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ScienceDirect
Neurochemistry International 52 (2008) 1332–1342
NEUROCHEMISTRY International
www.elsevier.com/locate/neuint

Partial recovery of dopaminergic pathway after graft of adult mesenchymal stem cells in a rat model of Parkinson's disease

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Received 31 January 2008; accepted 6 February 2008
Available online 10 March 2008

Abstract

Cellular therapy with adult stem cells appears as an opportunity for treatment of Parkinson's disease. To validate this approach, we studied the effects of transplantation of rat adult bone-marrow mesenchymal stem cells in a rat model of Parkinson's disease. Animals were unilaterally lesioned in the striatum with 6-hydroxydopamine. Two weeks later, group I did not undergo grafting, group II underwent sham grafting, group III was intra-striatal grafted with cells cultured in an enriched medium and group IV was intra-striatal grafted with cells cultured in a standard medium. Rotational amphetamine-induced behavior was measured weekly until animals were killed 6 weeks later. One week after graft, the number of rotations/min was stably decreased by 50% in groups III and IV as compared with groups I and II. At 8 weeks post-lesion, the density of dopaminergic markers in the nerve terminals and cell bodies, i.e. immunoreactive tyrosine hydroxylase, membrane dopamine transporter and vesicular monoamine transporter-2 was significantly higher in group III as compared with group I. Moreover, using microdialysis studies, we observed that while the rate of pharmacologically induced release of dopamine was significantly reduced in lesioned versus intact striatum in no grafted rats, it was similar in both sides in animals transplanted with mesemchymal stem cells. These data demonstrate that graft of adult mesemchymal stem cells reduces behavioral effects induced by 6-hydroxydopamine lesion and partially restores the dopaminergic markers and vesicular striatal pool of dopamine. This cellular approach might be a restorative therapy in Parkinson's disease.

Keywords: Autoradiography; Bone marrow; Dopamine transporter; Microdialysis; Tyrosine hydroxylase

1. Introduction

Parkinson's disease is a common neurological disorder characterized by progressive degeneration of dopaminergic neurons of the nigrostriatal pathway. The molecular mechanisms underlying the selective loss of these neurons involve a number a complex processes such as oxidative stress and immune/inflammatory factors, but still remain elusive (Anantharam et al., 2007; Zhu et al., 2007; Wang et al., 2007). The neuronal loss leads to deficiency in dopamine (DA) in the striatum, which is responsible for characteristic motor symptoms such as akinesia, bradykinesia, muscle rigidity and tremor at rest (Gibb and Lees, 1988). Pharmacological treatment with L-DOPA as well as surgical treatments such as deep brain stimulation (Olanow et al., 2000; Krause et al., 2001) improve parkinsonian symptoms but do not repair the dopaminergic pathway or prevent its degeneration. Therefore, novel cell therapy approaches such as cell grafting to restore the dopaminergic function are of interest. To this aim, different stem/progenitor cell populations have been tested in vivo such

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doi:10.1016/j.neuint.2008.02.003

as neural or neuronal progenitor cells (Gage et al., 1995; Dziewczapolski et al., 2003), neural stem cells (Isacson, 2002; Yang et al., 2002), fetal neurons (Lindvall et al., 1990; Clarkson, 2001) and embryonic stem cells (Freed et al., 2001; Björklund et al., 2002; Kim et al., 2002). Use of these cell types has been limited by ethical problems and difficulties in obtaining sufficient cell amounts. Moreover, trial outcomes have not been always positive (Freed et al., 2001).

Adult bone-marrow mesenchymal stem cells (MSC) show great promise for cell therapy because of their easy accessibility, multipotentiality (review by Dennis and Charbord, 2002) and immunomodulatory properties (Bartholomew et al., 2002). MSCs are capable of rapid expansion in culture and are able to differentiate in cells of mesodermic origin (osteocytes, chondrocytes, adipocytes and stromal cells following a vascular smooth muscle differentiation pathway) (Dennis and Charbord, 2002) and in cells of extra-mesodermic origin such as endodermic cells (hepatocytes) (Chagraoui et al., 2003; Sato et al., 2005). In addition, they can differentiate along a neuroectodermal pathway (Woodbury et al., 2000; Hermann et al., 2004; Dezawa et al., 2004; Wislet-Gendebien et al., 2005).

In this study, we grafted rat bone-marrow adult MSC cultured in an enriched or standard medium into the striatum in a rat model of Parkinson's disease obtained by striatal injection of 6-hydroxydopamine (6-OHDA) (Sauer and Oertel, 1994; Przedborski et al., 1995; Kirik et al., 1998; Deumens et al., 2002). As growth factors such as FGF2, FGF8, Shh and BDNF have been shown to allow the orientation of MSC towards a neuronal differentiation in vitro (Tsai and Kim, 2005; Fu et al., 2005), we used a sequential culture protocol with these factors (Jiang et al., 2002). However we obtained, in these experimental conditions, an incomplete engagement of MSC through the neuronal pathway, with lower expression of α-smooth-muscle actin, vimentin and Oct4 and higher expression of βIII-tubulin than cells in cultured in a standard medium. In addition, whatever the population of MSC used, animals transplanted showed partial functional recovery as assessed by decrease in induced rotational behavior. We therefore assessed in the striatum and substantia nigra, dopaminergic markers such as the immunoreactive tyrosine hydroxylase (TH), dopamine transporter (DAT), vesicular monoamine transporter ($VMAT_2$) and dopamine D_1 and D_2 receptors, and the functional release of DA in the striatum exclusively in animals grafted with MCS cultured in the enriched medium versus no grafted animals.

2. Experimental procedures

2.1. MSC isolation and cell culture

Adult male Sprague–Dawley rats were killed and femoral bones were removed. After flushing of femoral bone marrow, total nucleated cells were cultured at 2×10^4 cell/cm^2 in α-MEM medium with nucleotides (Gibco, Cergy Pontoise, France) supplemented with 20% fetal calf serum (FCS; Perbio, HyClone, Brebières, France), 1% (v/v) penicillin/streptomycin and 0.01% amphotericin B solution. Cells were cultured at 37 °C in a fully humidified atmosphere with 5% CO_2. Medium was changed twice a week until confluence. Then cells were detached using trypsine, washed twice in PBS and reseeded following the previous procedure. After three cell passages, adherent cells were considered to be MSC because of their phenotype (CD45−/31−/44+/73+/90+ cells) and their adipocytic, osteoblastic and chondrocytic differentiation potential. MSC from passages 4 to 6 were cultured for 3 weeks under two conditions: culture in standard medium as described above (medium was changed twice a week until cell confluence) or in neuronal differentiation medium (called enriched-medium) which consisted to add sequentially different cytokines to the standard medium: FGF2 (100 ng/mL) for the 1st week, FGF8 (10 ng/mL) and Shh (10 ng/mL) for the 2nd week and BDNF (10 ng/mL) (R&D, Lille, France) for the 3rd week. The medium was supplemented with BrdU (3 μg/mL) during the last 3 days of culture, in order to locate them in grafted animals. Cells were then harvested for real-time PCR and in situ immunofluorescence studies or for grafting in 6-OHDA-lesioned rats.

