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MODELING NIGROSTRIATAL DEGENERATION IN ORGANOTYPIC CULTURES, A NEW EX VIVO MODEL OF PARKINSON'S DISEASE

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Abstract

Parkinson's disease (PD) is the second most frequent neurodegenerative disorder afflicting 2% of the population older than 65 years worldwide. Recently, brain organotypic slices have been used to model neurodegenerative disorders, including PD. They conserve brain three-dimensional architecture, synaptic connectivity and its microenvironment. This model has allowed researchers a simple and rapid method to observe cellular interactions and mechanisms. In the present study, we developed an organotypic PD model from rat brains that includes all the areas involved in the nigrostriatal pathway in a single slice preparation, without using neurotoxins to induce the dopaminergic lesion. The mechanical transection of the nigrostriatal pathway obtained during slice preparation induced PD-like histopathology. Progressive nigrostriatal degeneration was monitored combining innovative approaches, such as diffusion tensor magnetic resonance imaging (DT-RMI) to follow fiber degeneration and mass spectrometry to quantify striatal dopamine content, together with bright-field and fluorescence microscopy imaging. A substantia nigra dopaminergic cell number decrease was observed by immunohistochemistry against rat tyrosine hydroxylase (TH) reaching 80% after 2 days in culture associated with a 30% decrease of striatal TH-positive fiber density, a 15% loss of striatal dopamine content quantified by mass spectrometry and a 70% reduction of nigrostriatal fiber fractional anisotropy quantified by DT-RMI. In addition, a significant decline of medium spiny neuron density was observed from days 7 to 16. These sagittal organotypic slices could be used to study the early stage of PD, namely dopaminergic degeneration, and the late stage of the pathology with dopaminergic and GABAergic neuron loss. This novel model might improve the understanding of PD and may represent a promising tool to refine the evaluation of new therapeutic approaches.

Keywords

Parkinson's disease; nigrostriatal organotypic slices; MRI; diffusion tensor imaging; dopamine; mass spectrometry

INTRODUCTION

Parkinson's disease (PD) is the second most common neurodegenerative disorder affecting 2% of the population older than 65 years, with 7–10 million people concerned worldwide (Lees et al., 2009; Gazewood et al., 2013). Clinically, the cardinal symptoms are tremor, rigidity of muscles, bradykinesia and loss of postural reflexes (Parkinson, 1817). The major symptoms are due, in part, to the lack of dopamine released in the striatum as a result of degeneration of dopaminergic neurons in the substantia nigra (SN). Dopaminergic neuronal death seems to be induced by α -synuclein aggregation, forming Lewy bodies which are toxic (Spillantini et al., 1997; Volles and Lansbury, 2003). At present, there is no cure for PD and treatments are merely symptomatic. Current therapy based on a dopamine replacement strategy consists mainly on the oral administration of the dopamine precursor L-3,4-dihydroxyphenylalanine (L-DOPA), but in long-term administration some secondary effects may appear (Ecker et al., 2009; Hauser, 2009). Novel drug and cell therapy approaches require extensive evaluation before routinely being used in humans (Poewe et al., 2012; Lindvall, 2013).

A wide variety of *in vitro* (Alberio et al., 2012) and *in vivo* models (Jackson-Lewis et al., 2012) have been developed to elucidate the pathogenesis, cell death mechanisms and to evaluate therapeutic strategies for PD. These PD models require a reproducible and well-characterized degeneration of the nigrostriatal dopaminergic system which is the main, but not the only pathway involved in PD. *In vivo* models can be broadly divided into genetic (Dawson et al., 2010) and neurotoxic models (Tieu, 2011). However, *in vivo* studies require high technical and financial resources and they do not allow to simultaneously test several conditions in the same animal. Furthermore, it results in an important difficulty to address different engraftment mechanisms when testing cell therapy approaches. In contrast, *in vitro* experiments with dopaminergic neurons are easy to develop and use but they do not allow the study of cell interactions with the microenvironment or with the host tissue.

Recently, organotypic brain slice cultures have been used for PD modeling. Since their introduction, organotypic brain cultures have become a useful tool to study physiological properties of tissues, to monitor both acute and chronic effects of drugs and to study neurological disorders as in global cerebral ischemia or PD (Daviaud et al., 2013). Striatum–mesencephalon and cortex–striatum–mesencephalon co-cultures were developed to study tyrosine hydroxylase (TH)-positive neurons, being TH the rate-limiting enzyme in dopamine synthesis (Ostergaard et al., 1990). Those co-cultures maintained brain morphological and electrophysiological characteristics but did not mimic PD hallmarks (Plenz and Kitai, 1996). In order to better simulate *in vivo* models in which nigrostriatal degeneration is caused by dopaminergic neurotoxin injection, organotypic slices were next treated or injected with rotenone (Stahl et al., 2009) or 6-hydroxydopamine (6-OHDA) (Cavaliere et al., 2010).

