimuli (tracking, staring), obsessive grooming (scratching, grooming) and stereotypias (side-to-side jumping, head checking, purposeless running, fiddling with bars). The maximal score was 90.

Homecage activity

The homecage activity was evaluated longitudinally using an activity digitalizing system (Phenorack, Viewpoint) as described previously (Neumane et al., 2012).

In vivo imaging acquisition

PET and MRI acquisition were performed at the imaging centre (CERMEP) under anaesthesia (atropine 0.05 mg/kg intramuscularly followed 15 min later by zoletil 15 mg/kg intramuscularly). Anatomical MRI acquisition consisted of a 3D T1-weighted sequence using a 1.5-T Magnetom scanner (Siemens). The anatomical volume covered the whole brain with 176 planes of 0.6 mm cubic voxels. PET imaging was performed using either a Siemens CTI Exact HR+ or a Siemens Biograph mCT/S64 scanner (due to a replacement of the scanner during the study; see Supplementary Table 2). The HR+ tomograph had a nominal in-plane resolution of 4.1 mm full-width at half-maximum. Tissular and head support 511 keV gamma attenuation was obtained by a 10-min transmission scan of 68Ge rotating rod sources before emission data acquisition. HR+ emission images were reconstructed with all corrections by a 3D filtered back projection algorithm (Hamming filter; cut-off frequency, 0.5 cycles/pixel) and a zoom factor of three. Reconstructed volumes were 63 slices (2.42-mm thickness, 128 × 128 matrices of 0.32 × 0.32 mm2 voxels), pixels in 63 2.42-mm spaced planes. The Biograph mCT had a spatial transverse resolution of 4.4 mm. Attenuation was obtained using a 1-min low-dose CT scan acquired before emission. Biograph mCT/S64 emission images were reconstructed using the Siemens ultraHD PET algorithm with 12 iterations, eight subsets and a zoom factor of 21. Reconstructed volumes were 109 slices (2.027-mm thickness, 256 × 256 matrices of 0.398 × 0.398 mm2 voxels).

For both PET scans, dynamic acquisition started with the intravenous injection of the radiotracers synthesized in the cyclotron unit at CERMEP. The following different tracers were used at baseline, before (pre-MDMA state) and after (post-MDMA state) MDMA: [11C]-N,N-dimethyl-2-[(2-amino-4-cyanophenylthio)benzylamine (DASB) for serotoniner transporter binding, [11C]-N-(3-iodoprop-2E-enyl)2-beta-carbomethoxy-3beta-(4-methylphenyl)nor trope (PE2I) for dopamine transporter binding and [11F]-fluoro-l-DOPA (DOPA) for studying amino acid decarboxylase activity (see Supplementary Table 1 for acquisition details).
**Image processing**

**Regions of interest delineation**

Region delineation (including the region of reference) was achieved by the propagation of the *M. fascicularis* maximum probability atlas using the MAXPROB method (Ballanger et al., 2013). Some cortical regions were grouped to get 10 cortical regions of interest. In total, we had 24 regions of interest (10 cortical and 14 subcortical) (Table 1). For each individual, PET scans were summed over frames, before being registered between PET acquisitions to provide an average PET image. Individual mean PET images were registered to their corresponding individual anatomical MRI, which was registered to the *M. fascicularis* MRI template. Transformations from native PET to individual MRI and individual MRI to template were then concatenated to provide direct (and inverse) affine transformations from PET native spaces to the template space. Because raphe nuclei are not defined in the atlas, the raphe region was defined by drawing a parallelepiped rectangle (3 × 4 × 6 mm) in the sagittal, coronal and axial planes, respectively centred at the coordinates \(x = 0; y = -13; z = -7\) in the template space. The position and size of this parallelepiped rectangle were chosen to encompass both dorsal and medial raphe, according to monkey stereotaxic coordinates (Saleem and Logothetis, 2006).

**Kinetic modelling**

PET studies were analysed by suitable tracer kinetic modelling at a regional, and at a voxel-based level. The parameters computed were the non-displaceable binding potential (BP\(_{ND}\)) of \(^{11}\)C-DASB and \(^{11}\)C-PE2I, using a simplified reference tissue model (Gunn et al., 1997), and the uptake rate (Ki, 10\(^{-3}\)/min) for \(^{18}\)F-DOPA calculated using frames recording between 30 and 90 min for the linearization and the Patlak graphical analysis (Patlak et al., 1983). The cerebellum (excluding the vermis) was considered as the reference area for the two models. Regional parametric values were obtained by modelling of the mean regional kinetics, extracted in the native PET spaces by the MAXPROB method. Whole brain parametric images were obtained by modelling the voxel kinetics. Parametric images were transformed to the common template space by the inverse transformation computed in the MAXPROB method.