2.2. RNA extraction and quantitative PCR

RNA was extracted using TRIZOL Reagent (Invitrogen, Cergy Pontoise, France) according to the manufacturer's instructions. To remove any contamination by DNA, extracts were treated with DNAse I (1 μL/μg RNA) in DNAse buffer 10× (1 μL/μg RNA). Sample integrity was checked by migration in 1.2% agarose gel (Invitrogen) stained by 0.05 μg/mL ethidium bromide (Invitrogen). Reverse transcription was performed for 50 min at 42 °C with 1 μg of total RNA. The reaction mixture contained 10 U reverse transcriptase superscript II (Invitrogen), 1 mM dNTP, 10 mM dithiotreitol (DTT) (Invitrogen) and 100 ng of random hexamers (Invitrogen) in a final volume of 20 μL. Real-time PCR was performed according to the manufacturer's instructions with use of the Platinum SYBR® Green qPCR SuperMix UDG (Invitrogen) with 0.5 ng or 10 ng cDNA for expression of β-actin housekeeping gene, and genes of interest such as nestin, βIII-tubulin, neurofilament M (NF-M200), NSE, TH, SM α-actin, vimentin, Oct 4, BDNF, GDNF, FGF2 and FGF8 (GenBank accession numbers: NM_012987, NM_031140, M18628, AF019973, NM_012740, NM_031004, NM_031140, NM_001009178, respectively).

Amplifications involved incubating the reaction mixtures at 50 °C for 2 min, 95 °C for 5 min following by 50 cycles at 95 °C (30 s), 58 °C (30 s) and 72 °C (30 s). Relative transcript level was determined by comparison of cycle threshold (C_t) values using the $2^{-\Delta\Delta C_t}$ method.

2.2.1. Primer sequences

Smooth muscle alpha actin (For, 5′-AGCCAGTCGCCATCAGGAAC-3′; Rev, 5′-TCATCACCAGCAAAGCCCG-3′), βIII-tubulin (For, 5′-AGTGCGGCAACCAGATAGGG-3′; Rev, 5′-GGGCACATACTTGTGAGAGGAGG-3′), NF-M200 (For, 5′-ACCCTGGAGATGGTGAATCACG-3′; Rev, 5′-TGGCGTAGCGGCATTTGAAC-3′), NSE (For, 5′-ACAAACAGCGTTACTTAGGCAAAGG-3′; Rev, 5′-TTCTCAGTCCCATCCAACTCCAG-3′), Oct4 (For, 5′-ATGGCTGGACACCTGGCTTC-3′; Rev, 5′-GTTGTCTGGCTGAACACCTTTCC-3′), TH (For, 5′-ACTGTGGAATTCGGGCTATG-3′; Rev, 5′-GACCTCAGGCTCCTCTGACA-3′), vimentin (For, 5′-CTACGAGGAGGAGATGAGGGAGTTG-3′; Rev, 5′-GCCAGAGAAGCATTGTCAACATCC-3′), nestin (For, 5′-TCGGGAGTGTCGCTTAGAGGTG-3′; Rev, 5′-GAGTCTCAAGGGTATTAGGCAAGGG-3′) and β-actin (For, 5′-GGAGCAGCTGGCAAAGCTTA-3′; Rev, 5′-AGGAGCGTACCCACGAGTGT-3′).

2.3. Immunocytochemistry studies

Cells were seeded on plastic chamber slides (Labtek, 8 wells, Nunc, Denmark) at 7×10^3 cells/well and were left for adherence during 24 h. After medium removal, cells were washed in PBS buffer (Gibco, Paris, France) for 10 min, fixed with paraformaldehyde (4%) and permeabilized in methanol (for 30 min at 4 °C). After two washes in PBS, cells were incubated for 30 min at RT with primary antibodies. Then after two washes in PBS, cells were incubated for 30 min with fluorescent secondary antibodies (alexa 488- or 594-conjugated goat anti-mouse, or alexa 488- or 594-conjugated goat anti-rabbit (1/400, Interchim, Montluçon, France). Preparations were mounted with VECTASHIELD® Mounting Medium containing DAPI (Abcys, Paris, France). Cells were observed on microscopy with a Leica DMR microscope (Leica Microsystems, Rueil-Malmaison, France). The percentage of positive cells for a given antibody was evaluated by counting 150 cells under 10× magnification in several random fields.

2.3.1. Primary antibodies

Rabbit anti-NF-M200 (1/25, N-4141, Sigma); mouse anti-βIII-tubulin (1/50, TuJ-1, R&D Systems); mouse anti-nestin (1/25, clone 401, BD Pharmingen); mouse anti-NSE (1/50, NSE-P1, Interchim); mouse anti-TH (1/50, TOH-P1, BD Pharmingen); mouse anti-vimentin (1/100, clone V9, Sigma); mouse anti-α-smooth muscle actin (1/50; clone 1A4, Sigma); mouse anti-BrdU (1/250, Sigma).

2.4. Unilateral striatal lesion with 6-hydroxydopamine

Adult female Sprague–Dawley rats weighing 250–300 g (Janvier, Le Genest-St-Isle, France) were used for the lesion and transplantation experiments. All procedures were carried out in accordance with the European Community Council Directive 86/609/EEC for the care of laboratory animals and after approval of the Regional Ethical Committee (INSERM37–002). Rats were kept on a 12-h light/dark cycle (temperature 22.4 ± 0.5 °C; hygrometry 40.3 ± 7.2%) and water and food were freely available. Ten minutes before surgery, animals were injected i.p. with desipramine (25 mg/kg) and pargyline (50 mg/kg) (Sigma, Saint Quentin Fallavier, France). Rats were then anesthetized with isoflurane (4%, 500 mL/min) and placed on a stereotaxic apparatus (Stoelting, Phymep, Paris, France). They were maintained under isoflurane 2% (500 mL/min) during surgery. The skull was exposed and small holes were made with a dental drill. Lesion was carried out by unilateral intra-striatal injection of 6-OHDA hydrochloride (2 mg/mL). A total of 20 μg of 6-OHDA was administered in two points of the right striatum (2 μg/μL in 0.01% ascorbic acid, pH 4.5 so 5 μL/point) with a Hamilton syringe (gauge 25, Hamilton, Massy, France) at a flow rate of 1 μL/min. Coordinates from bregma were: AP = +0.5 mm, $L = -2.5$ mm, $P = -5$ mm and AP = −0.5 mm, $L = -4.2$ mm, $P = -5$ mm according to Paxinos and Watson (1986); the syringe was left in place for 4 min after injection and then removed slowly to optimize toxin diffusion.

2.5. Behavioral test

One week post-lesion and then once a week during 4–6 weeks post-grafting, animal rotational behavior was tested in response to D-amphetamine sulphate injection (3 mg/kg i.p.) (Sigma). Animals were injected with amphetamine, placed into automated rotometer bowls (Imetronic, Pessac, France) and 15 min later, left and right full body turns were monitored by a computer during 90 min. Animals showing in average more than 10 ipsilateral turns per min were selected for experiments.

In a first set of experiments, 4 groups of 10 animals were lesioned. One week later, four homogenous groups were constituted according to their rotational behavior as follows: lesioned and no-grafted (group I, $n = 6$), lesioned and sham-grafted (group II, $n = 7$), lesioned and grafted with MSC cultured in enriched medium (group III, $n = 8$) and lesioned and grafted with MSC cultured in standard medium (group IV, $n = 7$).

In a second set of experiments, 2 groups of 10 animals were lesioned. One week later, two homogenous groups were constituted according to their rotational behavior as follows: lesioned and no-grafted (group I) and lesioned and grafted with MSC cultured in enriched medium (group II) for microdialysis studies.