However, as in animal models, 6-OHDA led to neuronal death mediated by oxidative stress inducing selective dopaminergic neuron degeneration but did not produce extra-nigral pathology or Lewy body-like inclusions (Schober, 2004; Cavaliere and Matute, 2011). More recently, whole rat brain sagittal organotypic slices were developed which maintained dopaminergic and cholinergic neurons, as well as a complex capillary network and long nerve fibers (Ullrich et al., 2011). In the same way, sagittal sections of mouse brain were performed. These slices maintained the integrity of neuronal pathways during 3 weeks in culture and were immersed in 6-OHDA to induce a dopaminergic pathway degeneration (Kearns et al., 2006).

In the present study, we developed a sagittal organotypic culture model that includes all the areas involved in the nigrostriatal pathway in a unique slice without using neurotoxins to induce the dopaminergic lesion. The goal was to induce progressive nigrostriatal degeneration in a single step by mechanical transection of the medial forebrain bundle (MFB) while preparing the slices, in order to obtain a simple reproducible PD *ex vivo* model. We next studied slice survival while cultured with different media to maximize slice viability. The dopaminergic nigrostriatal pathway whose fibers run within the MFB was characterized by immunostaining against TH. Furthermore, progressive nigrostriatal degeneration was monitored combining two innovative approaches, magnetic resonance imaging (MRI) and mass spectrometry, rarely used for this type of study in organotypic slices. Fractional anisotropy (FA) measurements by diffusion tensor magnetic resonance imaging (DT-RMI) were used to follow and characterize MFB fiber degeneration in the organotypic slices. Indeed, DT-RMI provides quantitative information on water diffusion which can be used to evaluate the biological tissue properties. Furthermore, a liquid chromatography–mass spectrometry (LC–MS/MS) method to quantify striatal dopamine content directly in organotypic tissue was developed in this study. This new *ex vivo* PD model may represent a promising tool to refine the evaluation of new therapeutic approaches as cell therapy or tissue engineering, among others.

EXPERIMENTAL PROCEDURES

Preparation of nigrostriatal organotypic slices

Animal care and use were in strict accordance with the regulations of the French Ministry of Agriculture and all animal procedures were approved by animal experimentation ethics committee of Pays de la Loire. Every effort was made to minimize animal suffering and the number of animals used.

Timed pregnant Sprague–Dawley rats were purchased from Janvier (Saint Berthevin, France) or from SCAHU (Service commun d'animalerie hospitalo-universitaire, Angers University, France). Postnatal 6–8-day-old pups were used to prepare organotypic slices according to the Stoppini method (Stoppini et al., 1991) with modifications. Pups were anesthetized by an intraperitoneal injection of 80 mg/kg of ketamine (Clorketam 1000, Vetoquiol, Lure, France) and 10 mg/kg of xylazine (Rompum 2%, Bayar Health Care, Kiel, Germany). Animals were sacrificed, brains were rapidly dissected and cerebral hemispheres were separated and glued onto the chuck of a water-cooled vibratome (Motorized Advance Vibroslice MA752, Campdem Instruments, Loughborough, UK) to be sagittally cut. Under

aseptic conditions, 400- μ m slices were cut in different configurations in order to obtain a progressive degeneration of the nigrostriatal pathway. Finally, sagittal sections were cut alongside of the midline, with a 14-degree angle of the razor blade, and placed in sterile ice-cold Gray's Salt Balanced Solution (Sigma–Aldrich, St. Louis, MO, USA) supplemented with 6.5 mg/L of glucose and antibiotics (100 U/ml penicillin, 0.1 mg/ml streptomycin, 0.25 μ g/ml amphotericin B) (Sigma–Aldrich, St. Louis, MO, USA) for 1 h. Typically, about 10 slices can be obtained per hemisphere. The first 2–3 slices and the two last brain slices did not contain the three main areas involved in the pathology and were discarded. Three to four slices per hemisphere were next transferred to 30-mm diameter semiporous membrane inserts (Millicell-CM, Millipore, Bedford, MA, USA) within a 6-well plate and put in culture at 37 °C, 5% CO₂. A total of about 30 rat pups and about 120 organotypic slices were necessary to perform the whole characterization. For each condition, a minimum of three slices taken from three different rat pups were used.

Nigrostriatal organotypic slice culture

Slices were cultured at the air–liquid interface and maintained using two different protocols. In the first protocol the slices were cultured during 16 days in a single medium: 50% Minimum Essential Medium Eagle (MEM) (Sigma–Aldrich, St. Louis, MO, USA), 25% Hank's Balanced Salt Solution (Hank's) (Sigma–Aldrich, St. Louis, MO, USA), 25% of decompemented horse serum (Gibco, Life Technologies, Paisley, UK), 6.5 mg/ml of glucose, 1 mM of L-glutamine (Sigma–Aldrich, St. Louis, MO, USA) and antibiotics. In the second protocol tested, the slices were conserved in two different media. From days 0 to 3, a serum-containing medium identical to first protocol culture media condition was used: 50% MEM, 25% Hank's, 25% of decompemented horse serum, 6.5 mg/ml of glucose, 1 mM of L-glutamine and antibiotics. From days 3 to 16, a serum-free medium was used: Neurobasal medium (Gibco, Life Technologies, Paisley, UK) supplemented with 6.5 mg/L of glucose, 1 mM of L-glutamine, 1 $\times$ B27 supplements (Gibco, Life Technologies, Paisley, UK) and antibiotics. The media was changed the first day after culture and then every 2 days during the entire culture period for both culture media protocols.

