

Combined 5-HT_{1A} and 5-HT_{1B} receptor agonists for the treatment of L-DOPA-induced dyskinesia

Ana Muñoz,¹ Qin Li,² Fabrizio Gardoni,³ Elena Marcello,³ Chuan Qin,² Thomas Carlsson,^{1,4} Deniz Kirik,⁴ Monica Di Luca,³ Anders Björklund,¹ Erwan Bezard^{5,*} and Manolo Carta^{1,4,*}

¹Neurobiology Unit, Wallenberg Neuroscience Center, Department of Experimental Medical Science, University of Lund, Sweden, ²Institute of Lab Animal Sciences, China Academy of Medical Sciences, Beijing, China, ³Department of Pharmacological Sciences and Centre of Excellence on Neurodegenerative Diseases, University of Milano, Italy, ⁴Brain Repair and Imaging in Neural Systems Unit, Section for Neuroscience, Department of Experimental Medical Science, University of Lund, Sweden and ⁵University Victor Segalen Bordeaux 2, Centre National de la Recherche Scientifique, Bordeaux Institute of Neuroscience, UMR 5227, Bordeaux, France

*These authors contributed equally to this work.

Correspondence to: Manolo Carta, Neurobiology Unit, Wallenberg Neuroscience Center, Department of Experimental Medical Science, University of Lund, 22184 Lund, Sweden and Erwan Bezard, University Victor Segalen Bordeaux 2, Centre National de la Recherche Scientifique, Bordeaux Institute of Neuroscience, UMR 5227, Bordeaux, France
E-mail: Manolo.Carta@med.lu.se and erwan.bezard@u-bordeaux2.fr

Appearance of dyskinesia is a common problem of long-term L-DOPA treatment in Parkinson's disease patients and represents a major limitation for the pharmacological management of the motor symptoms in advanced disease stages. We have recently demonstrated that dopamine released from serotonin neurons is responsible for L-DOPA-induced dyskinesia in 6-hydroxydopamine (6-OHDA)-lesioned rats, raising the possibility that blockade of serotonin neuron activity by combination of 5-HT_{1A} and 5-HT_{1B} agonists could reduce L-DOPA-induced dyskinesia. In the present study, we have investigated the efficacy of 5-HT_{1A} and 5-HT_{1B} agonists to counteract L-DOPA-induced dyskinesia in 1-methyl-4-phenyl 1,2,3,6-tetrahydropyridine (MPTP)-treated macaques, the gold standard model of Parkinson's disease. In addition, we have studied the ability of this treatment to prevent development of L-DOPA-induced dyskinesia in 6-OHDA-lesioned rats. The results demonstrate the existence of a potent synergistic effect between 5-HT_{1A} and 5-HT_{1B} agonists in their ability to dampen L-DOPA-induced dyskinesia in the MPTP-treated macaques. Sub-threshold doses of the drugs, which individually produced no effect, were able to reduce the abnormal involuntary movements by up to 80% when administered in combination, without affecting the anti-parkinsonian properties of L-DOPA. Furthermore, chronic administration of low doses of the 5-HT₁ agonists in combination was able to prevent development of dyskinesia, and reduce the up-regulation of FosB after daily treatment with L-DOPA in the rat 6-OHDA model. Our results support the importance of a clinical investigation of the effect of 5-HT_{1A} and 5-HT_{1B} agonists, particularly in combination, in dyskinetic L-DOPA-treated Parkinson's disease patients.

Keywords: L-DOPA; Dyskinesia; Parkinson's disease; Serotonin agonists; MPTP monkeys

Abbreviations: AIMs = abnormal involuntary movements; AUC = area under the curve; DA = dopamine; MPTP = 1-methyl-4-phenyl 1,2,3,6-tetrahydropyridine; 6-OHDA = 6-hydroxydopamine; PSD = post-synaptic density; s.c. = subcutaneous; TIF = Triton X-100-insoluble fraction

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Introduction

The appearance of dyskinesias represents the most troublesome side effect of long-term L-DOPA administration in Parkinson's disease patients and limits the use of L-DOPA in the advanced disease stage (Obeso *et al.*, 2000; Rascol *et al.*,

2000; Ahlskog and Muenter, 2001). In early stages of the disease, it is generally assumed that L-DOPA acts by being taken up into spared dopaminergic neurons and terminals, where it is converted to dopamine (DA), stored into synaptic vesicles and released in a physiologically regulated manner

(Cenci and Lundblad, 2006). As the dopaminergic degeneration progresses, fewer and fewer DA terminals can contribute to the conversion of peripheral administered L-DOPA. In this situation, other neuronal and non-neuronal cell types are suggested to play a role in DA production (Melamed *et al.*, 1980; Hefti *et al.*, 1981;). Among these, the serotonin neurons represent an interesting element because they express aromatic amino acid decarboxylase and vesicular monoamine transporter 2, which are responsible for conversion of L-DOPA to DA and storage of DA into synaptic vesicles, respectively (Arai *et al.*, 1994; Peter *et al.*, 1995). Several studies have shown that the serotonin neurons have the capacity to store and release DA after peripheral administration of L-DOPA, both *in vivo* and *in vitro* (Ng *et al.*, 1970, 1971; Hollister *et al.*, 1979). Tanaka and co-workers (1999) have shown that lesion of the serotonin system by intraventricular administration of the specific toxin 5,7-dihydroxytryptamine (5,7-DHT) reduced L-DOPA-derived extracellular DA by about 80% in hemiparkinsonian rats. A similar reduction in extracellular striatal DA level was also obtained following co-administration of the 5-HT_{1A} agonist (±)-8-OH-DPAT with L-DOPA (Kannari *et al.*, 2001), which also reduced up-regulation of prodynorphin and glutamic acid decarboxylase in the DA-denervated striatum, both of which are established markers of L-DOPA-induced motor complications (Tomiyama *et al.*, 2005).

We have recently demonstrated a causal link between the DA released from the serotonin neurons and the appearance of abnormal involuntary movements (AIMs) in the rat 6-hydroxydopamine (6-OHDA) model (Carta *et al.*, 2007). In these experiments, removal of the serotonin innervation by intraventricular injection of 5,7-DHT, or pharmacological silencing of the release from these neurons by a combination of 5-HT_{1A} and 5-HT_{1B} receptor agonists, resulted in a near-complete suppression of L-DOPA-induced dyskinesias in L-DOPA-primed 6-OHDA-lesioned rats. In addition, we showed that serotonin neuron transplants increased the pro-dyskinetic effect of L-DOPA by providing a 2- to 3-fold increase in the serotonin innervation of the host striatum and thus a possible additional source of dysregulated DA release (Carlsson *et al.*, 2007). These results suggest the DA released from serotonin terminals is the main pre-synaptic determinant of L-DOPA-induced dyskinesia in the rat Parkinson's disease model.

These results in dyskinetic rats suggested a potential use of 5-HT_{1A} and 5-HT_{1B} agonists, particularly in combination, for the treatment of L-DOPA-induced dyskinesia in Parkinson's disease patients. However, preclinical development of this approach required further validation in primate models of Parkinson's disease, such as the 1-methyl-4-phenyl 1,2,3,6-tetrahydropyridine (MPTP)-treated macaque. In the present study, we have therefore tested the efficacy of acute challenges with the 5-HT_{1A} and 5-HT_{1B} agonists, individually and in combination, to counteract L-DOPA-induced dyskinesia in dyskinetic MPTP-treated macaques. In addition, the effect of chronic administration of 5-HT_{1A} and 5-HT_{1B}

agonists was tested on the development of L-DOPA-induced dyskinesia in 6-OHDA-lesioned rats.

Materials and Methods

Monkey studies

Housing

Six female cynomolgus monkeys (*Macaca fascicularis*, Xierxin, Beijing, PR of China) were used. Animals were 5-years old and were previously treated with other drugs. However, 2 month wash-out with daily exposure to L-DOPA was undertaken to avoid possible interactions. They were housed in individual primate cages under controlled conditions of humidity, temperature and light (12 h light/12 h dark cycle, lights on at 8.00 a.m.); food and water were available *ad libitum*. Animal care was supervised by veterinarians skilled in the healthcare and maintenance of non-human primates. Experiments were carried out in accordance with European Communities Council Directive of November 24, 1986 (86/609/EEC) for care of laboratory animals.

Experimental parkinsonism and dyskinesia

Experiments were conducted according to previously published procedures and methods (Bezard *et al.*, 2003; Aubert *et al.*, 2005; Guigoni *et al.*, 2005). Monkeys received once daily i.v. injections of MPTP hydrochloride (0.2 mg/kg) until they displayed parkinsonian symptoms (mean number of injections = 15 ± 1) (Bezard *et al.*, 2001a). It took an average of 8 weeks for the bilateral parkinsonian syndrome to stabilize (i.e. consistent disability score over 2 consecutive weeks—median score = 8.5 with 7–10 range). Monkeys were then treated chronically with twice daily oral administration of Modopar (Roche, Basel, Switzerland, L-DOPA/carbidopa, ratio 4:1) for 6 months at a tailored dose designed to fully reverse the parkinsonian features (L-DOPA/carbidopa dose ranging between 15 mg/kg and 20 mg/kg, 3.7 mg/kg and 5 mg/kg, respectively) and developed dyskinesia.

Experimental design

L-DOPA/carbidopa (or its vehicle) was administered orally concomitantly with a subcutaneous (s.c.) administration of either vehicle, the 5-HT_{1A} agonist (±)-8-Hydroxy-2-dipropylaminotetralin hydrobromide [(±)-8-OH-DPAT, TOCRIS, UK] and/or the 5-HT_{1B} agonist, CP-94253 (TOCRIS) in the animal's home cage (Bezard *et al.*, 2003; Gold *et al.*, 2007). The animals were immediately transferred to an observation cage (dimensions—1.1 m × 1.5 m × 1.1 m) for a 250 min behavioural assessment (see below). Ten different treatments were employed; vehicle-vehicle, L-DOPA/carbidopa (dose ranging between 15 mg/kg and 20 mg/kg and 3.7 mg/kg and 5 mg/kg, respectively) vehicle, L-DOPA/carbidopa-8-OH-DPAT (0.05 mg/kg and 0.1 mg/kg), L-DOPA/carbidopa-CP-94253 (1.75 mg/kg and 2.5 mg/kg) and all combinations of L-DOPA/carbidopa-8-OH-DPAT-CP-94253. The 10 different treatments were randomly tested in each animal with a 3 day wash-out period in between.