2.6. Animal transplantations

Animals were anesthetized with isoflurane 4% (500 mL/min), placed on the stereotaxic apparatus and continued to be anesthetized with isoflurane 2% (500 mL/min).

2.6.1. Transplantation of MSC

Cells in suspension (NaCl 0.9%) were implanted at the same stereotaxic coordinates as the lesion points. A total of 90,000 cells were injected per point in a volume of 5 μL with a 25 μL Hamilton syringe (1.5 μL/min) (i.e., 180,000 cells per animal).

2.6.2. Sham graft

Animals were injected with 5 μL NaCl 0.9% in the same coordinates as the lesion points with a 25 μL Hamilton syringe (1.5 μL/min). A total of 10 μL NaCl 0.9% was injected per animal.

2.7. In vitro studies on brain sections

2.7.1. Preparation of brain sections

Eight weeks post-lesion, animals were killed and brains were removed and frozen into isopentane cooled at −35 °C (group I, $n = 6$; group III, $n = 8$). Coronal sections 16-μm thick were cut in a freezing microtome (−20 °C) and mounted on SuperFrostPlus® slides (CML, Nemours, France). Sections were kept at −80 °C until use. Immunohistochemical and autoradiographic experiments were performed on consecutive sections, either in the striatum (AP coordinates between +0.5 and −0.5 according to the atlas of Paxinos and Watson, 1986) or in the substantia nigra (AP coordinates between −5 and −6).

2.7.2. Anti-TH immunohistochemistry

Sections were fixed with 4% PFA (Merck, Fontenay-sous-Bois, France) for 30 min at room temperature and washed in PBS (0.1 M). Endogenous peroxidase activity was blocked with H_2O_2 (0.5%, 100 μL/section, 5 min). Slides were washed and incubated in a serum solution (10% goat serum, 0.1% tween and 2% gelatin in 0.1 M PBS) for 30 min, washed twice with PBS and then incubated in anti-TH antibody solution (rabbit polyclonal anti-rat, 1/500; Euromedex, Mundolsheim, France) for 3 h. Control sections were incubated with serum solution. For staining, a biotinylated rabbit anti-rabbit IgG and peroxidase-labeled streptavidin (ABC kit, Abcys, Paris, France) were used, then revealed with 3–3′-diaminobenzidine tetrachloride (DAB) (Sigma). Sections were dehydrated with 60°, 80°, 95° and 100° ethanol, fixed with histoclear and mounted with Pertex® mounting medium. Between 2 and 4 sections per animal were studied.

2.7.3. Localization of grafted cells by anti-BrdU immunohistofluorescence

Sections were fixed with 4% PFA for 30 min at room temperature and washed in PBS (0.1 M, 2× 10 min). Slides were washed in sodium buffer (SSC2X: 0.3 M NaCl, 0,03 M $Na_3C_6H_5O_7$, pH 7, 5 min) and incubated in a formamide solution (50% formamide in SSC2X) for 2 h, washed twice with SSC2X and then incubated in HCl 2N solution for 30 min. Acidification was neutralized in borate buffer (0.1 M, pH 8.5) and washed in PBS (0.1 M, 30 min and 5× 10 min). Slides were incubated in a serum solution (10% horse serum in 0.1 M PBS) for 30 min, washed with PBS and then incubated in anti-BrdU antibody solution (mouse monoclonal anti-BrdU, 1/1000, Sigma) overnight. Control sections were incubated with serum solution. After two washes in PBS, cells were incubated for 30 min with fluorescent secondary antibodies (alexa 488-conjugated goat anti-mouse (1/400, Interchim)). Preparations were mounted with VECTASHIELD® Mounting Medium containing DAPI (Abcys). Sections were observed on microscopy with a Leica DMR microscope.

2.7.4. In vitro autoradiographic studies of the DAT, $VMAT_2$, D_1 and D_2 receptors

Labeling of DAT with [^{125}I]-PE2I was carried out as previously described (Chalon et al., 1999). Briefly, brain sections were incubated in PBS buffer (0.1 M, pH 7.4) containing 137 mM NaCl, 2.7 mM KCl, 10.14 mM Na_2HPO_4, 1.76 mM KH_2PO_4 and 109 g/L D-saccharose together with 100 pM [^{125}I]-PE2I (specific activity 7.4×10^{13} Bq/mmol) for 90 min at room temperature. Sections were then washed twice in ice-cold buffer for 20 min and rinsed in distilled water at 4 °C. Unspecific binding was determined from adjacent sections incubated in the same solution supplemented with 100 μM cocaine (Cooper, France). After drying, slides were exposed to sensitive films (biomax MR, Kodak, VWR, France) with ^{125}I microscales (Amersham, Saclay, France) in X-ray cassettes for 24 h.

The $VMAT_2$ and dopaminergic D_1 and D_2 receptors were labeled with [^{3}H]-α-dihydrotetrabenazine ([^{3}H]-DTBZ, specific activity 7.4×10^{11} Bq/mmol) (ARC, St. Louis, MO, USA), *N*-methyl-[^{3}H]-SCH-23390 (specific activity 3.1×10^{12} Bq/mmol) and *N*-methyl-[^{3}H]-YM-09151–2 (specific activity 3.1×10^{12} Bq/mmol) (PerkinElmer, Courtaboeuf, France), respectively. Binding steps involved the same protocol as for [^{125}I]-PE2I binding, but unspecific binding was determined with reserpine (2 μM) (Sigma) for $VMAT_2$ and (+)-butaclamol (10 μM) for D_1 and D_2 receptors. Slides were exposed to sensitive films (biomax MR) with ^{3}H microscales (Amersham) in X-ray cassettes for 4 months for $VMAT_2$, 3 weeks for D_1 and 5 weeks for D_2 receptors. After exposure, films were revealed and fixed.

Quantification of autoradiographic images was performed using the Betavision + software (Biospace, Paris, France). For each animal, the proportion of total binding (TB) and nonspecific binding (NSB) was measured on three to four sections. The value of specific binding (SB) was determined as SB = TB − NSB. Results were expressed as the percentage of binding in the ipsilateral (right) versus controlateral (left) side.

The quantification of immunoreactive TH fibers was performed indirectly in the striatum by quantifying the white light absorption rate across the tissue with use of a computerized image analysis system (Sony) and the Histolab software (Microvision Instruments, Evry, Paris). In the substantia nigra pars compacta, the number of TH+ neurons was counted in the ipsilateral and controlateral sides using the Histolab software. Results were expressed in percentage of labeling in the ipsilateral versus controlateral side. Between two and four sections were quantified and the average was calculated for each animal.