Histological studies

At different times, ranging from 0 to 16 days, organotypic slices were washed with phosphate-buffered saline (PBS) (Lonza, Verviers, Belgium), fixed with 4% paraformaldehyde (PFA) (Sigma–Aldrich, St. Louis, MO, USA) in PBS pH 7.4 for 2 h and washed three times with PBS. Finally, slices were removed from membrane inserts and stored at 4 °C in PBS until use.

Immunofluorescence studies—Immunofluorescence was performed using antibodies against neuronal nuclei (NeuN) (clone A60, Merck Millipore, Billerica, MA, USA) and dopamine- and cAMP-regulated neuronal phosphoprotein (DARPP32) (BD Transduction Laboratories, Erembodegem, Belgium). Isotypic controls were performed for each antibody. Slices were incubated free-floating in PBS containing 1% Triton (PBST) (Sigma–Aldrich, St. Louis, MO, USA). After pre-blocking for 4 h with 4% bovine serum albumin (BSA, fraction V) (PAA Laboratories, Piscataway, NJ, USA), 10% normal goat serum (NGS) (Sigma–Aldrich, St. Louis, MO, USA) in PBST, slices were incubated for 48 h at 4 °C with

mouse anti-NeuN (20 µg/ml in 4% BSA PBST) or with mouse anti-DARPP32 (0.25 µg/ml in 4% BSA PBST). After three washes with PBS, the sections were incubated for 2 h with the horse affinity-purified biotinylated anti-mouse immunoglobulin G (IgG) secondary antibody (7.5 µg/ml, Vector Laboratories, Les Ulis, France) at room temperature. Then, slices were washed and incubated for 2 h with Streptavidin Fluorophores R488 or R547H (Interchim, Montluçon, France) diluted 1:200 in PBS. Finally, the sections were rinsed and mounted using fluorescent mounting medium (Dako, Carpinteria, CA, USA).

TH immunohistochemistry—To observe dopaminergic degeneration a mouse anti-rat TH antibody (clone 6D7, Covance, Emeryville, CA, USA) was used and isotypic controls were performed. After pre-blocking for 4 h with 4% BSA, 10% NGS in PBST, slices were incubated for 48 h at 4 °C with mouse anti-rat TH (10 µg/ml in PBST). After three washes with PBS, slices were incubated for 2 h with the goat affinity-purified biotinylated anti-rabbit IgG secondary antibody (7.5 µg/ml in PBST) (Vector Laboratories, Les Ulis, France). Quenching of peroxidase was made with 0.3% hydrogen peroxide (H₂O₂) (Sigma-Aldrich, St. Louis, MO, USA) in PBST, at RT for 20 min. After three washes, sections were then incubated with Vectastain ABC reagent (Vector Laboratories, Les Ulis, France) in PBS was made for 2 h. Finally, sections were washed and revealed with 0.03% H₂O₂, 0.4 mg/ml diaminobenzidine (Sigma, St. Louis, MO, USA) in PBS supplemented with 2.5% nickel chloride and dehydrated before mounting.

Quantification of TH-positive fibers and cells

TH-positive fiber density in the striatum at different time-points ranging from 0 to 16 days was quantified using the Metamorph[®] software. The TH fiber density was estimated by subtracting the striatum TH staining intensity minus the cortex TH staining intensity, which represents the background. TH staining was expressed in% considering that TH staining at day 0 (intact slice) represents 100%. Results were presented as mean differences±standard deviation and were calculated from two batches of three consecutive pictures in the center of the striatum and from six consecutive slices in the cortex above the striatum. Those pictures were taken from three different slices for each time-point.

The number of TH-positive neurons in the SN at different time-points ranging from days 0 to 16 was determined. Images were taken from three to four different slices for each time-point, in order to cover the whole SN. The number of positive-cells was manually counted assisted by Metamorph[®] analysis tool software. Results were presented as mean differences ±standard deviation.

Quantification of NeuN-positive neurons

The survival of total neurons in certain organotypic slice regions was estimated by immunofluorescence against NeuN. NeuN-positive neuron density was calculated, from days 0 to 16 using the Metamorph[®] software. At each time-point, six pictures taken from three different slices showing cortex, striatum and SN were used. NeuN staining was expressed in % considering that NeuN staining at day 0 (intact slice) represents 100%. Results were presented as mean differences±average deviation.