**Immunohistochemistry**

**Tissue preparation**

At the end of the experiments, the animals were deeply anaesthetized (ketamine at 1 mg/kg followed by a lethal dose of pentobarbitral) and perfused transcardially with 400 ml of saline (0.9% at 37°C) followed by 51 of 4% paraformaldehyde [in 0.1 M phosphate-buffered saline (PBS), pH 7.4 at 4°C] and 1 l of PBS with 5% sucrose. The brains were removed from the skull, rinsed in PBS complemented with 10% sucrose for 1 day and 20% sucrose for one further day, then frozen and cut into 50-µm thick sections coronally on a freezing microtome. Free-floating sections were conserved at −20°C in a cryoprotective solution containing 30% ethylene glycol, 30% glycerol and 0.1 M phosphate buffer.

**Immunostaining**

Free-floating sections were rinsed in Tris-buffered saline (TBS; 0.25 M Tris and 0.25 M NaCl, pH 7.5), incubated for 5 min in TBS containing 3% H\(_2\)O\(_2\) and 10% methanol, and then rinsed three times for 10 min each in TBS. After 15-min incubation in 0.2% Triton™ X-100 in TBS, the sections were rinsed three times in TBS. These were incubated for 72 h at 4°C with the following primary antibodies: anti-TH 1/5000 mouse monoclonal from Euromedex (catalogue number 22941), anti-serotonin 1/10 000 rabbit polyclonal from Euromedex (catalogue number 20080), anti-SERT (serotonin transporter) 1/200 goat polyclonal from Santa Cruz (catalogue number sc-14518) or anti-TPH2 1/800 sheep polyclonal from Millipore (catalogue number AB1541). After three rinses in TBS, the sections were then incubated for 2 h at 4°C with the corresponding secondary biotinylated antibody (1/500 from Abcys) in TBS. After being washed, the sections were incubated for 90 min at room temperature in avidin-biotin-peroxidase complex solution (final dilution, 1/50; Abcys). The sections were then rinsed twice in TBS and twice in Tris buffer (0.25 M Tris, pH 7.5) for 10 min each, placed in a solution of Tris buffer containing 0.1% 3,3’-diaminobenzidine (DAB; 50 mg/100 ml), and developed by H\(_2\)O\(_2\) addition (0.02%). The specificity of the immunostaining was assessed by omission of the primary antibody from the protocol. After processing, the tissue sections were mounted onto gelatin-alum-coated slides and dehydrated through graded alcohol to xylen for light microscopic examination using a computerized image analyser (Mercator, ExploraNova).

**Soma and fibre quantification**

The stained somas and fibres were plotted at ×6.3 and ×16 magnification, respectively after cartography of the different regions of interest. For each animal, TH+ cells were counted on nine regularly spaced sections encompassing the A8 (peri- and retrorubral area), A9 (pars compacta) and A10 (ventral tegmental area) dopamine regions as previously described (Mounayar et al., 2007; Neumane et al., 2012). The distribution of TPH2+ cells was also examined throughout five regularly spaced sections covering the antero-posterior extent of the raphe. The total number of TH+ and TPH2+ cells was estimated after correction by the Abercrombie method. SERT+, 5-HT+ and TPH2+ fibres were quantified in the regions of interest at five different anteriorities (AC +6; AC +0; AC −2; AC −5; AC −7). This was done by counting the number of fibres crossing the perimeter of five circles (diameter 100 µm) randomly distributed in each drawn region by the computer (Mercator, ExploraNova).