2.8. Microdialysis studies

The vesicular concentration of dopamine was investigated using in vivo microdialysis in one group of lesioned and no-grafted rats ($n = 6$) and in one group of lesioned and MSC-grafted rats ($n = 5$) at 6–8 weeks after lesion. Animals were anesthetized with isoflurane (4%, 500 mL/min), placed on a stereotaxic apparatus (Stoelting, Phymep, Paris, France) and maintained under isoflurane 1.5% (500 mL/min) during surgery. Two microdialysis guide cannula MAB6-20G (Phymep) were implanted into the left and right striatum (AP = 0, ML = −3.5, $P = -5$ and AP = 0, ML = +3.5, $P = -5$, from Bregma, respectively, according to the atlas of Paxinos and Watson, 1986) and permanently fixed to the skull. Cannulas MAB2-20G (Phymep) were removed and replaced by microdialysis probes MAB6/20/1 (Phymep). The rats were placed in a 120 CMA system for freely moving animals. The probes were perfused overnight at a constant rate of 0.8 mL/min using a micro-perfusion pump CMA/102 (Phymep) with an artificial fluid (Dulbecco's phosphate-buffered saline containing 138 mM NaCl, 0.9 mM $CaCl_2$, 0.5 mM $MgCl_2$, 2.7 mM KCl, pH 7.4). Experiment was performed 24 h after probes implantation. Right and left striata were perfused at a flow rate of 1.2 mL/min. The four first dialysates were taken each 20 min and used to define the basal release rate of DA. Then pharmacological stimulation was performed using tyramine infusion (0.8 mM) through the microdialysis probes during 30 min. Microdialysate samples were collected each 20 min during 4 h and kept at −80 °C. The DA concentration in dialysates was assayed by HPLC (system gold 118, Beckman, Villepinte, France) with electrochemical detection (Concorde, Waters, Saint-Quentin en Yvelines, France) and a catecholamine HB 3 μm column (100 mm × 3.2 mm) (Phymep). The mobile phase (pH 3) contained 20 mM of citric acid, 10 mM of sodium phosphate, 3 mM of octanesulfonic acid, 3.25 mM of heptanesulfonic acid, 0.1 mM of EDTA, 2 mM of potassium chloride, 3% of methanol and 7% of acetonitrile. Results were expressed as mean percentages of basal level ± S.E.M.

2.9. Statistical analysis

All values were expressed as mean ± standard error of the mean (S.E.M.). Statistical comparisons involved ANOVA and the Mann–Whitney nonparametric test.

3. Results

3.1. In vitro characterization of MSC

A pool of MSC cultured in the enriched or standard medium was no grafted but used for molecular characterization by Q-PCR. The expression of neuronal, mesenchymal, stem cells and neurotrophic marker genes of MSCs cultured in these two culture conditions is presented in Fig. 1. The transcription factor Oct4 was expressed in standard medium culture and significantly down-regulated in enriched medium. The mesenchymal genes α-smooth muscle actin (α-SM actin) and vimentin were down-regulated in enriched medium culture (Fig. 1.1); in this condition, all cells were positive for vimentin but only 60% were positive for α-SM actin (Fig. 1.2). By contrast neuronal genes, significantly the late neuronal expressed marker βIII-tubulin were up-regulated (Fig. 1.1); MSCs cultured in the enriched medium produced a number of neuronal markers: up to 2% of the cells were nestin positive, about 60% were βIII-tubulin positive (Fig. 1.2) and all were NF-M200 and NSE positive whereas TH or GFAP positive cells were not observed. Genes encoding neurotrophic factors as brain derived neurotrophic factor (BDNF), glial derived neurotrophic factor (GDNF) and fibroblasts growth factors (FGF2 and FGF8) were similarly expressed in cells of the both culture conditions. Enriched medium induced then a down-regulation of stemness and mesenchymal markers and an up-regulation of neuronal markers of MSCs.

3.2. Behavioral test

The intensity of rotational behavior evaluated 1-week post-lesion for 90 min following D-amphetamine injection was similar for the four groups (group I: 24.2 ± 1.7 turns/min, group II: 25.4 ± 2.3 turns/min, group III: 22.0 ± 2.1 turns/min, group IV: 23.7 ± 2.6 turns/min) (Fig. 2). One week post-grafting (i.e., 3 weeks post-lesion) rotational behavior did not differ significantly between group I (no-grafted) and group II (sham-grafted) (23.8 ± 2.1 and 25.1 ± 1.7 turns/min, respectively). By contrast, a significant 50% reduction was observed in groups III and IV (10.8 ± 1.7 and 14.1 ± 3.3 turns/min, respectively; $p < 0.05$ compared to the number of rotations before graft in these groups and $p < 0.05$ compared to the results of groups I and II at this time post-lesion). Animals grafted with MSC cultured in enriched or standard medium show therefore an improvement of rotational behavior induced by amphetamine injection. In both groups of grafted animals, this improvement was equivalent, remained stable for the next 5 weeks, and was statistically significant compared to lesioned animals no grafted or sham grafted.

As rotational behavior was similar in groups I and II for the 5 weeks post-grafting, and similar in groups III and IV, we exclusively compared groups I and III for the following experiments on brain sections.

3.3. Localization of grafted cells by immunohistofluorescence

In this experiment we used MSC cultured in an enriched medium supplemented with BrdU. Six weeks after graft, anti-BrdU immunohistochemistry was carried out in the striatum and substantia nigra. Positive BrdU ($BrdU^+$) cells were observed in the striatum exclusively close to the grafted coordinates (AP +0.5 from Bregma, Paxinos and Watson, 1986), as illustrated in Fig. 3. We observed that no $BrdU^+$ MSC was localized far from the grafted coordinates, into the intact striatum or in the substantia nigra.