Analysis of striatal dopamine content by mass spectrometry

Microdissected striata were dissociated by adding 200 μ l of 1 $\times$ reporter lysis buffer (Promega, Madison, WI, USA) followed by 14 s of ultrasonication. Then, 600 μ l of pure acetonitrile were added to precipitate proteins. After a centrifugation (10,000g, 15 min, 4 $^{\circ}$ C), samples were dropped onto an Ostro 96-well plate (Waters S.A., Saint-Quentin, France) following manufacturer's instructions to purify dopamine. Chromatography was performed using a Waters Alliance 2695 system (Waters S.A., Saint-Quentin-en-Yvelines, France) with a Uptishpere[®] C18 50DB 150 $\times$ 2.0 mm, 5 μ m column (Interchim, Montluçon, France). The column was heated up to 40 $^{\circ}$ C, the mobile phase was composed of water/formic acid 0.1% – methanol/formic acid 0.1% (95/5) from 0 to 8 min and then water/formic acid 0.1% – methanol/formic acid 0.1% (5/95) from 8 to 10 min with a return to normal from 10 to 11 min. The flow-rate was 0.3 mL/min. High-performance liquid chromatography (HPLC) effluent between 1 and 4 min was directed into a Quattro Micro triple quadrupole mass spectrometer (Waters S.A., Saint-Quentin, France). Ionization was achieved using electrospray in positive ion mode. The mass spectrometer operated in multiple reactions monitoring (MRM) mode. The (M H)⁺ m/z transitions for each compound were 137 and 91. A typical retention time of dopamine was found to be 1.50 min. Quantification was achieved with QuantLynx (Waters S.A., Saint-Quentin, France) by comparison of the observed peak area ratios of dopamine samples to a calibration curve obtained under the same experimental conditions.

Visualization of the MFB by magnetic resonance imaging

Imaging was performed on a Biospec Avance III MR scanner (Bruker Biospin, Wissembourg, France) using a 20-cm bore 7 T magnet equipped with a BGA12S gradient/shim system capable of 675 mT/m maximum gradient strength and a ¹H cryoprobe.

For the *in vivo* experiments, the pups were sedated with a mixture of isoflurane/O₂ (~0.1%–0.1 l/min) and wrapped in a cotton blanket positioned over a warm water blanket. After a rapid scout set of images and a first and second order shim over the brain, a two-segments 2D-Echo planar imaging sequence (repetition time TR/Effective echo time TE_{eff} 3500/30 ms) with 46 diffusion encoding directions, γ =3 ms, γ =17 ms, leading to a b =1000 s/mm² was performed. The geometrical parameters were fixed at FOV=14 mm $\times$ 14 mm. Three contiguous slices of 0.4 mm. The matrix size was defined at 128 $\times$ 96 leading to an in-plane resolution of 109 μ m $\times$ 146 μ m. As the number of experiment (N_{exp}) was fixed at 2, the total acquisition time was 22 min.

Organotypic slices were imaged at different times ranging from 0 to 16 days. Orthogonal scout proton images were obtained using a rapid acquisition with relaxation enhancement (RARE) sequence (TR=3.200 ms; (TE)=60 ms; RARE factor=4; FOV=1.5 $\times$ 1 cm; matrix 256 $\times$ 128; Two contiguous slices of 0.3 mm, N_{exp}=4). A localized first and second order shim was performed on the slice prior to diffusion imaging. A four-segment 2D-Echo planar imaging sequence (TR/TE_{eff} 3500/30 ms) with 46 diffusion encoding directions, γ =3 ms, γ =17 ms, leading to a b =1000 s/mm² was performed. The geometrical parameters were fixed at FOV=15 mm $\times$ 10 mm for a slice thickness of 300 μ m. The matrix size was defined at

128 × 96 leading to an in-plane resolution of 117 μm × 104 μm. As the number of repetitions was fixed at 4, the total acquisition time was 43 min.

Statistical analysis

Data are presented as the mean value of three independent experiments ± standard deviation (SD), unless otherwise stated. Significant differences between samples were determined using an analysis of variance (ANOVA) test, followed by a Scheffe post hoc test which indicated if conditions were significantly different. *P*-value was set to 0.05, unless otherwise stated.

RESULTS

Morphology of nigrostriatal organotypic slices

After testing different angles of slice, 400-μm sagittal organotypic slices were cut alongside the brain midline (Fig. 1A). Morphological analysis of organotypic brain slices cultured from neonatal tissue just after preparation showed that many brain areas could be clearly identified using bright-field microscopy like the hippocampus, cortex, caudate nucleus-putamen, globus pallidus, ventral pallidum, SN and cerebellum among others (Fig. 1B). The nigrostriatal pathway, which is involved in PD, is composed of dopaminergic fibers that have their cell bodies in the SN, which send axons through the MFB, to the striatum where dopamine is liberated. Striatum observation under bright-field microscope allowed to identify those dopaminergic fibers (Fig. 1C) by TH immunohistochemistry, just as two-photon fluorescence microscopy of TH-immunostained nigrostriatal slices at day 0 allowed to easily visualize the dopaminergic cell soma in the SN and the nigrostriatal pathway from the SN to the striatum (Fig. 1D).

Culture media choice and viability of nigrostriatal organotypic slices

Culturing conditions remarkably affected organotypic culture viability. We found that, when culturing the slices with culture media condition 1 that contains 25% of serum, the slices underwent progressive and rapid flattening after 5–7 days of culture. This deleterious effect of serum has already been described (Kim et al., 2013). However, when culturing the slices with culture media condition 2 where serum-containing media is switched to serum-free media 3 days after the initiation of the cultures, organotypic slice survival was enhanced and a better preservation of the original brain morphology was observed. Using these conditions, organotypic slices can be kept viable for periods longer than 2 weeks in culture. Thus, culture media condition 2 was selected for further experiments.