**Statistical analysis**

All statistical analyses were performed using Statistica software version 8 (www.statsoft.com). For PET, we used a mixed ANOVA with one between-subjects factor: group (MPTP recovered monkeys or MPTP symptomatic monkeys) × one within-subject factor: time at which the scan was performed (baseline, pre- or post-MDMA state). For immunohistochemical data, we used a one-way ANOVA [Group (control, MPTP recovered monkeys, MPTP symptomatic monkeys, MPTP recovered/MDMA monkeys)]. For homecage activity (during MDMA), Parkinson’s disease disability and dyskinesia,
<table>
<thead>
<tr>
<th>Region</th>
<th>11C-PE21 (BPND)</th>
<th>11C-DASB (BPND)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Baseline (n = 3)</td>
<td>Pre-MDMA</td>
</tr>
<tr>
<td>ACC</td>
<td>1.01 (±0.24)</td>
<td>0.33 (±0.06)</td>
</tr>
<tr>
<td>PCC</td>
<td>0.29 (±0.11)</td>
<td>0.47 (±0.08)</td>
</tr>
<tr>
<td>OFC</td>
<td>0.30 (±0.17)</td>
<td>0.66 (±0.05)</td>
</tr>
<tr>
<td>PFC</td>
<td>0.34 (±0.05)</td>
<td>0.26 (±0.03)</td>
</tr>
<tr>
<td>Insula</td>
<td>0.31 (±0.06)</td>
<td>0.15 (±0.05)</td>
</tr>
<tr>
<td>Sensorimotor</td>
<td>0.37 (±0.05)</td>
<td>0.20 (±0.03)</td>
</tr>
<tr>
<td>Parahipp/Enthorinal</td>
<td>0.03 (±0.10)</td>
<td>0.05 (±0.07)</td>
</tr>
<tr>
<td>Temporal</td>
<td>0.18 (±0.08)</td>
<td>0.24 (±0.05)</td>
</tr>
<tr>
<td>Parietal</td>
<td>0.30 (±0.04)</td>
<td>0.30 (±0.04)</td>
</tr>
<tr>
<td>Occipital</td>
<td>0.17 (±0.01)</td>
<td>0.14 (±0.11)</td>
</tr>
<tr>
<td>Basal ganglia</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Caudate, ant</td>
<td>7.13 (±0.72)</td>
<td>1.52 (±0.14)</td>
</tr>
<tr>
<td>Caudate, post</td>
<td>4.44 (±0.66)</td>
<td>1.37 (±0.04)</td>
</tr>
<tr>
<td>Put, ant</td>
<td>7.56 (±0.75)</td>
<td>3.05 (±0.26)</td>
</tr>
<tr>
<td>Put, post</td>
<td>6.35 (±0.63)</td>
<td>2.95 (±0.55)</td>
</tr>
<tr>
<td>Put, post vent</td>
<td>2.96 (±0.46)</td>
<td>2.68 (±0.60)</td>
</tr>
<tr>
<td>Vent striatum</td>
<td>4.65 (±1.21)</td>
<td>3.26 (±0.45)</td>
</tr>
<tr>
<td>Pallidum, ext</td>
<td>2.25 (±0.63)</td>
<td>2.21 (±0.08)</td>
</tr>
<tr>
<td>Pallidum, int</td>
<td>0.92 (±0.28)</td>
<td>0.79 (±0.30)</td>
</tr>
<tr>
<td>SN</td>
<td>1.28 (±0.40)</td>
<td>0.70 (±0.01)</td>
</tr>
<tr>
<td>Other areas</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Thalamus</td>
<td>0.75 (±0.12)</td>
<td>0.56 (±0.06)</td>
</tr>
<tr>
<td>Hippocampus</td>
<td>0.44 (±0.19)</td>
<td>1.80 (±0.17)</td>
</tr>
<tr>
<td>Amygdala</td>
<td>0.46 (±0.13)</td>
<td>0.34 (±0.05)</td>
</tr>
<tr>
<td>Raphe</td>
<td>0.84 (±0.14)</td>
<td>0.49 (±0.06)</td>
</tr>
<tr>
<td>Brainstem</td>
<td>0.84 (±0.14)</td>
<td>0.16 (±0.02)</td>
</tr>
</tbody>
</table>

*p < 0.05, **p < 0.01, ***p < 0.001 versus baseline; p < 0.05, **p < 0.01, ***p < 0.001 versus pre-MDMA state; *p < 0.05; **p < 0.01; ***p < 0.001 versus MPTP recovered monkeys.

ant = anterior; ACC = anterior cingulate cortex; OFC = orbitofrontal cortex; PCC = posterior cingulate cortex; PFC = prefrontal cortex; put = putamen; post = posterior; SN = substantia nigra; vent = ventral.
A double dopamine/5-HT lesion was induced by MPTP followed by MDMA (Fig. 1A). Due to progressive or acute MPTP intoxication, monkeys exhibited transient or persistent motor symptoms and were therefore divided into recovered (MPTP recovered) and symptomatic (MPTP symptomatic) monkeys (Fig. 1). As expected, compared to MPTP recovered monkeys, MPTP symptomatic monkeys exhibited a more severe parkinsonism associated to a stronger nigrostriatal dopamine lesion (Figs 1 and 2, Table 1 and Supplementary Figs 1 and 2). Additionally, the severity of parkinsonism also correlated with the serotonergic injury in the raphe (Fig. 1D and Supplementary Fig. 2C and D), confirming a possible alteration of the 5-HT system after MPTP. From the PET analysis, we did not observe a significant alteration of the serotonergic innervation in the basal ganglia after MPTP whatever the group of monkeys (Fig. 3 and Table 1). However, from post-mortem analysis, we found a drastic reduction of SERT + fibres in anterior striatal regions in MPTP symptomatic monkeys and a drastic sprouting of these fibres in MPTP recovered monkeys in both cortical and subcortical regions (Fig. 4 and Supplementary Table 2), extending a previous study (Neumane et al., 2012).

The acute effects driven by MDMA (administered 235 ± 25 days after the last MPTP injection) were pupillary dilatation (data not shown) and hypoactivity (~47% spontaneous homecage activity; post hoc, \( P < 0.001 \)) (Supplementary Fig. 3) associated with a slumped body posture and somnolence (data not shown). Two monkeys (MF4 and MF11) also presented myoclonic jerks in the tail and feet (data not shown). These behavioural responses stopped after cessation of acute MDMA injections.