Behavioural assessment

A battery of behavioural tests was performed as previously described (e.g. Bezard *et al.*, 2003; Guigoni *et al.*, 2005a,b; Gold *et al.*, 2007). A quantitative assessment of locomotor activity using computer-based passive infrared activity monitors (Excalibur, modified by the

Central Electronic Workshop, University of Manchester) was obtained every 5 min for 250 min (Bezard *et al.*, 2003; Gold *et al.*, 2007).

Non-parametric measures based on range of movement, bradykinesia and posture scales were made; parkinsonian condition (and its reversal) was assessed on a parkinsonian monkey rating scale (Imbert *et al.*, 2000) by *post hoc* analysis of video recordings by observers blinded to the treatment, in 10 min observation periods every 30 min for 250 min, as previously described (e.g. Bezard *et al.*, 2003; Guigoni *et al.*, 2005a,b). A score of 0 corresponds to a normal animal and a score above 6 to a parkinsonian animal.

The severity of dyskinesia was rated using the dyskinesia disability scale (Bezard *et al.*, 2003; Gold *et al.*, 2007) by *post hoc* analysis of video recordings, in 10 min observation periods every 30 min for 250 min. Both choreic (hyperkinetic, purposeless dance-like movements) and dystonic (sustained, abnormal muscle contractions) components of dyskinesia were rated: 0 = dyskinesia absent; 1 = mild, fleeting and rare dyskinetic postures and movements; 2 = moderate, more prominent abnormal movements, but not interfering significantly with normal behaviour; 3 = marked, frequent and, at times, continuous dyskinesia intruding on the normal repertoire of activity; or 4 = severe, virtually continuous dyskinetic activity replacing normal behaviour and disabling to the animal. All scales have been validated for inter- and intra-variability and differences in rating were discussed regularly to eliminate observer idiosyncrasy (Taylor *et al.*, 1994). For ethical and experimental reasons, animals were not killed at the end of behavioural experiments. However, historical data obtained with animals submitted to the very same regimen of MPTP and L-DOPA administrations have shown extreme lesions of the nigrostriatal pathway and comparable extent and pattern of denervation among the animals (Guigoni *et al.*, 2005b).

Rat studies

Housing

A total of 140 adult female Sprague–Dawley rats weighing 225–250 g were used in the present study (B&K Universal, Stockholm, Sweden). The animals were housed under a 12 h light/12 h dark cycle with free access to water and food. All experiments were performed according to the regulations set by the Ethical Committee for use of Laboratory animals at Lund University.

Experimental parkinsonism

All 6-OHDA injections were conducted under anesthesia induced by an injectable 20:1 mixture of Fentanyl and Dormitor (Apoteksbolaget, Sweden) using a stereotaxic frame (Stoelting, Wood Dale, Illinois) with an attached Hamilton syringe. The animals received 6-OHDA (Sigma-Aldrich AB, USA) injection into the medial forebrain bundle (MFB) (14 µg free base in 4 µl of saline containing 0.02% ascorbic acid) in order to achieve a complete lesion of the nigrostriatal pathway at the following coordinates (relative to bregma, see Paxinos and Watson (1998): AP: −4.4 mm, ML: −1.2 mm, DV: −7.8 mm. The toothbar was set at −2.4 mm. Injection speed was 1.0 µl/min and the syringe was kept in place for an additional 3 min before it was slowly retracted.

Experimental design

Three weeks after 6-OHDA injection, the rats were screened behaviourally in the amphetamine-induced rotation test (2.5 mg/kg i.p.). Animals exhibiting ≥ 6 full body turns/min towards the side of DA deficiency were included in the study. Animals were then allocated into two well-matched sub-groups (according to the amphetamine rotation) and received daily treatment with either L-DOPA methyl ester (6 mg/kg i.p. plus benserazide 10 mg/kg) individually, or in combination with the 5-HT_{1A} agonist (±)8-OH-DPAT and the 5-HT_{1B} agonist CP-94253 given s.c. at the same time of L-DOPA, for two weeks. At the end of this treatment (treatment period 1), animals received a low dose of apomorphine (0.02 mg/kg, s.c.) and tested for apomorphine-induced AIMs in order to investigate the sensitization state of the DA receptors. Treatments were then switched so that animals receiving L-DOPA only during treatment period 1 now received L-DOPA + 5-HT₁ agonists, while animals previously treated with L-DOPA + 5-HT₁ agonists were treated only with L-DOPA for an additional two weeks (treatment period 2). Animals were injected daily and tested every second day for L-DOPA-induced dyskinesia throughout the experimental periods 1 and 2 and then sacrificed for HPLC analysis of DA, serotonin and metabolites. Additional groups of animals were treated in an identical way and sacrificed at the end of the treatment period 1 for western blotting analysis and FosB immunostaining.

HPLC measurements

All animals were killed and striata were rapidly dissected out, frozen on dry ice and stored in −80°C freezer until analysis. At the time of the analysis, tissue was homogenized in 0.1 M perchloric acid and centrifuged at 10 000 r.p.m. for 10 min before filtering through minispin filters for additional 3 min at 10 000 r.p.m. The tissue extracts were then analysed by HPLC as described earlier (Carta *et al.*, 2006) with minor modifications. Briefly, 25 µl of each sample were injected by a cooled autosampler (Midas, Spark, Holland) into an ESA Coulochem III coupled with an electrochemical detector. The mobile phase (sodium acetate 5 g/l, Na₂-EDTA 30 mg/l, octane-sulfonic acid 100 mg/l, methanol 10%, pH 4.2) was delivered at a flow rate of 500 µl/min to a reverse phase C18 column (4.6 mm Ø, 150 mm length, Chrompack, Middleburg, The Netherlands). Peaks of DA, serotonin and metabolites were processed by the Azur Chromatographic Software (DataLab, France).

Amphetamine-induced rotation

Amphetamine-induced rotation was performed 3 weeks after the 6-OHDA injection to evaluate the extent of the DA lesion. Right and left full-body turns were recorded over 90 min, using automated rotometer bowls (AccuScan Instrument Inc., Columbus, Ohio), following an i.p. injection of 2.5 mg/kg of d-amphetamine sulphate (Apoteksbolaget, Sweden). The data are expressed as net full-body turns per minute, where rotation towards the side of the lesion was given a positive value.

L-DOPA- and Apomorphine-induced dyskinesia

In all tests, the AIMs were evaluated according to the rat dyskinesia scale described in detail previously (Lee *et al.*, 2000; Lundblad *et al.*, 2002). Briefly, the animals were placed individually in transparent plastic cages without bedding material and scored every 20 min following the injection of L-DOPA for the

entire time course of dyskinesias (about 120 min). The AIMs were classified into four subtypes according to their topographic distribution as forelimb (Li), orolingual (Ol) axial (Ax) and locomotive (Lo) behaviours. The forelimb and orolingual dyskinesia are predominantly seen as hyperkinesia, while the axial dyskinesia is essentially of a dystonic type. The locomotive dyskinesia was expressed as circling movements away from the lesioned side. Enhanced manifestations of normal behaviours, such as grooming, gnawing, rearing and sniffing were not included in the rating. The severity of each AIM subtype was assessed using scores from 0 to 4 (1: occasional, i.e. present <50% of the time; 2: frequent, i.e. present >50% of the time; 3: continuous, but interrupted by strong sensory stimuli; 4: continuous, not interrupted by strong sensory stimuli).

Dyskinesias were also evaluated after apomorphine injection (0.02 mg/kg, dissolved in saline containing 0.002% ascorbic acid; Apoteksbolaget, Sweden). Here, scoring was performed every 10 min using the same rating scale as for the L-DOPA-induced dyskinesias. The data are presented as integrated scores, area under the curve (AUC) in a raw data plot of total Ax + Li + Ol AIM scores (total AIMs \times interval of observation: $\times 20$ for L-DOPA; $\times 10$ for apomorphine).

Activity test

Locomotor activity was assessed (at day 3 of the treatment period 1) in open-field chambers, each equipped with a 16 \times 16 infrared photobeam system (dimensions 40.6 cm \times 40.6 cm \times 38.1 cm) using the Flex-Field Software system (San Diego Instruments, San Diego, CA). Animals were habituated for 1 h before the drugs were injected and the measurements begun.

Stepping test

The stepping test (Schallert *et al.*, 1992) was performed as previously described (Kirik *et al.*, 2001) with little modifications. Briefly, the rat was held by the experimenter fixing its hindlimbs with one hand and the forelimb not to be monitored with the other, while the unrestrained forepaw was touching the table. The number of adjusting steps was counted, while the rat was moved sideways along the table surface (90 cm in 5 s), in the forehand and backhand direction, for both forelimbs, and the average of the steps in the two directions was considered. Performance of the animals in the stepping test was assessed during treatment period 1 (after training sessions and reach of a stable performance) in the L-DOPA + 5-HT₁ agonists-treated group and in a group of naïve rats, after administration of L-DOPA + 5-HT_{1A} + 5-HT_{1B} agonists or L-DOPA only, respectively. On the day of the test (day 5 of treatment period 1) L-DOPA + 5-HT₁ agonists-treated and naïve rats were tested twice in baseline condition and two more times 60 min after administration of the drugs. Values are reported as an average of the two sessions on and off drug.