Using NeuN immunofluorescence, the survival of neurons within specific brain regions of the organotypic slices using media condition 2 was further evaluated (Fig. 2A). Between days 1 and 6, a decrease of less than 10% ± 5% of NeuN-positive cells in the cortex was observed (Fig. 2B). In contrast, a significant 60% ± 10% and 80% ± 1% decrease of NeuN staining occurred in the striatum and in the SN, respectively, between days 1 and 16 (Fig. 2B). *P*-value was set to 0.05 and experiments were performed with at least three brain slices from three different rat pups/condition.

Organotypic slice cultures display progressive degeneration of the nigrostriatal pathway

A thinning of the striatum within 3 days accompanied by a scarcity of fibers was observed by bright-field microscopy, predicting a reduction in dopaminergic terminals, which seemed to be total at day 16 (Fig. 3A). This dopaminergic deafferentation in the MFB was confirmed by immunochemistry against rat TH. A continuous loss of TH-positive fibers localized in this area was observed. At day 16, there were no TH-positive fibers left (Fig. 3A). A quantification of the striatal fiber diminution was made using Metamorph analysis tool. A $30\% \pm 20\%$ decrease of TH fibers was detected at day 2 compared to day 0, reaching a decrease of $40\% \pm 20\%$ at day 3. TH staining reached a $90\% \pm 15\%$ decrease at day 4 and disappeared until day $16 \pm 10\%$ (Fig. 3B). *P*-value was set to 0.05 and experiments were performed with at least three brain slices from three different rat pups/condition.

Moving upstream in the nigrostriatal circuit, the number of TH-immunoreactive neurons in the SN was evaluated at days 0, 2 and 16. An important decrease of dopaminergic neurons in this area was already observed after 24 h, reaching $80\% \pm 20\%$ after 48 h and being maximal after 16 days, with a $90\% \pm 15\%$ loss (Fig. 3C). *P*-value was set to 0.01 and $n=5$. The severe loss of DA nerve terminals in the striatum was accompanied with an important loss in the neurons of the SN.

Organotypic slice cultures showed loss of striatal dopamine content

Dopamine content in the striatum of the slices was measured by LC-MS/MS at different times after organotypic preparation. As can be seen in Fig. 3D, dopamine content in the striatum diminished in the time-period examined. After 3 days of culture a reduction of $30\% \pm 15\%$ of dopamine content was detected with a dopamine concentration decreasing from 65 ± 7 pg/ml at days 0 to 45 ± 5 pg/ml at day 3. Then, similar striatal dopamine content levels were found between days 3 and 16 (Fig. 3D). *P*-value was set to 0.05 and experiments were performed with at least three brain slices from three different rat pups/condition.

Delayed DARPP32-positive medium spiny neuron degeneration

An analysis by immunofluorescence of DARPP32-positive medium spiny neurons, the most numerous in the striatum, was performed at days 3, 6, 7, 11 and 16. Results showed that these neurons seemed to be only slightly affected by mechanical denervation before day 7. After 7 days in culture, a loss of about 50% of the striatal medium spiny neurons (MSNs) was detected; this loss may reach about 90% of the MSNs after 11 days in culture and seems to be total after 16 days. The loss of GABAergic MSCs observed from days 7 to 16 might not be due to the degeneration of the slice over the culture period since NeuN immunostaining showed that organotypic slices can be kept viable for periods longer than 2 weeks in culture (Fig. 4).

Organotypic slice cultures presented progressive loss of the MFB

Fiber MRI tractography was used to demonstrate the orientation and integrity of the MFB fibers *in vivo*. Cellular barriers encountered in highly directionally oriented tissues, such as white matter fibers, result in anisotropic water diffusion (smaller diffusion perpendicular than parallel to the fiber axis). The degree of directionality of diffusion can be quantified by

the measure of the FA parameter. FA value ranges from 0 to 1 when anisotropy increases (Shepherd et al., 2003, 2006b). MFB visualization using tractography was possible in living rats (Fig. 5A). Then, MRI tractography of ex vivo slices from days 0 to 16 was performed, and despite the thinness of the tissue, MFB fibers could be observed. A progressive reduction of the FA from days 0 to 7 was detected (Fig. 5B). It reached $40\% \pm 10\%$ at 24 h, $70\% \pm 15\%$ at 48 h and $80\% \pm 1\%$ at day 7. Afterward, the MFB could not be distinguished from the surrounding tissue and measurement could not be unambiguously performed (Fig. 5C). *P*-value was set to 0.01 and experiments were performed with at least three brain slices from three different rat pups/condition.

Furthermore, TH immunohistochemistry showed a rapid degeneration of dopaminergic fibers in the MFB as well. Just after slice preparation, MFB fibers were intact. Three days later, an important degeneration occurred and varicosities were evident. At day 14, no more dopaminergic fibers were detected in the MFB by TH immunohistochemistry (Fig. 5B).