The neurotoxic effect of MDMA in MPTP monkeys was addressed in vivo by PET imaging ~2 weeks after MDMA administration. The high \(^{11}\text{C}-\text{DASB} \text{BP} \text{ND} \) observed at the baseline and pre-MDMA state in rich 5-HT regions such as the striatum, the thalamus and the brainstem, was significantly reduced in all regions of interest (~50% on average) after MDMA in both MPTP recovered and MPTP symptomatic monkeys (Fig. 3 and Table 1). Overall, the MDMA lesion resulted in a lower \(^{11}\text{C}-\text{DASB} \text{BP} \text{ND} \) in all the regions of interest, in both MPTP symptomatic and MPTP recovered monkeys. To assess in vivo the specificity of the MDMA compound toward the 5-HT system, PET scans with \(^{11}\text{C}-\text{PE2I} \) and \(^{18}\text{F}-\text{DOPA} \) were also performed in the post-MDMA state. The remaining \(^{11}\text{C}-\text{PE2I} \text{BP} \text{ND} \) observed in the pre-MDMA state was not modified by MDMA (Fig. 2 and Table 1). Similarly, \(^{18}\text{F}-\text{DOPA} \) uptake was not further reduced by the MDMA (Supplementary Fig. 2).

The neurotoxic effects of MDMA were assessed by examining post-mortem tissues (Fig. 4). An important decrease of SERT+ fibres was demonstrated in MPTP recovered/MDMA monkeys at both cortical and subcortical levels compared to the control and MPTP recovered monkeys (Fig. 4 and Supplementary Table 2). Similar decreases of fibres were observed by using other serotonergic markers, such as 5-HT and TPH2 (the synthesizing enzyme of 5-HT) (Fig. 4). Regarding somas, we found that MDMA had no impact on the number of TPH2+ somas in the raphe region (\( P = 0.0629 \) and 0.0948 for dorsal and medial raphe, respectively) (80 ± 4% versus 101 ± 11% for MPTP recovered and MPTP recovered/MDMA monkeys, respectively) (Fig. 4D and indirectly shown on Fig. 1D). Similarly, MDMA did not affect the remaining number of TH+ somas in the nigral region (\( P = 0.118 \)) (69 ± 5% versus 71 ± 2% for MPTP recovered and MPTP recovered/MDMA monkeys, respectively) (Supplementary Fig. 1 and indirectly shown on Fig. 1C).

### Impact of the 5-HT lesion on cardinal motor symptom expression

The MDMA-driven 5-HT lesion did not evoke reappearance or worsening of tremor and akinetis/bradykinesia parkinsonian symptoms in MPTP recovered or MPTP symptomatic monkeys, respectively. However, the 5-HT lesion had an interesting and unexpected impact on rigidity (Fig. 5). For all MPTP recovered monkeys (6/6) exhibiting no residual rigidity before MDMA, we observed a reappearance of cogwheel rigidity (Fig. 5A and B). This reappearance had a specific pattern for each monkey as it could appear during or after MDMA and additionally be sustained (2/6) or transient (4/6), suggesting possible compensatory mechanisms. In any case, even when this effect was transient, the rigidity score was higher after MDMA lesion for MPTP recovered monkeys. For all MPTP symptomatic monkeys (5/5) exhibiting residual parkinsonian rigidity before MDMA, we first detected an immediate or progressive disappearance of cogwheel rigidity that lasted.
Figure 1 Study design and consequences of MPTP intoxication. (A) Experimental flowchart illustrating study design, treatments, timeline and group assignments. Control group involved naïve monkeys receiving neither MPTP, nor MDMA, nor L-DOPA. Behavioural analyses were carried out longitudinally. PET scans (red arrows) were performed in baseline, pre-MDMA and post-MDMA states. Post-mortem analyses were undertaken at the end of the protocols. Note that according to the progressive or acute MPTP intoxication, monkeys recovered (MPTPrec) from their motor symptoms or stayed symptomatic (MPTPsym). After MPTP, monkeys were further subdivided for L-DOPA (brown arrow) and/or MDMA (yellow box) treatments. (B) Evolution of motor score during the MPTP intoxication and the recovery or stabilization period for all MPTP-treated monkeys. Data represent the evolution of symptoms during and after the intoxication. Timelines were aligned such that Day 0 corresponds to the day on which the maximal motor score (motor peak) was obtained for each monkey. (C and D) The maximal motor score after MPTP negatively correlated with (C) the percentage of TH+ cell in A9 and (D) the percentage of TPH2+ cells in the dorsal raphe (DR) nucleus. Filled circles = MPTP recovered monkeys; open circles = MPTP symptomatic monkeys in D.
Figure 2 Impact of MPTP and MDMA intoxications on $^{11}$C-PE2I PET imaging. (A) Histogram representing the BP$_{ND}$ of $^{11}$C-PE2I in baseline ($n = 3$), pre-MDMA and post-MDMA states for MPTP recovered monkeys (MPTPrec, $n = 2$) and MPTP symptomatic monkeys (MPTPsym, $n = 4$) monkeys in subcortical areas. *$p < 0.05$, $**p < 0.01$, $***p < 0.001$ versus baseline. (B) MRI template (in grey) and $^{11}$C-PE2I PET images (in colour) on coronal and horizontal planes in baseline, pre-MDMA and post-MDMA states for MPTP recovered and MPTP symptomatic monkeys. Colours represent the level of BP$_{ND}$ using the cerebellum as the reference region (red indicates high BP$_{ND}$ whereas blue indicates low BP$_{ND}$ on the scale). (C) Negative correlations found between the maximal motor score after MPTP and $^{11}$C-PE2I BP$_{ND}$ in the pre-MDMA state in the anterior putamen, posterior caudate, ventral striatum and the ventral posterior putamen. Filled circles = MPTP recovered monkeys; open circles = MPTP symptomatic monkeys in C and D. ant = anterior; ACC = anterior cingulate cortex; BG = basal ganglia; cd = caudate; GPe = external globus pallidus; GPi = internal globus pallidus; OFC = orbitofrontal cortex; PCC = posterior cingulate cortex; PFC = prefrontal cortex; pu = putamen; post = posterior; SN = substantia nigra; SNc = substantia nigra pars compacta; Thal = thalamus; vent = ventral; VS = ventral striatum.
between 5 and 15 days after MDMA. We then observed a transient (2/5) or sustained (3/5) reappearance of rigidity until the onset of L-DOPA treatment, suggesting less reserve for recovery mechanisms. Of interest, three of five monkeys (MF10, MF12, MF9) exhibited a higher rigidity score than before MDMA. The variation of the rigidity score obtained before and after MDMA positively correlated with the ratio between $^{11}$C-DASB BP$_{ND}$ and $^{18}$F-DOPA Ki in the pre-MDMA state in the posterior putamen ($R^2 = 0.64$, $P < 0.01$; Fig. 5C) and in the external segment of the globus pallidus ($R^2 = 0.84$, $P < 0.01$; Fig. 5D). The higher the variation, the higher was the ratio, reflecting an imbalance between the dopamine and 5-HT injuries.