Estimation of FosB-positive cell numbers in striatum

An additional group of animals was subjected to the treatment period 1, as above. Treatment with 5-HT₁ agonists resulted in a significant protection from dyskinesia in this group as well (mean \pm SEM at the last treatment: 421 \pm 130 versus 56 \pm 28 in L-DOPA only and L-DOPA + 5-HT₁ agonists, respectively). Animals were sacrificed 48 h after the last treatment, as previously described (Carlsson *et al.*, 2005), the brains were removed and

processed for FosB immunostaining. Briefly, the brains were cut into 16 μ m thickness on a cryostat (HM500 M, Microm, Walldorf, Germany) and the sections were mounted on plus-charged glass slides (Superfrost +; Electron Microscopy Sciences, PA, USA). Striatal sections were processed for FosB immunohistochemistry, while additional striatal and midbrain sections were processed for tyrosine hydroxylase (TH) immunohistochemistry to verify the dopaminergic lesion. Sections were fixed for 30 min in 10% formalin and further rinsed with 3 \times KPBS + 0.25% Triton-X (KPBS/T). After pre-incubation for 1 h with 5% NHS (normal horse serum) in KPBS/T, slides were incubated overnight in room temperature with the corresponding primary antibody: FosB (1:15 000; goat polyclonal IgG; SC-48X; Santa Cruz, CA, USA) or TH (1:2000; mouse IgG; Chemicon, Millipore, USA, MAB 318). This was followed by 1 h incubation with the corresponding biotinylated secondary antibody (1:250, horse- α -goat, BA9500 or horse- α -mouse, BA2001; Vector Laboratories, Burlingame, CA, USA). After this incubation the slides were further incubated for 1 h in avidin-biotin-peroxidase solution (ABC Elite; Vector Laboratories) and visualized using the chromogen 3',3'-diaminobenzidine and 0.01% H₂O₂. Finally, the sections were dehydrated in ascending alcohol solution, cleared in xylene and coverslipped with Depex.

Two high-resolution images were captured for the FosB quantification, corresponding to +0.7 mm and -0.3 mm from bregma, using a Scanscope GL system with imagescope v8.2 software. The images were then imported into Canvas software and positive cells were marked in the whole striatum to give the total number of cells per section, as well as number of cells restricted to the lateral part of the striatum, which was defined as one-third of the length of the striatum in each section. Values are expressed as total number of positive cells in the two sections considered.

Western blot analysis of NMDA composition in the post-synaptic density

To investigate NMDA receptor composition at the level of the post-synaptic density (PSD), an additional group of animals was subjected to the treatment period 1, as above. Treatment with 5-HT₁ agonists resulted in a significant protection from dyskinesia in this group as well (mean \pm SEM at the last treatment: 607 \pm 23 versus 27 \pm 19 in L-DOPA only and L-DOPA + 5-HT₁ agonists, respectively). After the treatment period 1, animals were sacrificed 1 h after the last treatment, and striata analysed for NMDA receptor composition and distribution by western blotting after sequential centrifugation to separate different cellular compartments.

Subcellular fractionation was performed as reported previously with minor modifications (Gardoni *et al.*, 2001). Striata were homogenized in 0.32 M ice-cold sucrose containing the following (in mM): 1 HEPES, 1 MgCl₂, 1 EDTA, 1 NaHCO₃ and 0.1 PMSF, at pH 7.4, in the presence of a complete set of protease inhibitors (Complete; Roche Diagnostics, Basel, Switzerland) and phosphatases inhibitors (Sigma, St Louis, MO, USA). The homogenized tissue was centrifuged at 1000g for 10 min. The resulting supernatant (S1) was centrifuged at 13 000g for 15 min to obtain a crude membrane fraction (P2 fraction). The pellet was resuspended in 1 mM HEPES plus CompleteTM in a glass-glass potter and centrifuged at 100 000g for 1 h. The pellet (P3) was resuspended in buffer containing 75 mM KCl and 1% Triton X-100 and centrifuged at 100 000g for 1 h. The supernatant was stored and referred as Triton X-100-soluble fraction (S4). The final pellet (P4)

was homogenized in a glass–glass potter in 20 mM HEPES. Then, an equal volume of glycerol was added, and this fraction, referred as Triton X-100-insoluble fraction (TIF), was stored at -80°C until processing. TIF was used instead of the classical PSD because the amount of the starting material was very limited. The protein composition of this preparation was, however, carefully tested for the absence of pre-synaptic markers (i.e. synaptophysin) (Gardoni *et al.*, 2001). Similar protein yields were obtained in TIF purified from striata of all experimental groups, and the same amount of TIF protein was applied to SDS–PAGE and electroblotted for all samples. Nitrocellulose papers were blocked with 10% albumin in Tris-buffered saline (TBS) and then incubated for 2 h at room temperature with the primary antibodies: NR2A (diluted 1:1000), NR2B (diluted 1:1000), PSD-95 (diluted 1:2000), SAP97 (diluted 1:1000), in 3% albumin in TBS. After extensive rinsing in TBS/0.1% Tween-20, the nitrocellulose papers were then incubated with horseradish peroxidase-conjugated secondary antibodies [goat anti-rabbit for poly-clonal antibodies, diluted 1:10 000 (Pierce, Rockford, IL, USA); goat anti-mouse for monoclonal antibodies, diluted 1:20 000 (Pierce)], and then the antigen–antibody complex was revealed by enhanced chemiluminescence (ECL; Amersham Biosciences, Little Chalfont, UK).

Statistical analysis

Group comparisons were performed for the rat experiments using Mann–Whitney for analysis of dyskinesias and stepping test, and Kruskal–Wallis followed by Mann–Whitney for locomotor activity. One-way analysis of variance (ANOVA) was used for analysis of FosB and NR2B quantifications, followed by Bonferroni *post hoc* test. The Friedman non-parametric repeated measures analysis of variance, followed by Dunn's multiple comparisons test, was used to analyse the primate data, except for the activity data, which were analysed by ANOVA followed by Bonferroni. Statistics analysis was performed using SigmaStat statistical software version 2.0 for the rat experiments and the STATA program (Intercooled

Stata 9.0, Stata Corporation, College Station, TX, USA) for the primate experiment.

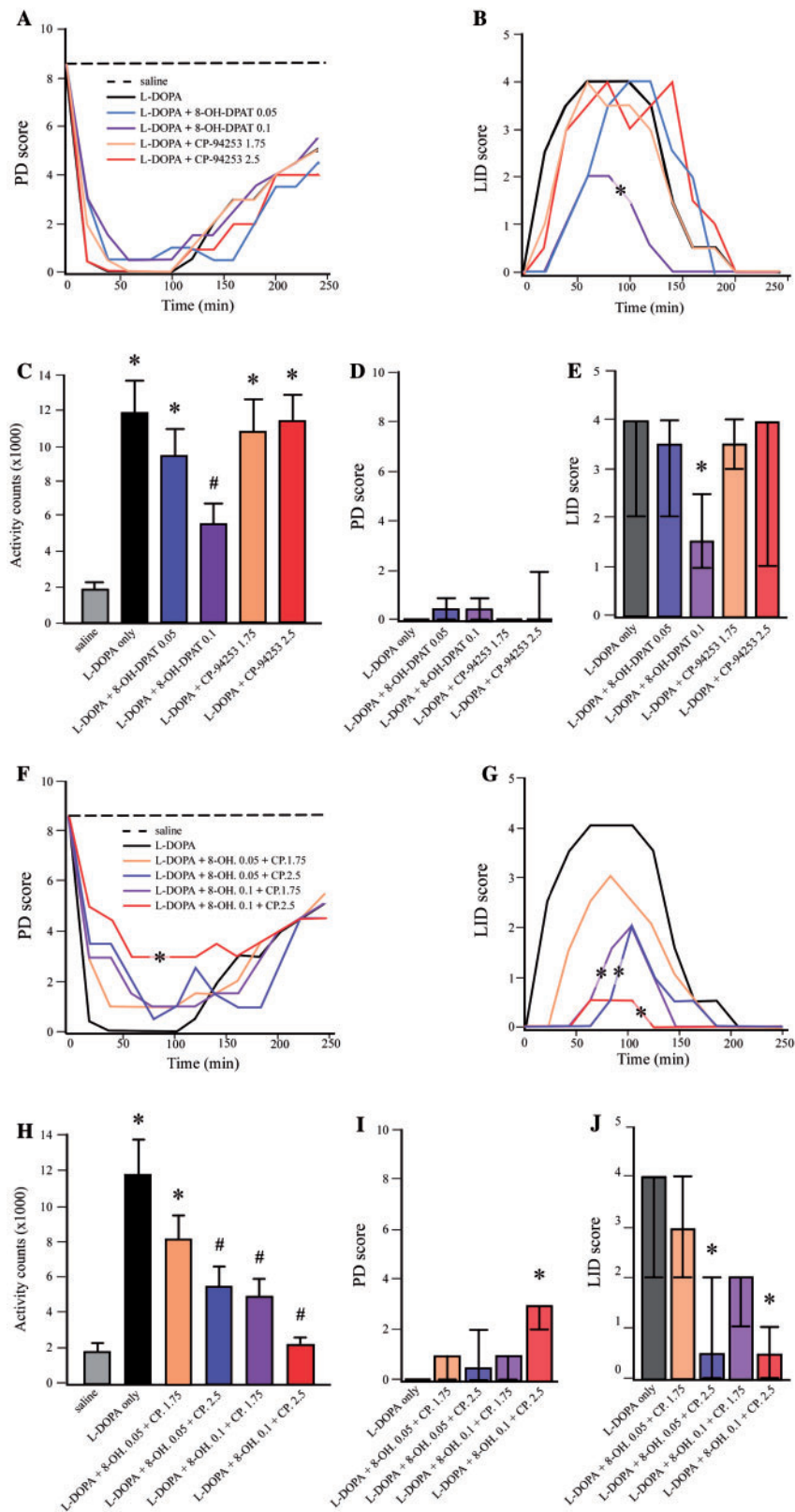
Results

Effect of serotonin receptor agonists on L-DOPA-induced dyskinesia in MPTP-treated macaques

To date, the MPTP-treated macaque represents the gold standard model of Parkinson's disease and shares several features with the disease in humans. Whether the serotonergic system plays an important role in dyskinesia manifestations after L-DOPA administration in this model is still an open issue. To investigate this point, six dyskinetic L-DOPA-primed MPTP-treated macaques were employed in this experiment. Each animal was subjected to injection of L-DOPA (at the minimal dose producing the maximal anti-parkinsonian effect), alone or together with the 5-HT_{1A} and 5-HT_{1B} agonists, individually and in combination. Two doses of the agonists were tested. Dyskinesia and parkinsonism were evaluated according to a modified rating scale, as previously described, and motor activity was measured by an automated system (Bezard *et al.*, 2001b, 2003).