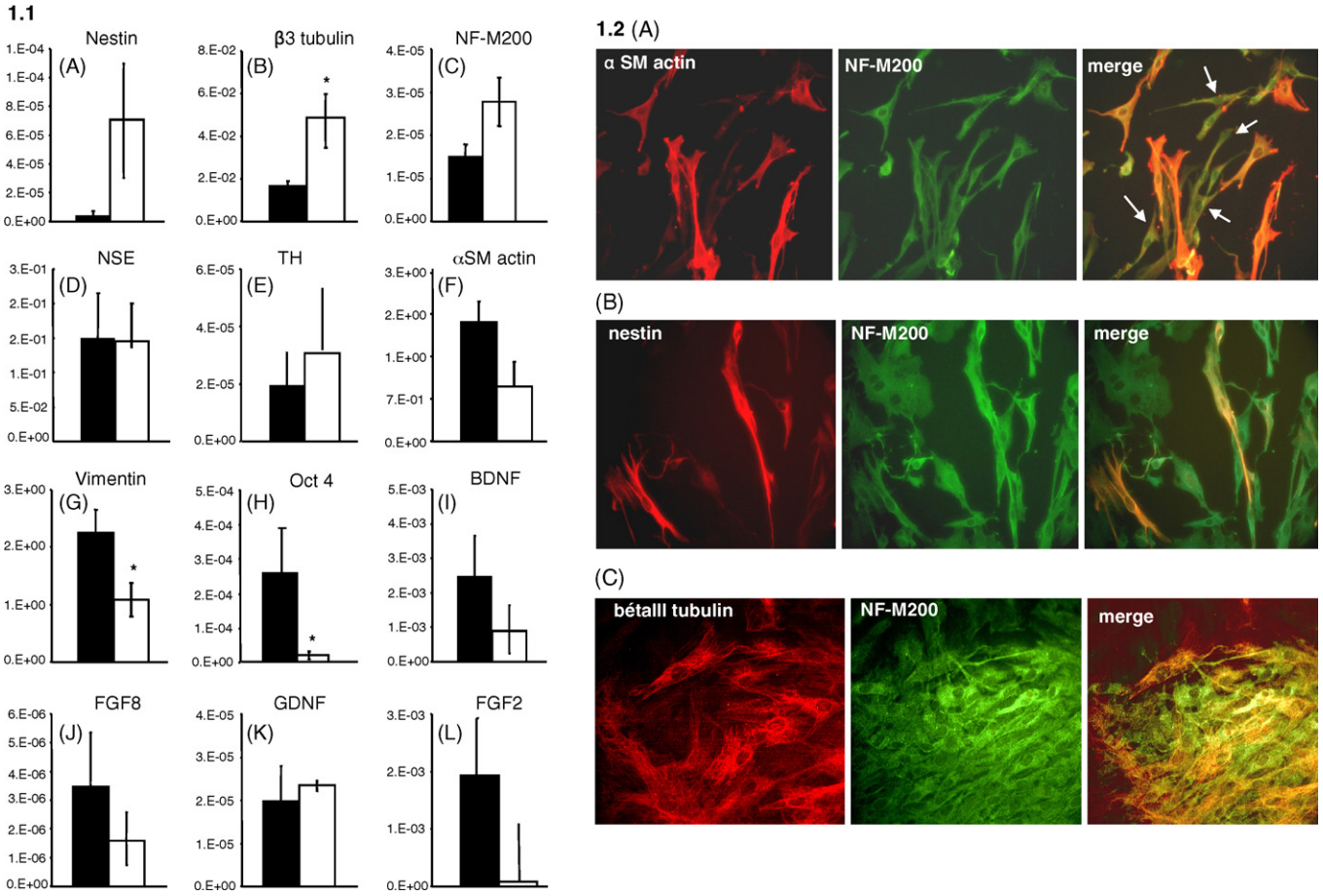

Fig. 1. Quantification of gene expression by real-time PCR (1.1) and in situ fluorescence studies of protein expression in MSC (1.2). Total RNA was extracted from MSC cultured in standard and enriched medium as described in Materials and Methods and subjected to Q-PCR. Results are expressed in relative quantities compared to expression of (-actin gene. Expression of gene of interest from MSC cultured in both condition were compared between us with the Mann–Whitney statistical test. Left column (black) is standard-medium culture and right column is enriched-medium culture (1.1). Fig. 1.2A, α smooth muscle actin (αSM actin, in red Alexa 594) and neurofilament M200 (NF-M200, in green Alexa 488); merged shows αSM actin-negative cells (white arrows) (magnification 20×). Fig. 1.2B, nestin (in red Alexa 594) and NF-M200 (in green Alexa 488); rare elongated cells express both markers (merged, white arrow) (magnification 20×). Fig. 1.2C, β3-tubulin (in red, Alexa 594) and NF-M200 (in green, Alexa 488), numerous cells express both markers (merged, white arrow) (magnification 20×). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of the article.)

3.4. Anti-TH immunohistochemistry

Anti-TH immunostaining in the ipsilateral (lesioned) striatum was 43.0 ± 6.4% that of the controlateral (intact) side in group I (as illustrated in Fig. 4A and B). In group III, the immunostaining was increased in the striatum (55.5 ± 6.7%) but not significantly different from that of group I ($p = 0.223$, Fig. 4A and C).

In the substantia nigra pars compacta of rats from group I, the number of TH + neurons was 24.2 ± 6.7% in the ipsilateral versus controlateral side (Fig. 4D and E). This percentage was significantly increased in group III (52.5 ± 8.2%; $p < 0.05$ as compared to group I) (Fig. 4D and F).

These results thus showed that grafting of MSC cultured in an enriched medium induced a slight increase of TH immunostaining in the striatum and a significant increase in the number of TH+ neurons in the substantia nigra pars compacta compared to no grafted animals.

3.5. Autoradiographic studies

Dopaminergic markers were quantified by autoradiographic studies on brain sections 6 weeks post-graft. Radiolabeling in the striatum of group I showed a DAT density (labeled with [^{125}I]-PE2I) of 17.7 ± 6.3% in the ipsilateral (lesioned) versus controlateral (intact) side (Fig. 5.1A and B). In group III, this density was significantly higher than in group I (44.6 ± 9.1%, $p < 0.05$) (Fig. 5.1A and C). In the substantia nigra DAT density was 23.4 ± 6.5% in group I (Fig. 5.1D and E) and significantly higher in group III (50.5 ± 6.8%, $p < 0.05$) on the ipsilateral side (Fig. 5.1D and F).

Radiolabeling of $VMAT_2$ in the striatum with [^{3}H]-DTBZ in group I was 17.9 ± 3.6% in the ipsilateral (lesioned) versus controlateral (intact) side (Fig. 5.2A and B). A significantly increased density was observed in group III (35.3 ± 5.1%, $p < 0.05$) (Fig. 5.2A and C). In the substantia nigra, the density of $VMAT_2$ was 36.4 ± 8.1% in group I on the ipsilateral side

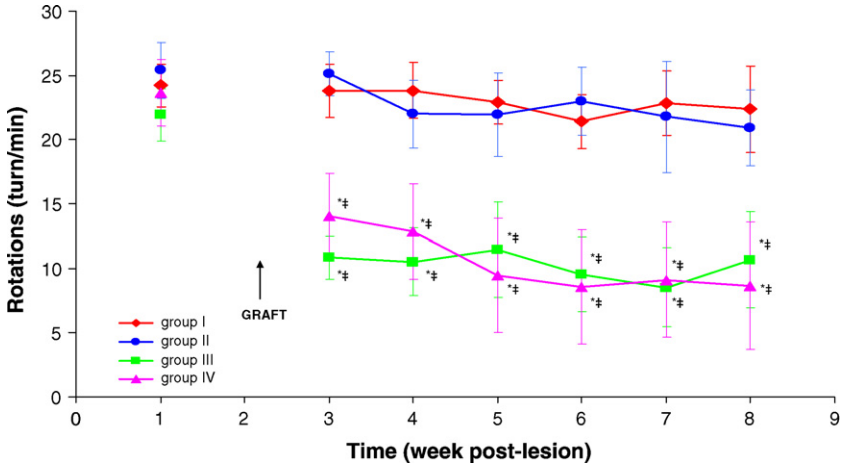

Fig. 2. Rotational behavior in response to amphetamine. Four groups of unilaterally lesioned rats were tested: no grafted (group I, $n = 6$), sham-grafted (group II, $n = 7$), grafted with MSC cultured in enriched medium (group III, $n = 8$) and grafted with MSC cultured in standard medium (group IV, $n = 7$). Results are expressed as means turns/min ± S.E.M.; $^{*}p < 0.05$, comparison between experimental groups (III and IV) and control groups (I and II); $^{\ddagger}p < 0.05$ comparison in a same group to the score obtained 1 week after lesion (Mann–Whitney test).

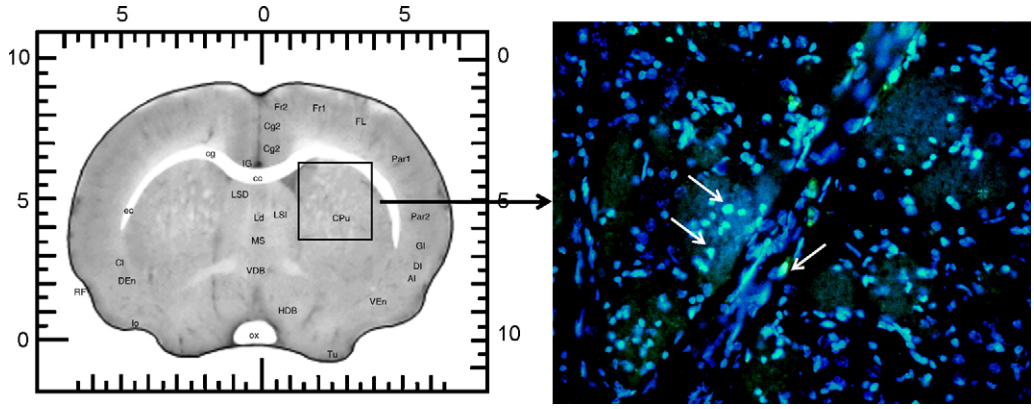

Fig. 3. Immunohistofluorescence study using anti-BrdU labeling into the striatum of grafted rats. Sections were mounted with medium containing DAPI and observed at 20× magnification. Positive MSC were defined by double binding with DAPI (blue) and BrdU (green) as observed on cells indicated by white arrows. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of the article.)