DISCUSSION

Organotypic cultures have great potential for disease modeling providing an ideal platform between *in vitro* and *in vivo* studies. They are easy to prepare and culture and interestingly, when sectioned appropriately, they retain the architecture and microenvironment of the original organ. Hence, organotypic brain slices have been used as models for neurological disorders such as stroke, epilepsy, cerebral ischemia or PD, among others (Davidaud et al., 2013). The present work sought to develop and characterize a new ex vivo model able to mimic early and late stages of PD based on the mechanical transection of the nigrostriatal pathway obtained during slice preparation. This is the first report that characterizes progressive nigrostriatal degeneration in organotypic slices using MRI and the evaluation of dopamine content using quantitative mass spectrometry together with immunohistochemistry and immunofluorescence.

Previous PD organotypic models used principally SN (Jaeger et al., 1989), ventral mesencephalon–striatum (Ostergaard et al., 1990), entorhinal–hippocampal (Diekmann et al., 1994), cortex–striatum (Plenz and Aertsen, 1996a,b) or complex triple slice cultures (Plenz and Kitai, 1996) that did not allow an easy study of all brain regions involved in this pathology. In the present work, brain sagittal slices were used since they contain in a unique slice many anatomical structures as corpus callosum, cortex, hippocampus, striatum, globus pallidus, SN and cerebellum among others. In addition, many of the original neuronal connections between brain structures are better preserved in brain sagittal slices (Ullrich et al., 2011). Furthermore, the subgranular zone of the dentate gyrus and the subventricular zone, two neural stem cell niches, are conserved which allows to study neurogenesis (Garzon-Muvdi and Quinones-Hinojosa, 2009). Sagittal sections remain the most complete type of organotypic slices since they allow the observation of cholinergic, GABAergic and dopaminergic neurons as well as of vascular brain capillaries or cortico-striatal and cortico-hippocampal nerve fibers (Kearns et al., 2006; Ullrich et al., 2011).

Organotypic slices are generally cultured using either, the roller tube culture method described by Gahwiler (1981) with a slice survival rate of up to 6–8 weeks, or the membrane

interface air-medium method developed by Stoppini et al. (1991), which offers a survival of about 2–3 weeks. The membrane interface technique that maintains brain slices on a porous membrane filter at the interface between medium and a humidified atmosphere was used in the present study, since this method provides an easier access to the slice culture preparation. Regarding the culture of the slices, the selected culture media protocol used a serum-free media from days 3 to 16. This allows having a well controlled media and studying solely the effect of added drugs or cell grafts on the behavior of brain cells. Ullrich et al. (2011) observed that, in their organotypic whole brain model, nerve growth factor or glial cell line-derived neurotrophic factor addition in the serum-containing media was necessary to support cholinergic or dopaminergic neuron survival, respectively. Moreover, with this culture media protocol whole slice survival seemed to be good enough with no significant flattening or distortion. In contrast, Ullrich et al. (2011) observed that, in their organotypic whole brain model, nerve growth factor or glial cell line-derived neurotrophic factor addition in the serum-containing media was necessary to support cholinergic or dopaminergic neuron survival, respectively. However, a culture media containing 25% of heat-inactivated horse serum was used in that study. It has been reported that serum-containing media may induce a strong degeneration of neurons in slice culture (Kim et al., 2013). In other studies (Kearns et al., 2006; Cavaliere et al., 2010) in which organotypic slices were cultured in a serum-free media with B27 supplements similar to our culture conditions, a long-term viability of dopaminergic neurons in organotypic culture was observed. Furthermore, we did not notice an important cell death, by NeuN staining, in the cortex or hippocampus, two regions that are not affected by the sectioning. This proved that a good neuronal survival was obtained, at least until 16 days, allowing the analysis of the pharmacological effects of injected drugs or cells on the organotypic slices during this time-period.

Previous studies of the nigrostriatal pathway used cultures and co-cultures with no loss of dopaminergic fibers that only allow assessing developmental, plastic or regenerative properties of DA neurons (Jaeger et al., 1989; Ostergaard et al., 1990; Diekmann et al., 1994; Plenz and Kitai, 1996; Plenz and Aertsen, 1996a; Ullrich et al., 2011). The single organotypic slices developed by Cavaliere et al. (2010) or Kearns et al. (2006) containing the nigrostriatal pathway could be conserved in culture about 12–14 days and 3 weeks, respectively, without SN dopaminergic neuron loss. This difference of survival could be explained by the fact that Kearns used mouse brain slices which are smaller and easier to keep in culture while Cavaliere used rat brain slices. The PD model developed by Kearns, uses 300–400 μm slices of the brain hemispheres cut in the parasagittal plane using a vibratome. To obtain the dopaminergic neuronal loss those slices were incubated in a 20 mM 6-OHDA bath for 10 min (Kearns et al., 2006). In the model developed by Cavaliere, axial slices of the hemispheres are performed with an angle of 45° compared to the base of the brain, therefore along the axis of the MFB without sectioning the fibers. Consequently slices can be conserved in culture with no dopaminergic degeneration. To obtain the dopaminergic neuronal loss, two protocols were tested. In the first one, slices were incubated in a 100 μM 6-OHDA bath for 60 min and in the second protocol, a transection in the middle of the MFB was performed (Cavaliere et al., 2010). After the 6-OHDA treatment, degeneration observed by Kearns et al. (2006) reached 46% in the SN and 60% in the

striatum, after 2 weeks in culture while Cavaliere et al. (2010) observed a 30% degeneration both in the SN and in the striatum within 24 h. The hand-operated denervation tested by Cavaliere induced a larger degeneration as 60% decrease in TH-positive neurons was observed both in the SN and in the striatum after 3–5 days *in vitro*. In the present model, MFB dopaminergic fiber death is induced during slice preparation. Indeed, the slice is performed along the nigrostriatal pathway, perpendicular to the one made by Cavaliere, so that the MFB fibers are sectioned at their base inducing the degeneration (Fig. 1).