As shown in Fig. 1B, the 5-HT_{1A} agonist (\pm)-8-OH-DPAT at 0.05 mg/kg and 0.1 mg/kg dose produced a dose-dependent reduction of L-DOPA-induced dyskinesia, which was significant at the higher dose (about 70% reduction in the AUC). Importantly, this effect was not accompanied by any reduction in the anti-parkinsonian action of L-DOPA (Fig. 1A). The 5-HT_{1B} agonist CP-94253, in contrast, failed to reduce L-DOPA-induced dyskinesia at any of the doses

Fig. 1 5-HT_{1A} and 5-HT_{1B} agonists effect on L-DOPA-induced dyskinesia in the MPTP-treated macaque. **(A)** 5-HT_{1A} ((\pm)-8-OH-DPAT; 0.05 mg/kg and 0.1 mg/kg) and 5-HT_{1B} (CP-94253; 1.75 mg/kg and 2.5 mg/kg) agonists in combination with L-DOPA had no negative impact upon parkinsonian disability score (as for panels B, F and G, median scores are shown without range for readability purpose, $F_{(7,964)} = 7.964$; $P = 0.09$). **(B)** Only 0.1 mg/kg (\pm)-8-OH-DPAT improved the L-DOPA-induced dyskinesia (LID) score ($F_{(7,14.306)} = 14.306$; $P = 0.006$; *different from L-DOPA). **(C)** Locomotor activity induced by 0.1 mg/kg (\pm)-8-OH-DPAT was no longer different from the OFF situation (grey column). Data expressed as mean \pm SEM (ANOVA followed by Bonferroni, $F_{(5,35)} = 21.705$, $P < 0.0001$; *different from saline, #different from L-DOPA, L-DOPA + CP-94253 1.75, L-DOPA + CP-94253 2.5). Highlight at $t = 80$ min of the impact upon **(D)** parkinsonian disability score (median/range: 0/0–0 for L-DOPA; 0.5/0–1 for 8-OH-DPAT 0.05; 0.5/0–1 for 8-OH-DPAT 0.1; 0/0–0 for CP-94253 1.75; 0/0–2 for CP-94253 2.5; $F_{(7,7.57)} = 7.57$, $P = 0.11$) and **(E)** LID score (median/range: 4/2–4 for L-DOPA; 3.5/2–4 for 8-OH-DPAT 0.05; 1.5/1–2.5 for 8-OH-DPAT 0.1; 3.5/3–4 for CP-94253 1.75; 4/1–4 for CP-94253 2.5; $F_{(7,14.082)} = 14.082$, $P = 0.007$; *different from L-DOPA) showing that only 0.1 mg/kg (\pm)-8-OH-DPAT improved LID. **(F)** Effect of combination of 5-HT_{1A} ((\pm)-8-OH-DPAT) and 5-HT_{1B} (CP-94253) agonists (8-OH-DPAT 0.05 + CP-94253 1.75; 8-OH-DPAT 0.1 + CP-94253 1.75; 8-OH-DPAT 0.05 + CP-94253 2.5; 8-OH-DPAT 0.1 + CP-94253 2.5) together with L-DOPA upon parkinsonian disability score ($F_{(7,16.068)} = 16.068$, $P = 0.002$). Combination at the highest doses (8-OH-DPAT 0.1 + CP-94253 2.5) worsened the Parkinson's disease score indicating an anti-L-DOPA effect ($P < 0.05$; *different from L-DOPA). **(G)** While the lowest doses in combination (8-OH-DPAT 0.05 + CP-94253 1.75) had no anti-dyskinetic effect, all three others (8-OH-DPAT 0.1 + CP-94253 1.75; 8-OH-DPAT 0.05 + CP-94253 2.5; 8-OH-DPAT 0.1 + CP-94253 2.5) significantly improved dyskinesia ($F_{(7,22.256)} = 22.256$, $P = 0.0002$; *different from L-DOPA). **(H)** The three combinations 8-OH-DPAT 0.1 + CP-94253 1.75, 8-OH-DPAT 0.05 + CP-94253 2.5 and 8-OH-DPAT 0.1 + CP-94253 2.5 significantly reduced the number of automated counts. [$F_{(5,35)} = 19.685$, $P < 0.0001$; *different from saline, #different from L-DOPA]. **(I)** Highlight at $t = 80$ min of the impact upon parkinsonian disability score, further confirmed that only the combination at the highest doses (8-OH-DPAT 0.1 + CP-94253 2.5) worsened the Parkinson's disease score indicating an anti-L-DOPA effect ($P < 0.05$; *different from L-DOPA) (median/range: 0/0–0 for L-DOPA; 1/0–1 for 8-OH-DPAT 0.05 + CP-94253 1.75; 0.5/0–2 for 8-OH-DPAT 0.05 + CP-94253 2.5; 1/0–1 for 8-OH-DPAT 0.1 + CP-94253 1.75; 3/2–3 for 8-OH-DPAT 0.1 + CP-94253 2.5; $F_{(7,19.835)} = 19.835$, $P = 0.0005$). **(J)** In the same way, highlight at $t = 80$ min of the impact upon LID score (median/range: 4/2–4 for L-DOPA; 3/2–4 for 8-OH-DPAT 0.05 + CP-94253 1.75; 0.5/0–2 for 8-OH-DPAT 0.05 + CP-94253 2.5; 2/1–2 for 8-OH-DPAT 0.1 + CP-94253 1.75; 0.5/0–1 for 8-OH-DPAT 0.1 + CP-94253 2.5; $F_{(7,19.54)} = 19.54$, $P = 0.0006$) showed that only the two combinations involving the highest dose of the 5-HT_{1B} agonist (8-OH-DPAT 0.05 + CP-94253 2.5; 8-OH-DPAT 0.1 + CP-94253 2.5) improved LID ($P < 0.05$; *different from L-DOPA).



tested (1.75 mg/kg and 2.5 mg/kg). CP-94253, however, was able to potentiate the effect of (\pm)-8-OH-DPAT (Fig. 1G). In line with our previous report in rats, we observed a synergistic effect between the two drugs. This was evident at doses of 0.05 mg/kg (\pm)-8-OH-DPAT plus 2.5 mg/kg CP-94253, which individually produced no or only a minor effect. At these doses, combination of the agonists was able to reduce dyskinesia by up to 80% (in the AUC) without any significant worsening of the parkinsonian score compared to L-DOPA-only (Fig. 1F and G). At higher doses, combination of the agonists was able to produce a near-complete abolishment of dyskinesia (about 95% reduction in the AUC). This effect, however, was accompanied by a 70% increase in the Parkinson's disease score compared with L-DOPA only (corresponding to 28% reduction in the anti-parkinsonian efficacy of L-DOPA). Further confirming this effect is the reduction in L-DOPA-induced motor activity (Fig. 1C and H). One should, however, be careful in interpreting locomotor activity counts, as they encompass both the normal and abnormal movements (Bezard *et al.*, 2003; Gold *et al.*, 2007). A reduction of the hyperkinetic component of dyskinesia or of dyskinesia themselves would thus reduce the number of counts (Fig. 1C—0.1 mg/kg (\pm)-8-OH-DPAT; Fig. 1H—0.05 mg/kg (\pm)-8-OH-DPAT plus 2.5 mg/kg CP-94253 and 0.1 mg/kg (\pm)-8-OH-DPAT plus 1.75 mg/kg CP-94253) but a larger decrease (e.g. Fig. 1H—0.1 mg/kg (\pm)-8-OH-DPAT plus 2.5 mg/kg CP-94253) also reflects the reduction of the anti-parkinsonian efficacy of L-DOPA. For a detailed highlight at peak dose dyskinesia, the effect of the agonists on Parkinson's disease (Fig. 1D and I) and LID (Fig. 1E and J) scores are also reported at the 80 min observation time point.

Effect of chronic administration of 5-HT_{1A} and 5-HT_{1B} agonists on the development of L-DOPA-induced dyskinesia and therapeutic efficacy of L-DOPA

The optimal anti-dyskinetic compound would be given in a chronic or sub-chronic fashion. We therefore questioned whether prolonged administration of the serotonin agonists could prevent development of dyskinesia in naïve animals or provide a long-lasting control of the side effect of L-DOPA once dyskinesias have been established. Ethical, practical and financial reasons make such a study very difficult to be performed in monkeys and we therefore used the 6-OHDA-lesioned rat model of L-DOPA-induced AIMs, the rodent analogue of L-DOPA-induced dyskinesia (Cenci *et al.*, 1998).

Two groups of 6-OHDA-lesioned rats ($n=11$ per group) were subjected to either daily chronic treatment with L-DOPA at the 6 mg/kg dose (plus Benserazide 10 mg/kg), or to the same L-DOPA regimen plus a combination of 5-HT_{1A} and 5-HT_{1B} agonists. The doses of the agonists, (\pm)-8-OH-DPAT 0.05 mg/kg and CP-94253 1.0 mg/kg, were chosen based on our previous study (Carta *et al.*, 2007)

and was shown to acutely reduce L-DOPA-induced dyskinesia by up to 80% in MFB-lesioned rats. As illustrated in Fig. 2A, the control group, receiving only L-DOPA, showed a fast onset of AIMs and only two animals were non-dyskinetic after 2 weeks of treatment (data not shown). In the L-DOPA + 5-HT₁ agonists group, in contrast, only three animals developed some mild dyskinesia, which we defined here as low responders to the serotonin agonists treatment, while 8 out of 11 rats were completely free of dyskinesia (see values of 14th injection of treatment period 1 in Fig. 2C). Overall, the 5-HT₁ agonists-treated group was significantly protected from dyskinesia compared with the L-DOPA only-treated group at the end of treatment period 1 (Fig. 2A).