Figure 3 Impact of MPTP and MDMA intoxications on $^{11}$C-DASB PET imaging. (A) Histograms representing the BP$_{ND}$ of $^{11}$C-DASB in baseline ($n = 7$), pre-MDMA and post-MDMA states for MPTP recovered monkeys (MPTPrec, $n = 7$) and MPTP symptomatic monkeys (MPTPsym, $n = 4$) monkeys in subcortical brain areas. $^5p < 0.05$, $^6p < 0.01$, $^7p < 0.001$ versus baseline; $^$p < 0.05, $^**p < 0.01$, $^***p < 0.001$ versus pre-MDMA state; $^*$p < 0.05, $^**$p < 0.01 versus MPTP recovered monkeys. (B) MRI template (in grey) and $^{11}$C-DASB PET images (in colour) on coronal, horizontal and sagittal planes in baseline, pre-MDMA and post-MDMA states. Colours represent the level of BP$_{ND}$ using the cerebellum as the reference region (red indicates high BP$_{ND}$ whereas pink indicates low BP$_{ND}$ on the scale). ant = anterior; ACC = anterior cingulate cortex; BG = basal ganglia; cd = caudate; GPe = external globus pallidus; GPi = internal globus pallidus; OFC = orbitofrontal cortex; PCC = posterior cingulate cortex; PFC = prefrontal cortex; pu = putamen; post = posterior; SN = substantia nigra; SNC = substantia nigra pars compacta; Thal = thalamus; vent = ventral; VS = ventral striatum.
Figure 4 Impact of MPTP and MDMA intoxications on serotonergic markers assessed by immunohistochemistry on post-mortem tissue. (A) Brain regions successively numbered, in which SERT-positive fibres were quantified, at five different anteriority levels according to the anterior commissure. (B and C) Schematic representation of SERT + fibre density (B) in MPTP recovered (MPTPrec, n = 4) and MPTP symptomatic (MPTPsym, n = 4) monkeys compared to control monkeys (n = 4) and (C) in MPTP recovered/MDMA monkeys (MPTPrec-
Impact of 5-HT lesion on L-DOPA-induced motor and non-motor effects

As expected, L-DOPA counteracted the parkinsonian disability score and induced dose-dependent dyskinesias in MPTP symptomatic monkeys (Fig. 6). In MPTP recovered monkeys, L-DOPA triggered dose-dependent behavioural hyperactivity (Fig. 7A) and neuropsychiatric-like behaviour (Fox et al., 2010) (Fig. 7B). Interestingly, we found that the neuropsychiatric-like score was positively correlated with $^{11}$C-DASB BPND before MDMA in the ventral posterior putamen ($R^2 = 0.79$, $P < 0.01$; Fig. 7C). The higher the binding before MDMA, the higher was the score. When looking at specific types of behaviour, the hallucinatory-like response score was positively correlated.