(Fig. 5.2D and E). A significant increase was observed in group III (62.0 ± 7.6%, $p < 0.05$) (Fig. 5.2D and F).

In the striatum, all groups showed similar binding of [^{3}H]-SCH-23390 to dopamine D_1 receptors in the ispsilateral versus controlateral side (Table 1). In the substantia nigra pars reticulata, the binding in the lesioned side was 96.0 ± 3.0% in group I, with a significant decrease in group III. As well, in the striatum, all groups showed similar binding of [^{3}H]-YM-

Table 1
Autoradiographic studies of the dopamine D_1 receptors using [^{3}H]-SCH-23390 and D_2 receptors using [^{3}H]-YM-09151-2

Cerebral area	Group	Percentage of binding in the ipsi- (lesioned) versus controlateral (intact) side	
		D_1	D_2
Striatum	I	84.2 ± 8.1	88.4 ± 4.3
	III	90.6 ± 4.6 ($p = 0.64$)	85.8 ± 3.8 ($p = 0.56$)
Substantia nigra pars compacta	I	–	5.3 ± 3.6
	III	–	18.4 ± 5.4 ($p < 0.05$)
Substantia nigra pars reticulata	I	96.0 ± 3.0	–
	III	65.9 ± 2.5 ($p < 0.05$)	–

The binding was measured in the striatum for D_1 and D_2 receptors, in the substantia nigra pars reticulata for D_1 receptors and in the substantia nigra pars compacta for D_2 receptors. Results are expressed as the percentage of binding in the ipsilateral (lesioned) compared to the controlateral (intact) side. Group I, $n = 6$, group III, $n = 8$. p values are in comparison to group I, using the Mann–Whitney test.

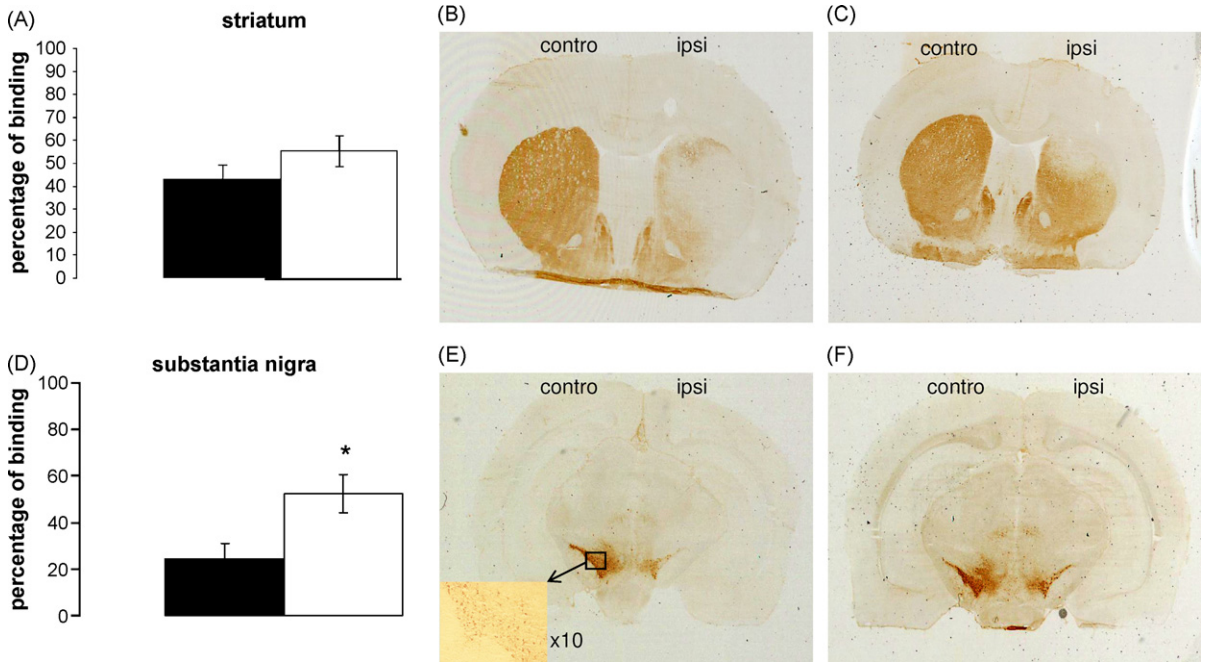

Fig. 4. Immunoreactive TH in the striatum and substantia nigra of animals grafted or not with MSC cultured in enriched medium. Immunoreactivity was assessed in the striatum by quantification of the density of TH+ fibers (4A) and in the substantia nigra (4D) by counting TH+ neuronal cells bodies. Results are expressed as the percentage of anti-TH staining in the ipsilateral (lesioned, right striatum) compared to the controlateral (intact, left striatum) side. Group I (■), $n = 6$; group III (□), $n = 8$. Errors bars are S.E.M.; $*p < 0.05$, significantly different from group I (Mann–Whitney test). Images are representative striatum staining in group I (4B) and group III (4C); substantia nigra staining in group I (4E) and group III (4F).

09151-2 to dopamine D_2 receptors (Table 1). In the substantia nigra pars compacta, group I showed weak binding ($5.3 \pm 3.6\%$) in the ipsilateral versus controlateral side, with significantly increased binding in group III ($18.4 \pm 5.4\%$, $p < 0.05$).

Autoradiographic studies showed therefore a partial and significant recovery of dopaminergic presynaptic markers in grafted animals compared to no grafted or sham grafted animals.

3.6. Microdialysis studies

Intra-striatal infusion of tyramine induced release of DA in both striata of each animal (Fig. 6). The extracellular DA level measured before tyramine infusion corresponds to the basal level (varying between 5 and 15 nmol in different animals), and then tyramine was infused through the microdialysis canula in order to induce a release of dopamine from the vesicular storage pool (Fairbrother et al., 1990). In no grafted group (Fig. 6A), DA level at the maximal release effect was $1163 \pm 228\%$ in the left (intact) striatum and $452 \pm 83\%$ in the right (lesioned) striatum, showing a reduction of 61% on the lesioned versus intact side ($p < 0.05$). By contrast, the DA level at maximal release effect was no statistically different in the left (intact) and right (lesioned) striatum in the grafted group ($955 \pm 115\%$ and $752 \pm 76\%$, respectively) (Fig. 6B). These microdialysis studies showed that graft of MSC cultured in an enriched medium in the striatum of lesioned rats are able to partially restore tyramine-induced DA release in the striatum.

4. Discussion

Partial functional recovery was already observed after MSC grafting in animal models of CNS injury such as brain ischemia (Zhao et al., 2002; Chopp and Li, 2002), traumatic brain injury (Mahmood et al., 2001) or spinal cord injury (Hofstetter et al., 2002). A clinical assay in patients with ischemic stroke showed the feasibility, safety and potential efficacy of intravenous injection of autologous MSC (Bang et al., 2005). All these data and the possible efficacy of MSC grafting in a mouse MPTP model of parkinsonism (Li et al., 2001) led us to test the potential of MSC striatal grafting as a therapeutic strategy for Parkinson's disease.