After the first 2 weeks of treatment (treatment period 1), animals were challenged with a sub-threshold dose of apomorphine (0.02 mg/kg), in absence of any agonist treatment, and tested for dyskinesia to rule out possible masking rather than protective effect of the 5-HT₁ agonists on L-DOPA-induced dyskinesia. This experiment revealed a lower degree of DA receptor sensitization in the animals previously treated with L-DOPA + 5-HT₁ agonists compared to the control group (Fig. 2B). Forty-eight hours after the apomorphine challenge, treatments were switched so that the animals previously receiving only L-DOPA now received L-DOPA plus agonists, and the animals previously receiving the combined treatment were given L-DOPA alone (treatment period 2). As shown in Fig. 2C, animals now on the combined treatment showed a significant reduction in the abnormal movements for the entire experimental period, with a maximal 90% reduction at the 4th administration. During the second week, however, we observed gradual partial loss of efficacy, likely due to the well-known phenomenon of internalization of the pre-synaptic 5-HT receptors upon repeated exposure to agonists (El Mansari *et al.*, 2005; Kuan *et al.*, 2008). Nevertheless, at the end of treatment period 2, animals appeared still relatively protected, since the dyskinesia score was significantly different than the level seen in the same animals at the end of treatment period 1. Interestingly, the L-DOPA-treated animals, which were high responders to the agonists during treatment period 1, appeared to be protected from a full development of dyskinesia when they received L-DOPA only during treatment period 2, while the three low responders in the treatment period 1 developed high dyskinesia score (Fig. 2C).

After treatment period 2, the animals receiving L-DOPA plus agonists were treated with L-DOPA only and then given a 3-week washout period and tested again with L-DOPA and L-DOPA plus agonists ($n=9$, two animals that were non-dyskinetic during period 1 were not included). As shown in Fig. 2D, this drug-free period was sufficient to allow a complete recovery from the receptor desensitization induced by the chronic agonists treatment. The result of this test suggests that autoreceptor desensitization is a reversible phenomenon upon discontinuation of the drug treatment.

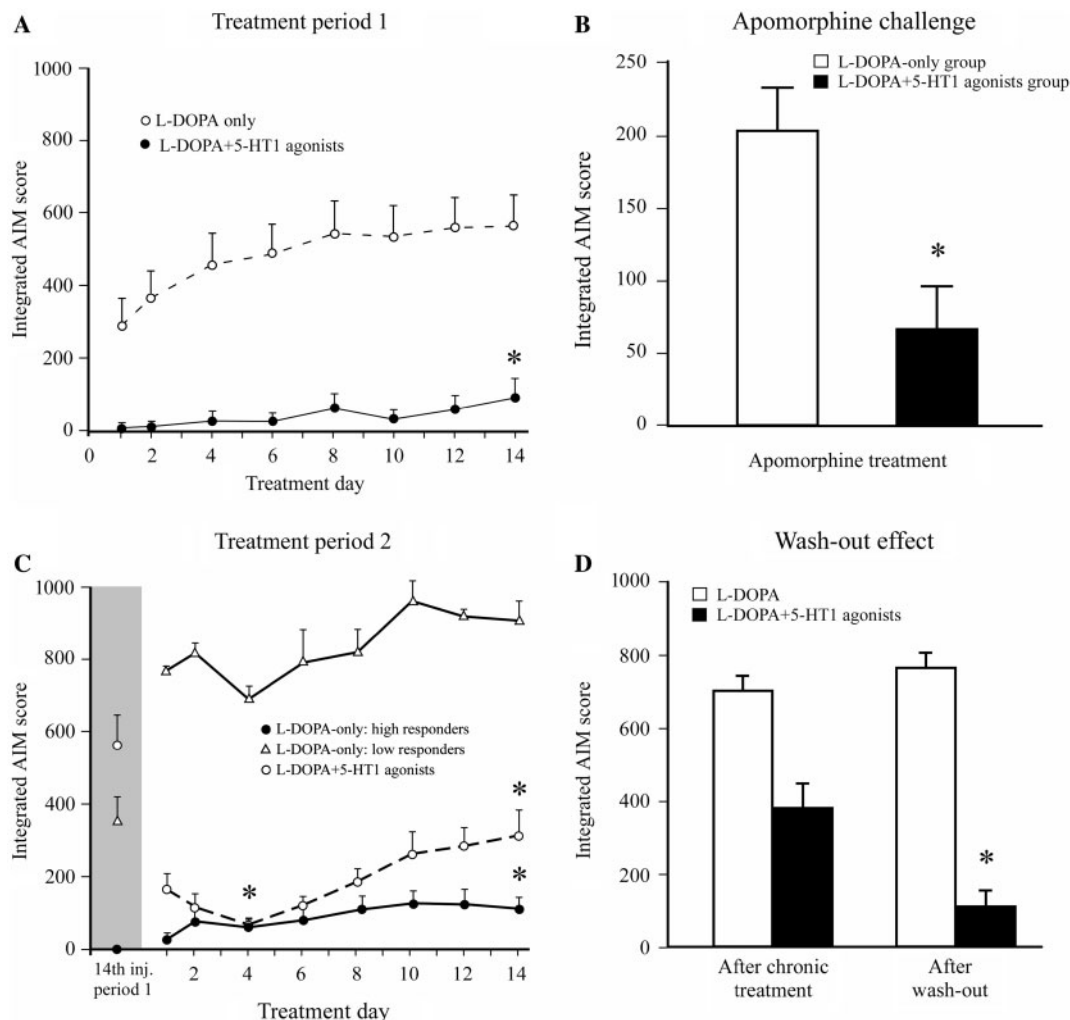


Fig. 2 Chronic effect of 5-HT_{1A} + 5-HT_{1B} agonists on L-DOPA-induced dyskinesia in 6-OHDA-lesioned rats. Co-administration of 5-HT_{1A} + 5-HT_{1B} agonists with L-DOPA resulted in a significant inhibition of the development of dyskinesia (reported as the sum of limb, axial and orolingual movements) at the end of the treatment period I [(A); mean \pm SEM at the 14th administration: 557 ± 84 versus 96 ± 52 in the L-DOPA only and L-DOPA + 5-HT_{1A} + 5-HT_{1B} agonists-treated groups, respectively; $P = 0.001$ in the Mann–Whitney test; $n = 11$ per group]. Three animals, however, appeared to be low responders to the agonists treatment and developed some degree of dyskinesia, while the other eight rats were completely free of dyskinesia (see values of the 14th injection in the treatment period I in (C)). The apomorphine (0.02 mg/kg, s.c.)-induced dyskinesia test revealed a significant lower sensitization state of the DA receptors in the L-DOPA + 5-HT_{1A} + 5-HT_{1B} agonists-treated group [(B); mean \pm SEM: 203 ± 31 versus 66 ± 20 in the L-DOPA only and L-DOPA + 5-HT_{1A} + 5-HT_{1B} agonists-treated groups, respectively; $P = 0.02$ in the Mann–Whitney test; $n = 11$ per group). Treatments were then switched during treatment period 2 (C). The results showed a significant reduction of dyskinesia in the L-DOPA + 5-HT_{1A} + 5-HT_{1B} agonists-treated group compared with the last score at the end of the treatment period I, which was maximal (90% reduction) at the 4th administration (mean \pm SEM: 56 ± 29 versus 557 ± 84 in the L-DOPA + 5-HT_{1A} + 5-HT_{1B} agonists at the 4th administration of treatment period 2 and L-DOPA only at the 14th administration of treatment period I, respectively; $P = 0.009$ in the Mann–Whitney test; $n = 11$ for L-DOPA + 5-HT_{1A} + 5-HT_{1B} agonists group) and still present at the last treatment (mean \pm SEM: 308 ± 72 versus 557 ± 84 in the L-DOPA + 5-HT_{1A} + 5-HT_{1B} agonists at the 14th administration of treatment period 2 and L-DOPA only at the 14th administration of treatment period I, respectively; $P = 0.039$ in the Mann–Whitney test). In addition, only the three low responders to the agonists of the treatment period I developed high dyskinetic score, while a significant protection was observed in the L-DOPA-treated group previously responsive to the agonists treatment compared to the L-DOPA-treated group at the end of the treatment period I [(C); mean \pm SEM: 109 ± 33 versus 557 ± 84 in the L-DOPA only at the 14th administration of treatment period 2 and L-DOPA only in the 14th administration of treatment period I, respectively; $P = 0.008$ in the Mann–Whitney test; $n = 8$ for high responders, $n = 3$ for low responders). After a 3-week washout 5-HT₁ agonists treatment regained a full efficacy [(D); mean \pm SEM: 377 ± 69 versus 111 ± 42 under tolerance and after wash-out, respectively; $P = 0.007$ in the Mann–Whitney test; $n = 9$).

In agreement with our previous report (Carta *et al.*, 2007), all 6-OHDA-lesioned animals employed in these experiments had near-complete depletion of striatal DA (>99% compared to the intact side) as measured by HPLC,

while serotonin tissue levels were unaffected by 6-OHDA lesions, or administration of the agonists (data not shown).