Figure 5 Impact of MPTP and MDMA intoxications on parkinsonian rigidity. (A) Example of the evolution of the rigidity score before and after MDMA for one MPTP recovered/MDMA monkey without rigidity (MF11) and one MPTP symptomatic monkeys/MDMA monkey with rigidity (MF10) before MDMA (Day 1 corresponds to first day of MDMA administration). (B) Evolution of the rigidity score (mean ± SEM) before, during and after MDMA for each group of monkeys (MPTP recovered/MDMA versus MPTP symptomatic/MDMA); (C and D) Positive correlations found between the variation of the rigidity score with $^{11}$C-DASB BPND/$^{18}$F-DOPA Ki ratio within the posterior putamen (C) and the external segment of the globus pallidus (D).

Figure 4 Continued
MDMA, $n = 4$) compared to MPTP recovered monkeys ($n = 4$). (D) Photomicrographs at low ($\times 4$) or high ($\times 16$) magnification of coronal sections at the level of the dorsal posterior putamen (AC-5) and the dorsal raphe (AC-15) exemplifying the SERT, 5-HT and TPH2 labelling obtained for one monkey of each experimental group [MF15 (control), MF18 (MPTP symptomatic monkeys), MF1 (MPTP recovered monkeys) and MF4 (MPTP recovered monkeys-MDMA)]. Scale bar = 200 μm.
with the $^{11}$C-DASB BP$_{ND}$ before MDMA within the posterior ventral putamen (Fig. 7D; $R^2 = 0.58$, $P < 0.05$) while the repetitive grooming score positively correlated with it within the external pallidum (Fig. 7E; $R^2 = 0.60$, $P < 0.05$).

The MDMA-driven 5-HT lesion had a strong impact on the L-DOPA-induced responses. For MPTP symptomatic/MDMA monkeys, dyskinesias were fully abolished or strongly reduced [area under the curve (AUC) 40 mg/kg $-92\%$, 80 mg/kg $-77\%$; post hoc, $P < 0.001$] in response to low or high doses of L-DOPA, respectively (Fig. 6A and B). It was noteworthy that the anti-parkinsonian effect of L-DOPA was preserved despite the MDMA lesion (Fig. 6D). For MPTP recovered/MDMA monkeys, L-DOPA administered at low doses failed to evoke significant behavioural hyperactivity (Fig. 7A). Only the highest dose triggered an enhancement (+34%, $P < 0.001$). Similarly, after MDMA lesion, only the highest dose of L-DOPA triggered neuropsychiatric-like behaviour significantly (+113%, $P < 0.01$), but this response mainly consisted of repetitive grooming and stereotypies (Fig. 7B).

**Figure 6** Behavioural impact of L-DOPA treatment and MDMA intoxication on MPTP symptomatic monkeys. (A) Dyskinesias in response to increasing doses of L-DOPA during L-DOPA treatments (before and after MDMA) for MPTP symptomatic monkeys (MPTPrec, $n = 4$). $^aP < 0.05$, $^bP < 0.01$, $^cP < 0.001$ versus first L-DOPA before MDMA. (B) Areas under the curve (AUC) of dyskinesias scores. $^aP < 0.05$, $^bP < 0.01$, $^cP < 0.001$ between doses; $^aP < 0.05$, $^bP < 0.01$, $^cP < 0.001$ versus L-DOPA before MDMA. (C and D) Parkinsonian disability score in OFF and ON during chronic L-DOPA treatment (C) before and (D) after MDMA. $^aP < 0.05$, $^bP < 0.01$, $^cP < 0.001$ versus OFF state.