The characterization of the progressive nigrostriatal degeneration was achieved by combining different techniques. MRI studies showed a 70% MFB fiber FA decrease in 2 days, suggesting fiber degeneration. This method has been used to address brain tissue structure on sliced and fixed samples (Shepherd et al., 2006a; Flint et al., 2010) and to demonstrate early pathological changes in parkinsonian patients (Yoshikawa et al., 2004), but to the best of our knowledge, this is the first report describing nigrostriatal pathway degeneration in an organotypic slice PD model by DT-RMI. TH immunostaining quantification revealed a rapid loss of 80% of the dopaminergic cell bodies in the SN and of 30% of striatal fiber density at 2 days. A 15% loss of striatal dopamine content quantified by mass spectrometry after 2 days was also observed, as a result of the terminal loss in the striatum. Those results suggest that in our model, the dopaminergic degeneration occurs first in the SN area, which then affects the MFB and striatal fibers. It is difficult to correlate this observation with PD pathology as the primary cellular locus of dysfunction is a controversial issue (Kordower et al., 2013). This anterograde fiber degeneration confirms the report of Beirowski et al. (2005) showing this directionality in fiber degeneration after sectioning. The relatively quick dopaminergic nigrostriatal pathway degeneration allows obtaining a therapeutic window of around 14 days to study novel therapeutic approaches.

The lesion seems to be quite localized at first to the nigrostriatal pathway, but from days 7 to 16 an important loss of striatal GABAergic MSN occurred. The late GABAergic pathway degeneration observed after 8 days in culture could be due to the damage induced after the sagittal slice preparation as previously reported by Cavaliere et al. (2010). This strong striatal MSN death explains the important decrease of NeuN expression in the striatum as GABAergic MSN represents about 90% of the striatal neurons. Thus, this new *ex vivo* PD model could be used to model early and late stages of the pathology. Actually, from days 0 to 8, a degeneration of the nigrostriatal pathway occurred associated with a dopamine loss in the striatum which corresponds to an early stage of the disease. Then, from days 8 to 14, a degeneration of GABAergic neurons appears which matches the later PD phase.

In spite of the high complexity of PD, experimental models that mimic as close as possible the symptoms and the temporal progression of the disease are needed. Obviously, the model presented in this study differs from a chronic neurodegenerative disorder. Indeed in the human pathology, neurodegeneration is very progressive, occurs over several years and declares mainly in people over 60 years. In our model, the neurodegeneration is observed in 2 weeks, in 7-days-old rat pups which differs from the human pathology. However, despite their limitations, this model offers a good possibility to choose the degree of degeneration and therefore the stage of the disease. Furthermore a new therapeutic approach can be tested in only a few weeks allowing a rapid screening. Recently also adolescent and adult rats have

been used and even human post-mortem tissue slices have been kept alive for a maximum time of 1 week (Finley et al., 2004; Su et al., 2011; Fernandez-Bueno et al., 2012).

Moreover, in PD, dopaminergic neuronal death may be induced by the formation of Lewy bodies mainly composed of α -synuclein aggregation and other aggregated proteins (Volles and Lansbury, 2003; Spillantini et al., 1997). Unfortunately, Lewy body formation was not analyzed in our model. However, it has also been shown that neuronal death in the SN may occur in part by an important overproduction of reactive oxygen species (ROS) which may lead to Lewy bodies formation and toxicity (Blandini, 2013). Thus, since a 2 μ M concentration of ROS was detected in intact hippocampal organotypic slices (Ganesana et al., 2012), ROS formation may be re-created in our model during sagittal slice preparation as well. Indeed, the slice preparation induces an important lesion and a little inflammation of the slice can be observed during the first day in culture. It could be interesting to study this possibility in future studies.

As dopaminergic degeneration has been completely characterized using several techniques, this allows choosing the degree of dopaminergic denervation before starting the screening of new treatments. Indeed, this model would be useful to test both neuroprotective and neuroregenerative strategies. In addition, the model might be a useful tool to understand the fundamental molecular mechanism underlying degeneration. Within this line, axonal regeneration (Ishihara et al., 2011) and glial response (Ziemka-Nalecz et al., 2013) after axotomy could be studied in the model presented here. Moreover, using MRI that produces detailed images of a living brain tissue over time without using radioactive ligands (Petridou et al., 2006), fiber tracts can be visualized in organotypic slices and pathway integrity assessed by calculation of FA and the study of neuronal metabolism using magnetic resonance spectroscopy (Delli Pizzi et al., 2013). This could be used to evaluate the neuroregenerative potential of axonal growth therapeutics among others (Ullrich et al., 2011). To this end, efficacy changes over time can be evaluated using the same treated organotypic slice, thus reducing costs. As demonstrated in this study, LC-MS/MS can also be used to quantify a neurotransmitter. In addition the association of mass spectrometry and microdissection allows performing this neurotransmitter analysis in a chosen area and not only in the culture media (Krabbe et al., 2009).