In order to investigate the impact of the serotonin agonist treatment on the therapeutic effect of L-DOPA,

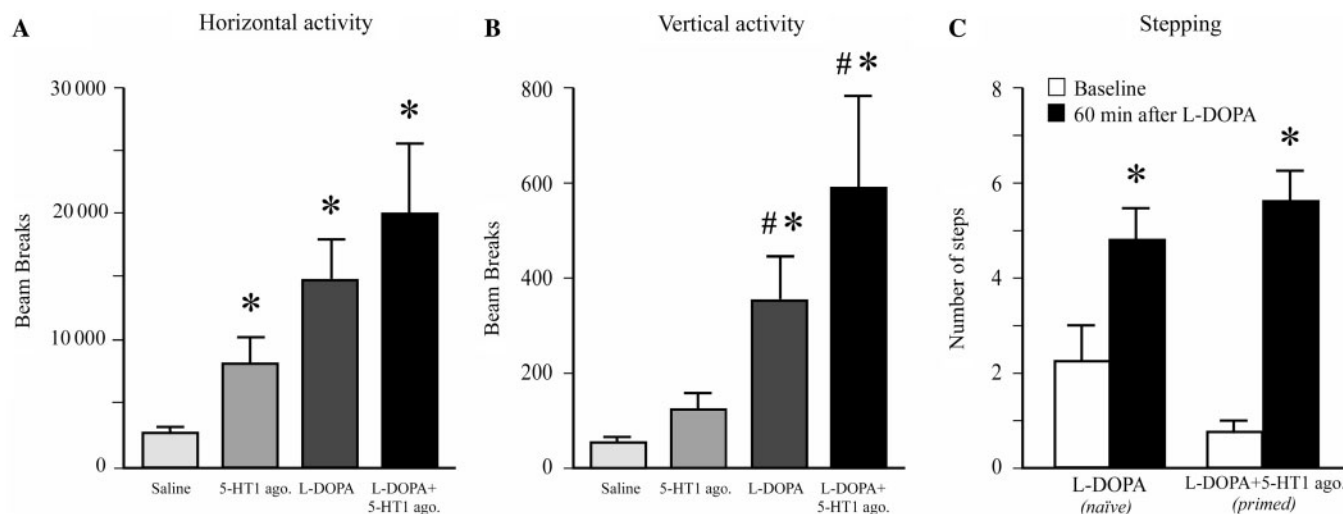


Fig. 3 Effect of the 5-HT_{1A/1B} agonists on the therapeutic effect of L-DOPA in 6-OHDA-lesioned rats. Co-administration of the agonists with L-DOPA did not reduce the ability of L-DOPA to improve horizontal (mean \pm SEM: 19 887 \pm 5524, 14 576 \pm 3255, 7967 \pm 2150 and 2500 \pm 313 for L-DOPA + 5-HT_{1A} + 5-HT_{1B} agonists, L-DOPA only, agonists only and saline, respectively; $P < 0.05$ for L-DOPA + 5-HT_{1A} + 5-HT_{1B} agonists versus saline, L-DOPA only versus saline and 5-HT_{1A} + 5-HT_{1B} agonists only versus saline in the Kruskal–Wallis followed by Mann–Whitney test; *different from saline) and vertical activity (mean \pm SEM: 586 \pm 195, 351 \pm 94, 121 \pm 33 and 52 \pm 12 for L-DOPA + 5-HT_{1A} + 5-HT_{1B} agonists, L-DOPA only, 5-HT_{1A} + 5-HT_{1B} agonists only and saline, respectively; $P < 0.05$ for L-DOPA + 5-HT_{1A} + 5-HT_{1B} agonists versus saline, L-DOPA only versus saline, L-DOPA only versus 5-HT_{1A} + 5-HT_{1B} agonists only in the Kruskal–Wallis followed by Mann–Whitney test; *different from saline, # = different from agonists). In addition, performance of the animals in the stepping test was tested after L-DOPA administered with or without the 5-HT_{1A} + 5-HT_{1B} agonists (C). The results show that co-administration of the 5-HT_{1A} + 5-HT_{1B} agonists did not reduce the ability of L-DOPA to improve the number of steps of the parkinsonian forelimb (mean \pm SEM: 2.3 \pm 0.8 versus 4.8 \pm 0.7 in baseline condition and 60 min after L-DOPA, respectively in the naïve group, $P = 0.017$ in the Mann–Whitney test; 0.7 \pm 0.2 versus 5.7 \pm 1.0 in baseline condition and 60 min after L-DOPA, respectively in the L-DOPA + 5-HT_{1A} + 5-HT_{1B} agonists group, $P < 0.001$ in the Mann–Whitney test; *different from baseline).

we tested motor activity during treatment period 1. Neither horizontal nor vertical activities were affected by co-administration of the agonists with L-DOPA compared with L-DOPA only (Fig. 3A and 3B). In addition, L-DOPA-induced improvement in the forelimb used in the stepping test was also unaffected by the 5-HT₁ agonist treatment (Fig. 3C). These results are in line with our previous report (Carta *et al.*, 2007) showing preservation of the therapeutic effect of L-DOPA in the cylinder test of partial 6-OHDA-lesioned rats after co-administration with the same 5-HT agonists.

Effect of the chronic administration of the 5-HT_{1A} + 5-HT_{1B} agonists on the number of FosB-positive cells in striatum

Swings in striatal DA release and consequent pulsatile stimulation of striatal DA receptors generated by intermittent L-DOPA administration have been suggested to be responsible for a cascade of events in the post-synaptic striatal neurons, which eventually result in changes in gene expression and appearance of dyskinesia. Thus, induction of FosB, has been proposed as a marker of the cellular changes underlying the side effects of L-DOPA (Andersson *et al.*, 1999). To investigate whether co-treatment with the serotonin receptor agonists may affect L-DOPA-induced up-regulation of this marker, one additional group of

6-OHDA-lesioned rats was included and subjected to the same treatment as above. After treatment period 1, animals were sacrificed, brains removed and sectioned for FosB immunostaining. A significant correlation between AIM score and number of FosB-positive cells was found (coefficient of correlation, $r = 0.72$ and 0.76 for the total striatum and the lateral part, respectively). As shown in Fig. 4, co-administration of 5-HT_{1A} + 5-HT_{1B} agonists with L-DOPA resulted in a significant reduction of FosB-positive cells compared with the L-DOPA-treated dyskinetic animals, particularly in the lateral part of the striatum, which has been suggested to play an important role in the induction of dyskinesia (Cenci *et al.*, 1998; Andersson *et al.*, 1999). Interestingly, all animals in the dyskinetic L-DOPA-treated group had higher numbers of FosB-positive cells than any of the ones in the L-DOPA + 5-HT₁ agonist-treated group, with an average reduction of 48% and 61% when considering the entire striatum or the lateral part, respectively. As expected, a sub-group of L-DOPA-treated rats did not develop dyskinesia. In these animals, the number of FosB-positive cells was similar to that found in the drug naïve group, except in one case, where the number of FosB-positive cells was similar to the dyskinetic animals. This animal, however, in contrast to the other non-dyskinetic animals, had a high-rotational response to the administration of L-DOPA, which may explain FosB induction. It is worth to note that FosB-positive nuclei in the dyskinetic

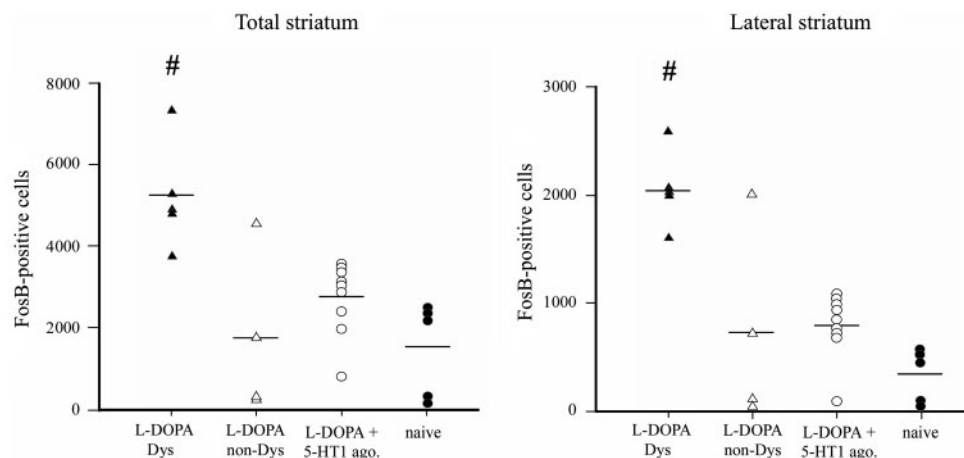


Fig. 4 Effect of the chronic administration of the 5-HT_{1A} + 5-HT_{1B} agonists on the number of FosB-positive cells in striatum. Significant correlation between AIM score and number of FosB-positive cells was found in animals exposed to the treatment period I (coefficient of correlation r was 0.72 and 0.76 for the total striatum and the lateral part, respectively; two brain sections were considered and all striatal FosB-positive cells were counted). Co-administration of the 5-HT_{1A} + 5-HT_{1B} agonists with L-DOPA ($n = 9$) resulted in a significant reduction of FosB-positive cells compared with the L-DOPA-treated dyskinetic animals ($n = 9$), when considering both the entire striatum (mean \pm SEM: 5258 ± 569 , 1783 ± 1001 , 2749 ± 289 and 1528 ± 517 in the dyskinetic L-DOPA, non-dyskinetic L-DOPA, L-DOPA + 5-HT_{1A} + 5-HT_{1B} agonists treated and naïve groups, respectively; ANOVA followed by Bonferroni test: $F_{(3,19)} = 8.992$; $P < 0.001$ for dyskinetic L-DOPA versus naïve, $P = 0.003$ for dyskinetic L-DOPA versus non-dyskinetic L-DOPA, $P = 0.01$ for dyskinetic L-DOPA versus L-DOPA + 5-HT_{1A} + 5-HT_{1B} agonists) and the lateral part (mean \pm SEM: 2048 ± 157 , 712 ± 458 , 790 ± 99 and 330 ± 112 in the dyskinetic L-DOPA, non-dyskinetic L-DOPA, L-DOPA + 5-HT_{1A} + 5-HT_{1B} agonists treated and naïve groups, respectively; ANOVA followed by Bonferroni test: $F_{(3,19)} = 13.418$; $P < 0.001$ for dyskinetic L-DOPA versus naïve, $P = 0.002$ for dyskinetic L-DOPA versus non-dyskinetic L-DOPA, $P < 0.001$ for dyskinetic L-DOPA versus L-DOPA + 5-HT_{1A} + 5-HT_{1B} agonists) (# different from non-dyskinetic L-DOPA, L-DOPA + 5-HT_{1A} + 5-HT_{1B} agonists and naïve).

group appeared also to be more intensely stained than in the agonist-treated group, suggesting a higher amount of FosB expression per cell (data not shown).