**Discussion**

This is the first time that a double lesion monkey model has been developed to more closely mimic the dopamine and 5-HT lesions occurring in the brains of parkinsonian patients (Huot et al., 2011a; Politis, 2014; Politis and Niccolini, 2015). Despite the limitations of this toxin-based animal model (Ricaurte et al., 2000; Porras et al., 2012) (MPTP being not selective of dopamine neurons and MDMA injuring only 5-HT fibres), the implication of the presynaptic serotonergic system could be addressed for the first time in the parkinsonian non-human primate, calling the dysfunction of raphe neurons in parkinsonism to mind (Huot et al., 2011a; Del Tredici and Braak, 2012; Politis, 2014; Politis and Niccolini, 2015). Three major findings emerged from this work: (i) the injury of the 5-HT innervating system impacted the expression of rigidity; (ii) L-DOPA-induced dyskinesias were abolished after 5-HT injury; and (iii) neuropsychiatric-like behaviours driven by L-DOPA were also impaired after 5-HT injury.
Figure 7 Behavioural impact of L-DOPA treatment and MDMA intoxication on MPTP recovered monkeys. (A) Homecage activity and (B) neuropsychiatric-like behaviours in response to increasing doses of L-DOPA during L-DOPA before and after MDMA for MPTP recovered monkeys (MPTPrec, n = 3). $^{5}p < 0.05$, $^{5}$p < 0.01, $^{55}$p < 0.001 versus baseline; *$^{p} < 0.05$, **$^{p} < 0.01$, ***$^{p} < 0.001$ versus L-DOPA before MDMA. (C) Positive correlation found between the neuropsychiatric-like score and $^{11}$C-DASB BPND before MDMA within the posterior ventral putamen. Each monkey is represented by a specific symbol. MRI template (in grey) and $^{11}$C-DASB PET images (in colour) on horizontal plane at the level of the ventral posterior putamen in monkeys (MF9, MF5 and MF8) presenting a light, moderate, severe score, respectively for neuropsychiatric-like response before MDMA. Colours represent the level of BPND using the cerebellum as the reference region (red indicates high BPND whereas pink indicates low BPND on the scale). (D) Positive correlation found between the hallucinatory-like response score and $^{11}$C-DASB BPND before MDMA within the posterior ventral putamen. (E) Positive correlation found between the repetitive grooming score and $^{11}$C-DASB BPND before MDMA within the external globus pallidus (GPe).
Among the cardinal motor symptoms expressed by parkinsonian patients, only tremor has been clearly linked to 5-HT dysfunction so far (Huot et al., 2011a; Politis, 2014; Politis and Niccolini, 2015). Although not always concordant regarding the form of tremor involved, PET imaging studies have shown that a diminished availability of 5-HT1A receptors in the raphe or of SERT in the thalamus correlates with the severity of resting tremor (Huot et al., 2011a; Politis, 2014; Politis and Niccolini, 2015). A reduced availability of SERT in the caudate, putamen and raphe correlates with the severity of action and postural but not resting tremor (Loane et al., 2013), suggesting that different pathological mechanisms might be involved in the different forms of tremor. Resting tremor was replaced by action tremor in our MPTP-treated macaques (resting tremor is the only symptom seldom reproduced except in the MPTP-treated African green monkey) (Mounayar et al., 2007; Porras et al., 2012) and not altered by 5-HT lesion. Bradykinesia was not induced or worsened after MDMA in recovered or symptomatic monkeys, respectively. The striatal blockade of 5-HT transmission in recovered monkeys does not induce bradykinesia either (Neumane et al., 2012). This is consistent with a human study showing no SERT dysfunction in bradykinetic parkinsonian patients (Loane et al., 2013). The only symptom for which we found an impact after MDMA lesion was rigidity. The expression of rigidity was modified by the serotonergic lesion and this effect strongly depended on the previous serotonergic and dopaminergic innervation state, especially in the posterior putamen and external segment of the pallidum (transient appearance mostly in recovered monkeys that have greater compensatory mechanisms and transient disappearance followed by reappearance in symptomatic monkeys that can no longer compensate). Strengthening those preclinical results, we also observed that the greater 5-HT denervation in the putamen (assessed by 11C-DASB binding), the greater the rigidity of parkinsonian patients (S. Thobois, unpublished results). Such a link between 5-HT dysfunction and rigidity is novel and might have been hidden or under-valued because (i) the scoring of rigidity is seldom in non-human primates (Imbert et al., 2000; Mounayar et al., 2007; Porras et al., 2012); (ii) akinesia/bradykinesia and tremor are the most prominent symptoms in parkinsonian patients (Kang et al., 2005; Uitti et al., 2005); or (iii) akinesia and rigidity are often associated in parkinsonian patients (akinetic-rigid subtype) and related to the lesion of the dopamine system (Jellinger, 1999; Politis, 2014).

A causal demonstration of 5-HT fibres mediating 1-DOPA-induced dyskinesias in non-human primates is given in our present work, which shows for the first time, a complete abolition of 1-DOPA-induced dyskinesias after the MDMA-driven 5-HT terminals injury. This work strengthens the view that MDMA is effective in reducing 1-DOPA-induced dyskinesias in rats (Bishop et al., 2006), monkeys (Iravani et al., 2003; Huot et al., 2011b) and early-onset parkinsonian patients (BBC, 2001) by demonstrating the underlying and causal physiopathological mechanism, i.e. the serotonergic hyperinnervation, abolished by MDMA. The lesion of the 5-HT system or the administration of 5-HT1A agonists suppresses 1-DOPA-induced dyskinesias in rats (Carta and Tronci, 2014). There is also a beneficial role of administrating 5-HT1A or 5-HT1AR agonists in dyskinetic monkeys or parkinsonian patients, although a possible worsening of parkinsonian symptoms (Carta and Tronci, 2014; Politis et al., 2014). In the same vein, the use of antidepressants acting at the SERT level has been shown to offer an interesting antidyskinetic strategy (Conti et al., 2014; Mazzucchi et al., 2015), highlighting again the deleterious role of the presynaptic 5-HT fibres (Carta and Tronci, 2014; Cenci, 2014; Politis et al., 2014). Finally, as the inclusion of 5-HT neurons in the grafted foetal dopamine transplant significantly worsens 1-DOPA-induced dyskinesias in the rat model of Parkinson’s disease (Carta and Tronci, 2014), particular attention has been focused on the possible influence of the dopamine:5-HT ratio detected in early parkinsonian patients (Suwijn et al., 2013) and parkinsonian rats (García et al., 2011) on 1-DOPA-induced dyskinesia expression. In parkinsonian patients, the future development of dyskinesias is not associated with a higher SERT to dopamine transporter (DAT) ratio at early stages (Suwijn et al., 2013). In the rat, the inclusion of 5-HT neurons is not critical as long as there are sufficient dopamine neurons (García et al., 2011), but our MPTP symptomatic dyskinetic monkeys, which mimic an advanced stage of Parkinson’s disease, may certainly present an imbalance in favour of 5-HT. It is noteworthy that in grafted-parkinsonian patients, dyskinesias are mediated by 5-HT fibres in recovered monkeys (Politis et al., 2010) and present a high striatal SERT/DAT ratio (Politis et al., 2011).