During the past decade, many studies have demonstrated that MSC are multipotent stem cells differentiating along mesodermic pathways (osteoblastic, chondrocytic, adipocytic, stromal) (Dennis et al., 1999). More recently, experiments have shown that bone-marrow MSC can also differentiate following a neural or neuronal pathway (Sanchez-Ramos et al., 2000; Wislet-Gendebien et al., 2003, 2005). The growth factors FGF2 (Tsai and Kim, 2005), FGF8 and Shh (Fu et al., 2005), and BDNF are able of directing stem cells to the neuronal pathway. As was previously reported (Jiang et al., 2002), we used a sequential exposure to these growth factors. We showed that the enriched medium culture induced two changes: a decreased level of mesenchymal markers such as α-SM actin and a down-regulation of Oct4. In accordance with previous results in different species (Tondreau et al., 2004; Minguell et al., 2005; Deng et al., 2006), we demonstrated at the RNA and protein level that rat MSC express neuronal protein such as nestin,

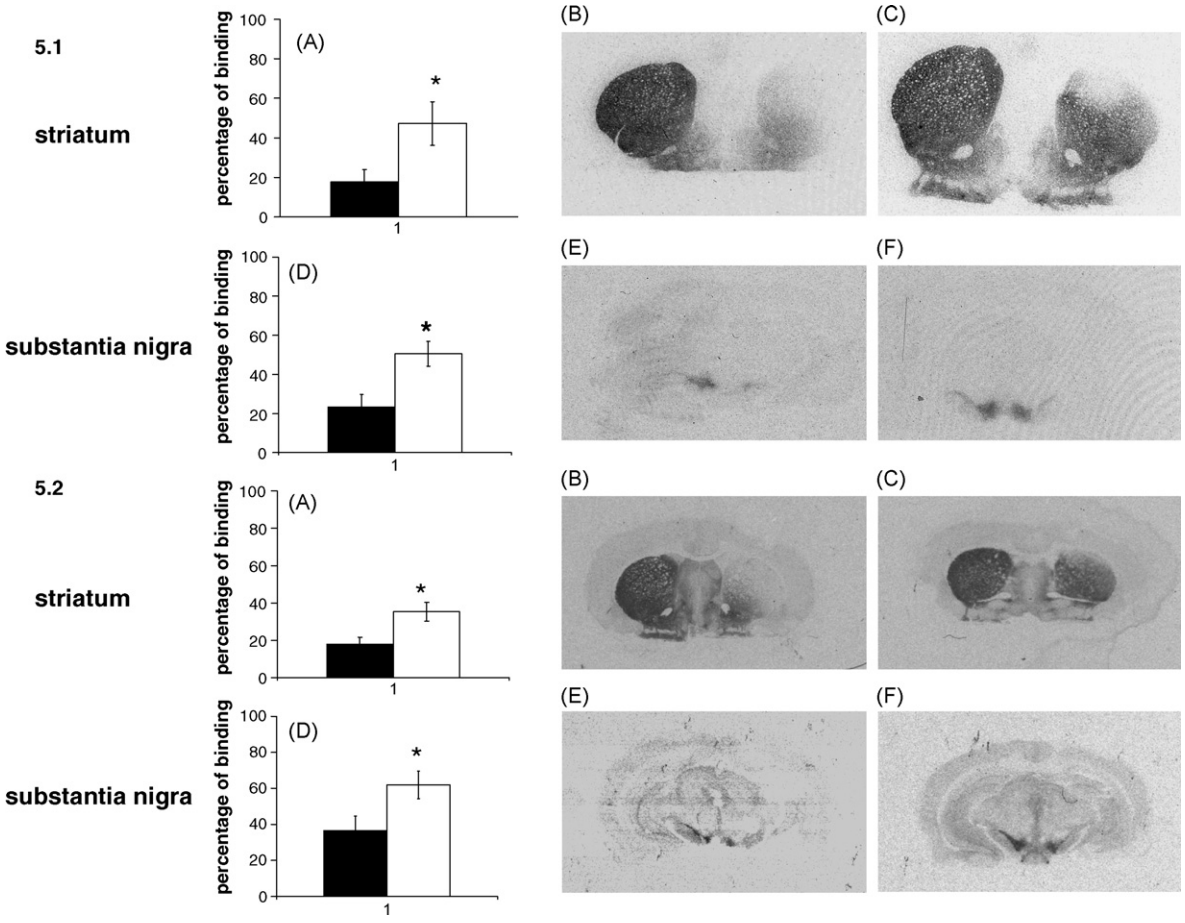

Fig. 5. Autoradiographic study of DAT with [^{125}I]-PE2I (5.1) and $VMAT_2$ with [^{3}H]-DTBZ (5.2) in animals grafted or not with MSC cultured in enriched medium. The binding was measured in the striatum (5.1A, 5.2A) and in the substantia nigra (5.1D, 5.2D). Results are expressed as the percentage of binding in the ipsilateral (lesioned, right side) compared to that in the controlateral (intact, left side) side. Group I (■), $n = 6$; group III (□), $n = 8$. Errors bars are S.E.M.; $^{*}p < 0.05$, significantly different from group I (Mann–Whitney test). Representative images show striatum staining in group I (5.1B and 5.2B) and group III (5.1C and 5.2C); substantia nigra staining in group I (5.1E and 5.2E) and group III (5.1F and 5.2F).

NF-M200 or βIII-tubulin before any specific differentiation process. In our system, we did not obtain a complete expression of neuronal markers, possibly because of the low concentration of Shh as compared to the high concentration (up to 500 ng/mL) used by others (Yan et al., 2005). As well, during the differentiation process, we used fetal calf serum, which inhibits nestin expression and could block in part neural differentiation (Wislet-Gendebien et al., 2003, 2005). These

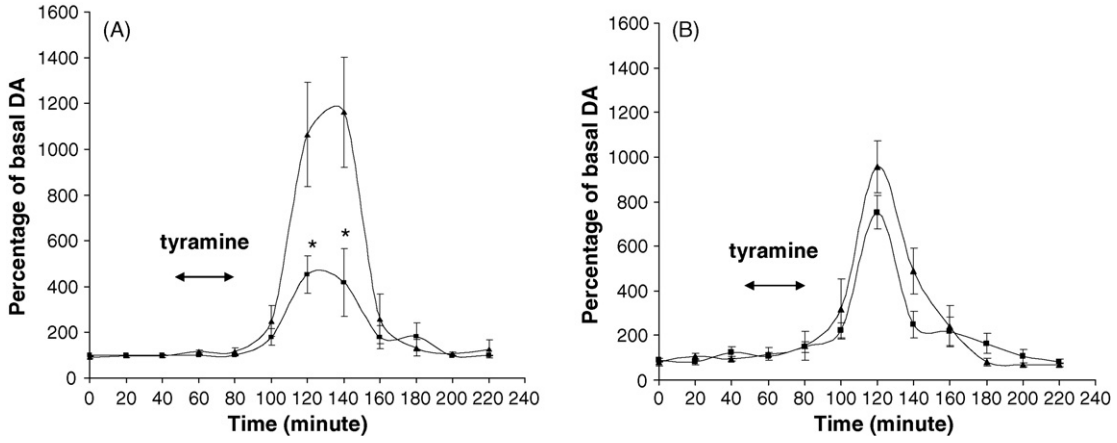

Fig. 6. Microdialysis studies showing the vesicular storage pool of dopamine in animals grafted or not with MSC cultured in enriched medium. (A), release of DA (% basal level) in the lesioned (■) and intact (▲) striatum after a tyramine challenge (0.8 mM through the microdialysis probe) in the group of no grafted animals ($n = 6$). (B), release of DA (% basal level) in the lesioned (■) and intact (▲) striatum after a tyramine challenge (0.8 mM through the microdialysis probe) in the group of enriched MSC-grafted animals ($n = 5$). Results are expressed as means % of basal values ± S.E.M.; $^{*}p < 0.05$, comparison between both striatum with the Mann–Whitney test.

findings agree with an incomplete engagement through the neural pathway.