Alteration in NMDA receptor subunits composition

Physiological stimulation of the DA receptors is known to be important for regulation of trafficking of striatal NMDA receptors (Dunah and Standaert, 2001). Accordingly, DA denervation and un-physiological, pulsatile stimulation of striatal DA receptors, induced by intermittent L-DOPA administration, have been reported to alter synaptic levels of NMDA receptor subunits and associated PSD proteins (Fiorentini *et al.*, 2006; Gardoni *et al.*, 2006). To investigate whether modulation of pre-synaptic DA release by means of 5-HT₁ agonists may affect synaptic levels of NMDA receptor subunits and PSD proteins, an additional group of animals was subjected to the treatment period I and western blot analysis was carried out in different cellular fractions. Consistent with the earlier studies, we observed in the 6-OHDA-lesioned rats a down-regulation of the NR2B subunit content in the TIF, which is enriched in PSD proteins, in absence of a parallel alteration of the NR2A subunit in the same samples (Fig. 5A and B). Induction of dyskinesia by chronic, daily treatment with L-DOPA was accompanied by a significant up-regulation of the NR2B subunit in the same cellular compartment, and co-

administration with the 5-HT₁ agonists resulted in a further significant up-regulation of these subunits in the non-dyskinetic (agonist-treated) rats compared with all other experimental groups. In line with previous studies (Fiorentini *et al.*, 2006; Gardoni *et al.*, 2006), this alteration is interpreted to reflect a redistribution of the subunits between different cellular compartments (synaptic versus extra-synaptic), since the total tissue content was not affected (data not shown). The content of the NR2A subunit in the TIF, in contrast, was similar in all experimental groups (Fig. 5A). Furthermore, no modification of PSD-95 and SAP97 members of PSD-MAGUKs proteins in the TIF was induced by any of the treatments (Fig. 5A).

Discussion

The present results show that 5-HT_{1A} and 5-HT_{1B} agonists have a synergistic effect in suppressing L-DOPA-induced dyskinesia in the MPTP-treated macaque. Sub-threshold doses of the drugs, which individually produced very little, insignificant effects, were able to reduce dyskinesia severity by up to 80% when administered in combination, without affecting the anti-parkinsonian properties of L-DOPA. In addition, we show that daily co-administration of the 5-HT_{1A} and 5-HT_{1B} agonists can prevent development of L-DOPA-induced abnormal involuntary movements and associated striatal alterations, such as striatal

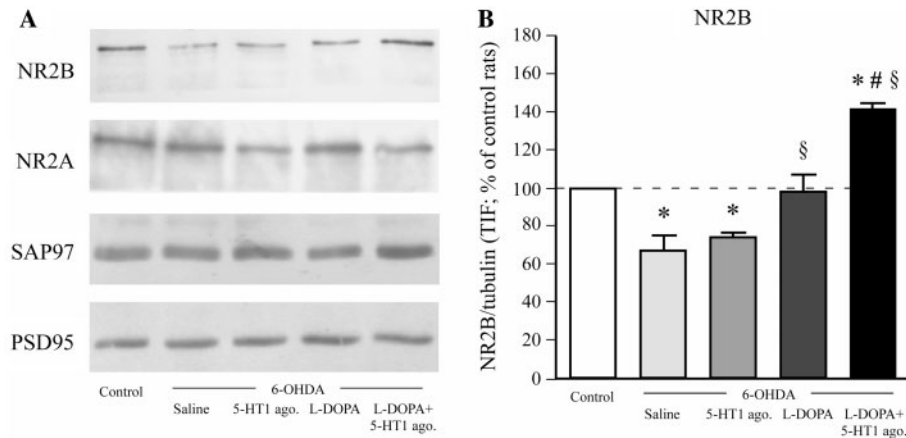


Fig. 5 Effect of the chronic administration of the 5-HT_{1A} + 5-HT_{1B} agonists on the NMDA receptor subunits composition. Animals were subjected to the treatment period I and sacrificed for quantification of the NMDA receptor subunits NR2B and NR2A, as well as for quantification of the PSD proteins PSD95 and SAP97. 6-OHDA lesions induced down-regulation of the NR2B subunit content in the TIF. Chronic daily treatment with L-DOPA was accompanied by a significant up-regulation of the NR2B subunit in the same cellular compartment. In addition, serotonin agonists (with L-DOPA) treatment resulted in a further significant up-regulation of these subunits in the non-dyskinetic (agonist-treated) rats compared with all other experimental groups (mean \pm SEM, percentage respect to intact control sides: 67 ± 18 , 74 ± 6 , 98 ± 20 and 140 ± 9 for 6-OHDA/naïve ($n = 5$), 6-OHDA/5-HT_{1A} + 5-HT_{1B} agonists ($n = 6$), 6-OHDA/L-DOPA ($n = 6$) and 6-OHDA/L-DOPA + 5-HT_{1A} + 5-HT_{1B} agonists ($n = 6$), respectively; ANOVA followed by Bonferroni: $P = 0.017$ for 6-OHDA versus control, $P = 0.014$ for 6-OHDA/5-HT_{1A} + 5-HT_{1B} agonists versus control, $P = 0.009$ for L-DOPA + 5-HT_{1A} + 5-HT_{1B} agonists versus L-DOPA, $P = 0.029$ for L-DOPA versus 6-OHDA and $P < 0.001$ for L-DOPA + 5-HT_{1A} + 5-HT_{1B} agonists versus 6-OHDA). This alteration seems to reflect a redistribution of the NR2B receptor between the cellular compartments since no change in the total homogenate was detected (mean \pm SEM, percentage respect to intact control sides: 94 ± 38 , 104 ± 20 , 92 ± 23 and 88 ± 16 for 6-OHDA/naïve, 6-OHDA/5-HT_{1A} + 5-HT_{1B} agonists, 6-OHDA/L-DOPA and 6-OHDA/L-DOPA + 5-HT_{1A} + 5-HT_{1B} agonists, respectively). No modification of NR2A, PSD95 and SAP97 in the TIF was induced by any experimental treatment performed, suggesting that alteration was specific for NR2B. (*different from control; #different from L-DOPA; §different from 6-OHDA/saline).

FosB induction, without reducing the therapeutic effect of L-DOPA in the rat Parkinson's disease model. These data extend our previous observations pointing to the serotonin system as a critical element in L-DOPA-induced dyskinesia (Carta *et al.*, 2007) and highlight the potential clinical application of 5-HT_{1A} and 5-HT_{1B} for the management of L-DOPA-induced dyskinesia in Parkinson's disease patients.

Effect of 5HT_{1A/1B} treatment in 6-OHDA-lesioned rats

Physiological regulation of extracellular DA levels is normally maintained by the presence of the D₂ auto-receptor and DA transporter on the pre-synaptic DA terminals. These elements represent a feedback controlled mechanism able to fine tune the level of DA in the synaptic cleft (Venton *et al.*, 2003; Cragg and Rice, 2004). We have recently suggested (Carta *et al.*, 2007) that lack of this feedback control mechanism of DA release from the serotonin terminals is responsible for the high swings in synaptic DA after L-DOPA administration, which have been associated with un-physiological, pulsatile stimulation of the DA receptors in the striatal neurons and appearance of dyskinesia (Chase, 1998; de la Fuente-Fernandez *et al.*, 2004a, b). Indeed, in the present study, in agreement with the suggested mechanism of action (i.e. modulation of pre-

synaptic release of DA from the serotonin terminals) administration of 5-HT₁ agonists resulted in a significant protection from development of dyskinesia and reduced up-regulation of FosB in striatal target neurons (Fig. 2A and 4), a well-established marker of L-DOPA-induced DA receptor sensitization (Andersson *et al.*, 1999). In addition, physiological stimulation of striatal DA receptor is required for normal trafficking of NMDA receptor subunits in striatal target neurons (Dunah and Standaert, 2001). Accordingly, DA denervation affects synaptic level of NR2 subunits and their interaction with PSD proteins (Gardoni *et al.*, 2006). In line with previous observations (Gardoni *et al.*, 2006), we found here an up-regulation of the NR2B subunits in the triton-insoluble fraction in the non-dyskinetic, agonists-treated rats (Fig. 5). In contrast to the previous report, however, a significant increase of the NR2B subunit was also found in the L-DOPA-treated dyskinetic rats compared with lesioned, saline-treated animals. Different experimental conditions, such as the L-DOPA dose (6 mg/kg versus 10 mg/kg), the gender employed in these studies (female versus male) and different lesion protocols (MFB versus nigral 6-OHDA injections) might account for these discrepancies. Nevertheless, up-regulation of the NR2B subunit at the post-synaptic membrane compared with the intact control side of the brain appears to be a characteristic feature of the non-dyskinetic animals. Given the suggested role of the striatal NMDA receptors

in the induction and maintenance of dyskinesias, re-distribution of the striatal NMDA receptor NR2B subunits between synaptic and extra-synaptic compartments might play a role in the prevention of dyskinesia upon 5-HT₁ agonist administration. The results highlight the interplay between striatal pre- and post-synaptic alterations in L-DOPA-induced dyskinesia and confirm previous reports pointing to the NR2B subunits as a critical receptor in dyskinesia (Hadj Tahar *et al.*, 2004; Gardoni *et al.*, 2006).

It is worth noting that chronic administration of the 5-HT_{1A} and 5-HT_{1B} agonists from the very first dose of L-DOPA appears to provide a long-term protection against development of dyskinesia. In fact, animals treated with the agonists during the treatment period 1, and highly responsive to the treatment, appeared to be resistant to the induction of dyskinesia when they received L-DOPA during the treatment period 2 (Fig. 2A and C). Further investigations would be required to investigate the molecular basis of this event.