One striking and unexpected result we have obtained is that our MPTP-recovered monkeys chronically treated with 1-DOPA did not develop dyskinesias, but rather hyperactive and neuropsychiatric-like behaviours, which were both abolished by subsequent MDMA lesion. These findings are particularly interesting as it demonstrates that serotonergic fibres also play a significant role in the pathophysiology of non-motor symptoms driven by 1-DOPA. As recovered monkeys are the ones exhibiting a sprouting of 5-HT fibres after MPTP (Mounayar et al., 2007 and present work), one can speculate that these fibres sustain the expression of behavioural disorders via aberrant processing of exogenous 1-DOPA and release of dopamine as false neurotransmitter in non-motor regions, as it is the case in motor regions (putamen) for 1-DOPA-induced dyskinesias (Politis et al., 2014). The positive correlations that we found before MDMA between (i) the presynaptic 5-HT innervation in the posterior ventral putamen and the severity of the hallucinatory-like response; and (ii) the presynaptic 5-HT innervation in the external pallidum and the severity of repetitive grooming, and the abolition of these non-motor symptoms after MDMA, strengthen this hypothesis. It would be interesting to investigate whether lesioning the serotonergic fibres in the posterior ventral putamen and external pallidum would
abolish the hallucinatory-like response and grooming, respectively in response to l-DOPA. Of note, the limbic part of the external pallidum is a region from which stereotyped behaviours (grooming and licking/biting) can be pharmacologically evoked in the monkey (Grabli et al., 2004; Sgambato-Faure et al., 2014; Tremblay et al., 2015). As for the ventral posterior putamen, it is a structure connected to the inferotemporal cortex involved in the visual recognition and discrimination of objects (Baizer et al., 1993; Middleton and Strick, 1996). From human studies, it has been shown that serotonin dysfunction is involved in the physiopathology of psychosis (Zahodne and Fernandez, 2008). Atypical antipsychotic, quetiapine and clozapine reduce l-DOPA-induced psychosis and psychotic-like behaviour without a significant increase in parkinsonian disability (Fox et al., 2008; Gottwald and Aminoff, 2011). A role for 5-HT2A receptors in mediating visual hallucinations via the ventral visual pathway in Parkinson’s disease has also been suggested (Ballanger et al., 2010; Huot et al., 2010). One can expect that the administration of selective 5-HT2A antagonists inside the posterior ventral putamen would abolish the hallucinatory-like response after l-DOPA. The slower degeneration of serotonergic (versus dopaminergic) terminals as Parkinson’s disease progresses could therefore be a risk factor for occurrence of these l-DOPA-induced non-motor symptoms.

Altogether, these findings provide an important conceptual advance by demonstrating that lesioning the presynaptic serotonergic fibres impacts the expression of rigidity and abolishes the l-DOPA-induced dyskinesia and neuropsychiatric-like behaviour in the macaque model of Parkinson’s disease. Outside of the context of Parkinson’s disease, these findings highlight the possibility that serotonergic fibres may play a deleterious role not only in movement disorders but also in behavioural disorders. By using this double-lesioned model, it will be possible to investigate the contribution of the serotonergic system in the expression of the non-motor symptoms associated to the disease itself rather than to its dopamine replacement therapy (as in the present study with l-DOPA). One important question that remains to be investigated is whether MPTP recovered/MDMA double-lesioned monkeys develop an apathetic-like or an anxious-like state. Another important feature of this model is that by reversing the sequential use of the toxins (MDMA before MPTP), one can address the impact of an early serotonergic lesion on the appearance and severity of the parkinsonian motor and non-motor symptoms. Therefore, this double-lesioned model opens important new preclinical research avenues that may lead on new potential therapeutic targets.

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Supplementary material

Supplementary material is available at Brain online.

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