The present study was carried out to further investigate the potential of MSC in behavioral and neurochemical recovery in a 6-OHDA-lesioned rat model of Parkinson's disease characterized by a severe but partial and stable lesion of the nigro-striatal dopaminergic pathway. The severity of the 6-OHDA lesion depends on factors such as the dosage, the site and the number of injections (Kirik et al., 1998). Thus, the partial lesion model we used could represent a symptomatic stage of Parkinson's disease with a mean 75% loss of TH-positive neurons in substantia nigra accompanied by a 40–60% loss of TH-positive fibers in the striatum, depending on the area. Dopaminergic nigro-striatal nerve terminal density as assessed by the DAT ligand PE2I, showed a mean 80% reduction in the striatum. A similar reduced density of this dopaminergic marker was observed in the substantia nigra, showing that intrastriatal injection of 6-OHDA provoked a loss of both striatal nerve terminals and nigra cell bodies, as has been described in this type of model (Sauer and Oertel, 1994; Przedborski et al., 1995). The reduced density of $VMAT_2$ in both the striatum and substantia nigra confirmed this loss of dopaminergic nerve terminals and cell bodies. By contrast, few modifications were observed in the striatal density of both dopamine D_1 and D_2 receptors. No modification in density of D_1 receptors had already been observed in this type of model (Przedborski et al., 1995), whereas an increased density in D_2 receptors can occur after 6-OHDA lesion (Przedborski et al., 1995; Xu et al., 2005). Because our autoradiographic studies were performed at 8 weeks post-lesion, we can hypothesize that the hypersensitivity of striatal D_2 receptors, which is a compensatory mechanism occurring in the early stage of lesion, was no more present at this late stage. The great reduction in D_2 receptor binding observed in the substantia nigra could be explained by the loss of cell bodies where these receptors are localized as autoreceptors.

MSC cultured in an enriched medium allowed them to acquire some characteristics orientated toward neural cells compared to MSC cultured in a standard medium. The effects of the intra-striatal grafting of MSC was assessed by follow-up with the rotation-stimulated test, evaluation of dopaminergic markers at the nerve-terminal and cell-body levels, and functional release of DA after pharmacological challenge. One week after grafting, the number of rotations per min was 40–50% reduced in both grafted groups comparing to the no grafted and sham groups, the reduction being maintained during 6 weeks post-grafting. In the second experiment aimed to microdialysis studies, we observed a similar reduction in the rotational behavior (data not shown).

At 6 weeks post-grafting, the examination of dopaminergic markers (i.e., TH, DAT and $VMAT_2$) on the lesioned side showed an overall increase in density as compared to that in the no grafted group. In the striatum, a moderate 15–20% recovery was found for TH and $VMAT_2$ and reached 30% for DAT. The higher recovery of DAT as compared to TH and $VMAT_2$ in grafted animals could be explained by a higher sensitivity of the binding method and/or by a better sensitivity of DAT as a marker of dopaminergic neurons in the injured striatum (Ito et al., 1999; Inaji et al., 2005). In the substantia nigra, a 20–25% recovery was observed in grafted animals for the markers of cell bodies and dopamine D_2 receptors. An unexpected result was observed for the dopamine D_1 receptors, which were significantly reduced in density in the substantia nigra pars reticulata of grafted group as compared to the no grafted group. This apparent response to grafting remains to be elucidated.

Extracellular DA levels from the presynaptic vesicular pool measured simultaneously in the lesioned and intact striata of no grafted and grafted animals demonstrated that the transplantation had a recovery effect in the tyramine-stimulated release of DA. Such in vivo effect had already been found in rat models of Parkinson's disease after transplantation of fetal neurons (Rioux et al., 1991) or embryonic stem cells (Rodriguez-Gomez et al., 2007), but never after MSC grafting.

The present study thus demonstrated functional (behavioral) and neurochemical recovery after grafting of adult bone-marrow MSC. This finding could be interpreted as a sparing of dopaminergic neurons and/or as a sprouting from the remaining intact fibers in the striatum. Concerning the effects observed in the substantia nigra pars compacta (increase of TH staining, DAT and D_2R), it can be hypothesized that the graft of MSC might have prevent the progressive neuronal dead. Indeed, it has been shown in the model we used that 2 weeks after 6-OHDA lesion, a percentage of intoxicated dopaminergic neurons did no more express dopaminergic markers such as TH, but are not yet dead, this dead being progressive (Sauer and Oertel, 1994). According to the behavioral test, this recovery occurred as soon as 1 week after grafting and remained stable during 6 weeks. We were able to detect a BrdU signal in the site of transplantation, thus showing that cells derived from the grafted population were still present 6 weeks after graft. However, the hypothesis of in situ differentiation of transplanted cells seems unlikely because of the short delay between the grafting and behavioral recovery. Indeed, transplantation of undifferentiated ES cells in a rat model of Parkinson's disease has been shown to improve amphetamine-induced rotational behavior beginning at 7 weeks after grafting, this effect being explained by in situ differentiation of ES cells in functional dopaminergic neurons (Björklund et al., 2002). The second hypothesis is that transplanted MSC had produced neurotrophic factors, chemokines or cytokines as has been described in vitro (Sensebé et al., 1997). In our culture conditions, MSC express RNA encoding neurotrophic factors in vitro (BDNF, GDNF, FGF2 and FGF8). This finding could result in a blocking effect of neuron degeneration accompanied or not by a sprouting into the striatum, leading to an early and long-lasting reduction of behavioral response to amphetamine, as has been observed after intra-striatal administration of glial cell line-derived neurotrophic factor (GDNF) in a 6-OHDA lesion model close to the one we used (Kirik et al., 2000, 2001).

In conclusion, this study demonstrate that transplantation of a population of adult bone-marrow MSC induced partially in a neural pathway is able to restore in part the dopaminergic function of the nigrostriatal pathway leading to an early improvement of behavior, an increased density of dopaminergic

markers, and a in vivo recovery of DA release, in a rat model of Parkinson's disease. The causes of these improvements need to be explored. In the future, grafting MSC (autologous or allogeneic) could represent a simple and efficient treatment of Parkinson's disease in humans.

Acknowledgements

This work was supported by grants from EFC-CA and IFR135, Tours, by the Région Centre (Programmes de Recherche Interorganismes) and was funded in part by the EC-FP6 project Diagnostic Molecular Imaging (DiMI), LSHB-CT-2005-512146-3. We thank Professor C. Andrès for his precious help in molecular biology studies, and Guillaume Gatefait and Alain Langonné for technical assistance.

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