Recently, Eskow *et al.* (2007) have found protection from development of dyskinesia in the same animal model by chronic administration of the partial 5-HT_{1A} agonist buspirone. 5-HT_{1A} agonists, however, are known to act not only on pre-synaptic serotonin receptors, but also on post-synaptic receptors located in other brain areas, such as the pre-frontal cortex (Yamada *et al.*, 1988; Ceci *et al.*, 1994; Knobelmann *et al.*, 2000). Activation of these receptors are known to dampen the activity of the glutamatergic neurons projecting to the striatum. Reduced glutamatergic activity is thus an alternative mechanism that may account for the anti-dyskinetic effect found by these authors (Antonelli *et al.*, 2005; Mignon and Wolf, 2005; Carta *et al.*, 2007). Activation of post-synaptic 5-HT_{1B} receptors has also been suggested to produce anti-dyskinetic effect by dampening release of GABA in striatum and substantia nigra pars reticulata (Zhang *et al.*, 2008).

Activation of the post-synaptic 5-HT₁ receptors has been linked to induction of serotonin syndrome both in rats and non-human primates, an effect that may seriously compromise motor function (Goodwin *et al.*, 1986; Smith and Peroutka, 1986; Yamada *et al.*, 1988). In our experimental conditions, doses of the agonists were chosen, based on our previous work (Carta *et al.*, 2007), in order to target the pre-synaptic serotonin receptors. Indeed, the 5-HT₁ agonists were unable, at the present doses, to produce reduction of apomorphine-induced dyskinesia in a group of MFB-lesioned dyskinetic rats (data not shown). Thus, at low doses, the efficacy of the 5-HT₁ agonists in counter-acting L-DOPA-induced dyskinesia is suggested to be due to the dampening effect on the release of DA from the serotonin neurons rather than to reduced glutamatergic input into the striatum (Carta *et al.*, 2007). Importantly, administration of the agonists did not appear to reduce the therapeutic effect of L-DOPA, as assessed in tests of general motor activity and from the performance in the stepping test (Fig. 3).

Effect of 5HT_{1A/1B} treatment in MPTP-treated macaques

MPTP-treated monkeys provide the best available animal model of Parkinson's disease, which shares several features with the human disease. In the present study, we took advantage of this model in order to investigate the validity of the serotonin agonist approach in primates. Interestingly, we report here a similar synergistic effect between the two agonists as the one previously shown in the rat model. Thus, combining doses, which individually produced no effect [(±)-8-OH-DPAT 0.05 mg/kg and CP-94253 2.5 mg/kg], we could observe a marked decrease in the abnormal movements (by about 80% reduction in the AUC) (Fig. 1G). Importantly, this effect was obtained without any significant worsening of the therapeutic effect of L-DOPA (Fig. 1F). Combination of the same dose of CP-94253 (2.5 mg/kg) with a higher dose of (±)-8-OH-DPAT (0.1 mg/kg) produced a near-complete suppression of L-DOPA-induced dyskinesia (about 95% reduction in the AUC). However, this combination produced 28% reduction in the therapeutic efficacy of L-DOPA, although a significant therapeutic value of L-DOPA was still retained relative to the baseline impairment (Fig. 1F).

Previous primate studies have given conflicting results on the possibility of reducing the release from the serotonin neurons by 5-HT_{1A} or 5-HT_{1B} receptor agonists without interfering with the efficacy of L-DOPA medication. Indeed, a partial reduction of L-DOPA-induced dyskinesia in MPTP-lesioned marmosets treated with (+)-8-OH-DPAT has been reported, but this effect was accompanied by worsening of parkinsonism (Iravani *et al.*, 2006). Others, however, have shown that the partial 5-HT_{1A} agonist Sarizotan can reduce L-DOPA-induced dyskinesia in MPTP-treated macaques without significantly deteriorating the efficacy of L-DOPA (Bibbiani *et al.*, 2001). The different species employed and/or differences in the magnitude of MPTP-induced DA depletion might account for the discrepancies between these studies. Indeed, preservation of a residual DA innervation could have profound consequences on the therapeutic efficacy of L-DOPA when DA release from serotonin neurons is silenced. Spared DA terminals are likely to serve as a buffer for L-DOPA-derived DA after intermittent L-DOPA administration, and mediate a physiological, feedback-regulated release of DA.

The decreased anti-parkinsonian effect of L-DOPA reported by Iravani and colleagues after (+)-8-OH-DPAT treatment could, at least in part, also be due to the high dose of 5-HT_{1A} agonist needed to obtain a significant anti-dyskinetic effect, when given alone. Indeed, these authors observed signs of serotonin syndrome after administration of (+)-8-OH-DPAT in MPTP-lesioned marmosets, which may have contributed to the appearance of hypokinesia and dystonia in their animals as we previously observed in MPTP-treated animals when using high doses of comparable drugs (Bezard *et al.*, 2006). In the same study,

the authors observed a similar reduction in the anti-parkinsonian effect of the D₂/D₃ direct agonist pramipexole after co-administration with (+)-8-OH-DPAT, an observation which may suggest a post-synaptic-related side effect due to the high dose of the drug. Different properties of the drugs employed might also account for the discrepancies between our results and those reported by Iravani and colleagues. Indeed, (+)-8-OH-DPAT was notably less potent than (±)-8-OH-DPAT in counter-acting dyskinesia in our 6-OHDA-lesioned rats (data not shown).

A 5-HT_{1B} receptor agonist has also been tested individually in MPTP-treated dyskinetic marmosets (Jackson *et al.*, 2004). However, the anti-dyskinetic effect of the compound employed resulted in a diminished therapeutic effect of L-DOPA. We did not observe any detrimental effect of CP-94253 individually at the doses used here in the MPTP-treated macaques, although these doses neither resulted in anti-dyskinetic effect. Higher doses may produce different effects. However, this goes beyond the purpose of the study, since the sub-threshold dose already produced a near-complete suppression of dyskinesia when combined with (±)-8-OH-DPAT.

In line with the present results, elevation of the serotonergic tone induced by the psychotropic drug MDMA has been also found to produce reduction of the pro-dyskinetic effect of L-DOPA in MPTP-treated marmosets by mechanism partly involving 5-HT_{1A/1B} receptors (Iravani *et al.*, 2003).

Clinical implications

Pharmacological blockade of serotonin neuron activity by 5-HT_{1A} and 5-HT_{1B} receptor agonists, particularly in combination, might have potential clinical application in Parkinson's disease patients. Indeed, the 5-HT_{1A} partial agonist Sarizotan has been recently tested for its anti-dyskinetic properties, not only in MPTP-treated monkeys, but also in Parkinson's disease patients (Olanow *et al.*, 2004; Goetz *et al.*, 2007). However, despite promising results in the earlier clinical investigations, a large phase III clinical trial was recently terminated for lack of efficacy. Although a detailed report of the phase III trial has not been published yet, there are several factors that may account for the failure to obtain any significant anti-dyskinetic effect in this study. First, the dose of drug chosen for this trial might have been too low to provide the necessary blockade of DA release from the serotonin terminals. Second, Sarizotan has also some antagonistic properties on the DA receptors, which might explain in part the side effects observed in the early trial, particularly the worsening of the parkinsonism (Olanow *et al.*, 2004). In light of the preclinical results discussed above, and assuming that the serotonin terminals play a similar role in mediating L-DOPA-derived DA release in humans, it is also possible that targeting the 5-HT_{1A} receptors alone is

not sufficient to provide a significant control of the excessive swings in DA release and therefore of dyskinesias. According to our rodent and primate data, simultaneous activation of the 5-HT_{1A} and 5-HT_{1B} receptors should result in a more potent effect and in a better control of the motor side effects of L-DOPA medication, at doses acting mainly on the pre-synaptic receptors.

It is worth noting that the MPTP-treated primate model resembles the end stage of the disease in humans. In such a situation, the therapeutic effect of L-DOPA might partly depend on DA released from the serotonin terminals. The partial reduction of the efficacy of L-DOPA observed with the higher doses of the serotonin agonist in our monkey experiment seems to support this view. Less advanced Parkinson's disease patients are likely to retain some residual DA innervation, which can mediate L-DOPA-derived DA production and sustain the therapeutic effect of the drug. In such cases, even a complete blockade of DA release from the serotonin terminals should not have any major impact on the therapeutic effect of L-DOPA. If this is correct, careful selection of patients would be necessary in order to ensure maximal benefit from serotonin agonist treatment. Our rodent data showing prevention of dyskinesia upon chronic treatment with the serotonin agonists also speaks in favour of an early intervention as a way to avoid the development of troublesome dyskinesia and maintain intact therapeutic value of L-DOPA. Finally, as 5HT_{1A} agonists have shown neuroprotective efficacy in clinically relevant experimental designs using various animal models of Parkinson's disease including the MPTP-treated macaque (Bezard *et al.*, 2006), an early introduction of such drugs could potentially slow down the neurodegenerative process, further supporting the case of this class of drugs.

Possible induction of tolerance upon repeated administration of the serotonin agonists is a concern for this approach. Indeed, desensitization of pre-synaptic 5-HT_{1A} receptor is a well-known phenomenon linked to chronic administration of serotonin reuptake inhibitors, and explains the delayed therapeutic efficacy of these drugs (El Mansari *et al.*, 2005; Kuan *et al.*, 2008). Our data indicate that development of desensitization may reduce the anti-dyskinetic effect of the combined agonist treatment over time. In a clinical setting, this suggests that treatment sessions followed by wash-out periods may be needed to avoid this effect.

In conclusion, the results of the present study highlight the critical role of the serotonin system in the induction of L-DOPA-induced dyskinesia in the rat, as well as monkey Parkinson's disease model and the ability of serotonin 5-HT₁ receptor agonists to counteract this side effect. The demonstration of a synergistic effect between 5-HT_{1A} and 5-HT_{1B} receptor agonists in primates may have interesting clinical implication for the treatment of L-DOPA-induced dyskinesia in Parkinson's disease patients, where targeting of the 5-HT_{1A} alone has not produced the expected efficacy.

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