In summary, this model represents a promising tool to quickly and efficiently test new and better treatments for PD, including cell therapy or tissue engineering. The development and optimization of simple ex vivo models with precise control of the extracellular environment and a complete characterization of the neurodegeneration, allows studying not only the therapeutic effect of the treatment under evaluation but also stem cell responses and the role of the microenvironment. A better comprehension of all the parameters involved in the therapeutic effect is thus obtained. Using this model, a comparative study between two stem cell types associated with microparticles for PD treatment is currently being performed (Daviaud et al., 2011, unpublished results).

CONCLUSION

We have prepared and characterized a relevant ex vivo model of PD using innovative and conventional techniques. This new model allows to study dopaminergic, cholinergic or GABAergic neurons as well as blood vessels or glial cells and their interactions. It is easy to obtain, and the dopaminergic degeneration is reproducible and does not involve complicated surgical process, and does not need neurotoxin injection. Furthermore, this model can be used to study the early stage of the pathology, namely dopaminergic neurodegeneration, or later stage of the disease with dopaminergic and GABAergic neuron loss.

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Abbreviations

6-OHDA	6-hydroxydopamine
BSA	bovine serum albumin
DARPP32	dopamine- and cAMP-regulated neuronal phosphoprotein
DT-RMI	diffusion tensor magnetic resonance imaging
FA	fractional anisotropy
H₂O₂	hydrogen peroxide
Hank's	Hank's Balanced Salt Solution
IgG	immunoglobulin G
LC-MS/MS	liquid chromatography–mass spectrometry
MEM	Minimum Essential Medium Eagle
MFB	medial forebrain bundle
MRI	magnetic resonance imaging
MSN	medium spiny neuron
NeuN	neuronal nuclei
NGS	normal goat serum
PBS	phosphate-buffered saline
PBST	phosphate-buffered saline containing 1% Triton
PD	Parkinson's disease
RARE	rapid acquisition with relaxation enhancement
ROS	reactive oxygen species

SN	substantia nigra
TH	tyrosine hydroxylase

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Fig. 1. Morphology of nigrostriatal organotypic slices shortly before preparation. (A) Schematic view of organotypic culture preparation. (B) Morphological integrity of nigrostriatal organotypic slice. (C) Bright-field observation of the dopaminergic medial forebrain bundle (MFB) fibers emerging from the SN to the striatum. (D) Representative images showing fluorescence and bright-field images of TH-immunostained nigrostriatal pathway.

Fig. 2.

Viability of nigrostriatal organotypic slices. (A) Immunofluorescence against neuronal nuclei (NeuN) showing four brain regions: cortex, hippocampus, striatum and substantia nigra (SN) at day 1 at the top and at day 16 at the bottom. (B) Quantification of the density of NeuN-positive cells in cortex, striatum and SN shows no important loss of staining in the cortex after 16 days in culture while NeuN expression decreased by 60% and 80% in the striatum and the SN respectively. *Significantly different results with $n=3$ and P -value=0.05.

Fig. 3.

Organotypic slice cultures display progressive anterograde degeneration of the nigrostriatal pathway. (A) Bright-field microscope observation (top) and TH immunostaining (bottom) allowed to observe dopaminergic fibers of the nigrostriatal pathway at days 0, 2, 3 and 16 (from left to right). A slimming of dopaminergic fibers of the MFB and the striatum is observed shown by the red arrows. (B) Striatal TH-positive fiber quantification. TH density in the striatum was of 70% at day 2, 60% at day 3 and 10% at day 4 compared to day 0 ($n=3$). (C) Dopaminergic neuron quantification in the SN. The number of dopaminergic neurons remaining in the SN reached 20% at day 2 and 15% at day 14 ($n=5$; P -value=0.01). (D) Quantification of dopamine content in the striatum by LC-MS/MS. This quantification was based on the addition of the two masses of dopamine: M91 and M137. A 30% loss of

striatal dopamine content was observed ($n=3$). *Significantly different results with P -value=0.05 unless otherwise stated.

Fig. 4. Immunofluorescence against DARPP32, which highlights GABAergic medium spiny neuron (MSN) presence. Before day 6, no major differences were found, while a slight loss of DARPP32-positive neurons was detected at day 7. This decrease reached a significant level at day 11 and was total at day 16. Right column represents a 10-fold magnification of the area of the striatum in the insert.

Fig. 5.

Organotypic slice cultures presented a progressive loss of dopaminergic fibers in the medial forebrain bundle. (A) Observation of the medial forebrain bundle in living rat by MRI. (B) Observation of the medial forebrain bundle in organotypic slices from days 0 to 7 (from top to bottom) by MRI (indicated by the arrows) and immunochemistry against rat TH. (C) Anisotropy fraction quantification of the MFB fibers. A loss of anisotropy of 40% was observed after 24 h, 70% after 3 days and reached 80% after 7 days. *Significantly different results with $n=3$ and P -value=0.01.