Xenotransplantation
Official Journal of the International Xenotransplantation Association

Long-term immunosuppression for CNS mouse xenotransplantation: effects on nigrostriatal neurodegeneration and neuroprotective carotid body cell therapy.

Journal:	*Xenotransplantation*
Manuscript ID	XEN-18-O-0009.R1
Manuscript Type:	Original Article
Date Submitted by the Author:	n/a
Complete List of Authors:	Villadiego, Javier; Instituto de Biomedicina de Sevilla, Cell Therapy and Molecular Physiology Lab; Universidad de Sevilla, Fisiología Médica y Biofísica Romo-Madero, Sonia; Instituto de Biomedicina de Sevilla; Universidad de Sevilla, Fisiología Médica y Biofísica García-Swinburn, Roberto; Instituto de Biomedicina de Sevilla; Universidad de Sevilla, Fisiología Médica y Biofísica Suárez-Luna, Nela; Instituto de Biomedicina de Sevilla; Universidad de Sevilla, Fisiología Médica y Biofísica Bermejo-Navas, Alfonso; Instituto de Biomedicina de Sevilla; Universidad de Sevilla, Fisiología Médica y Biofísica Echevarría, Miriam; Instituto de Biomedicina de Sevilla; Universidad de Sevilla, Fisiología Médica y Biofísica Toledo-Aral, Juan; Instituto de Biomedicina de Sevilla; Universidad de Sevilla, Fisiología Médica y Biofísica
Keywords:	Parkinson's disease, Immunosuppression, Xenotransplantation, Carotid Body, Neurodegeneration.
Topics:	Organs - Other, Cells - Other (e.g. neural), Transplantation - small animal models, Immunosuppression

Long-term immunosuppression for CNS mouse xenotransplantation: effects on nigrostriatal neurodegeneration and neuroprotective carotid body cell therapy.

Javier Villadiego[1,2,3*,#] Sonia Romo-Madero[1,2,*], Roberto García-Swinburn[1,2], Nela Suárez-Luna[1,2,], Alfonso Bermejo-Navas[1,2,], Miriam Echevarría[1,2,] and Juan J. Toledo-Aral[1,2,3,#].

1. Instituto de Biomedicina de Sevilla-IBiS, Hospital Universitario Virgen del Rocío/CSIC/Universidad de Sevilla. Sevilla, 41013. Spain.
2. Departamento de Fisiología Médica y Biofísica, Universidad de Sevilla. Sevilla, 41009. Spain.
3. Centro de Investigación Biomédica en Red sobre Enfermedades Neurodegenerativas (CIBERNED), Spain.

*JV and SR-M contributed equally to this work.

[#]Address correspondence to Dr. Juan J. Toledo-Aral or Dr. Javier Villadiego, Instituto de Biomedicina de Sevilla-IBiS, Avda. Manuel Siurot s/n, 41013 Sevilla, Spain. Tel: (34) 955923035; Fax: (34) 955923101; E-mail: juanjo@us.es or fvilladiego@us.es

Running title: Long-term immunosuppression & antiparkinsonian xenografts.

Abstract

Background: The use of long-term immunosuppressive treatments on neural transplantation has been controversial during the last decades. Although nowadays there is a consensus about the necessity of maintaining a permanent state of immunosuppression to preserve the survival of cerebral grafts, little is known about the effects that chronic immunosuppression produces both on the neurodegenerative process and on transplants function.

Methods: Here we establish a new immunosuppressive protocol, based on the discontinuous administration of cyclosporine A (15 mg/Kg; s.c.) and prednisone (20 mg/Kg; s.c.), to produce long-term immunosuppression in mice. Using this treatment, we analyse the effects that long-term immunosuppression induces in a chronic 1-methyl-4-phenyl-1,2,3,6,-tetrahydropyridine (MPTP) model of parkinsonism and on the neuroprotective and neurorestorative anti-parkinsonian actions exerted by rat carotid body (CB) xenografts.

Results: This protocol preserves the survival of rat CB xenotransplants maintaining the general wellness of the grafted mice. Although permanent immunosuppression does not prevent the MPTP-induced cell death of nigral neurons and the consequent degeneration of dopaminergic striatal innervation, allowing for its use as Parkinson's disease (PD) model, it reduces the microglial activation and slightly declines the striatal damage. Moreover, we reported that chronic administration of immunosuppressant drugs does not alter the neuroprotective and restorative anti-parkinsonian actions of rat CB xenografts into parkinsonian mice.

Conclusions: This new immunosuppressive protocol provides a new murine model to assay the long-term effects of cerebral xenografts and offer a pharmacological alternative to the commonly used genetic immunodeficient mice, allowing the use of genetically modified mice as hosts. In addition it will permit the experimental analysis of the effects produced by human CB xenografts in the chronic PD murine model, with the final aim of using CB allografts as an option of cell therapy in PD patients.

Keywords: Parkinson's disease, Immunosuppression, Xenotransplantation, Carotid Body, Neurodegeneration.

Abbreviations:

Carotid body, CB

Cyclosporine A, CsA

3,4-dihydroxyphenylacetic acid, DOPAC

Dopamine, DA

Glial cell line-derived neurotrophic factor, GDNF

Glial fibrillary acid protein, GFAP

homovanillic acid, HVA

6-hydroxydopamine, 6-OHDA

Ionized calcium-binding adapter molecule 1, Iba1

1-methyl-4-phenyl-1,2,3,6,-tetrahydropyridine, MPTP

Parkinson's disease, PD

Prednisone, Pred

Substantia nigra pars compacta, SNpc

Tyrosine hydroxylase, TH

1. Introduction

Parkinson's disease (PD) is characterized by the progressive degeneration of dopaminergic substantia nigra pars compacta (SNpc) neurons projecting to the striatum. This continuous loss of nigral neurons lead to a dopamine (DA) deficit in the striatum that correlates with the typical motor symptoms.[1-4] Although pharmacological and surgical therapies are currently used to palliate the symptoms, to date there is no cure for the disease.[5] During the last decades the replacement of the lost dopaminergic input by the transplantation of dopamine-releasing cells has been proposed as a treatment in PD patients.[6-8] Among the different cell types tested, the allograft of foetal ventral mesencephalic neurons provided the best clinical benefit.[9-13] However, the clinical efficacy of these grafts has been questioned by two double-blind trials that showed few clinical benefits with the appearance of dyskinesia in some of the grafted patients.[14,15] The variability in the clinical outcome obtained in the different trials has been attributed to different causes such as patient selection, tissue preparation or graft location. Nevertheless, the graft immunogenicity has emerged as a critical factor that could compromise the clinical benefit, since in the two double-blind trials that failed to show efficacy the patients were not immunosuppressed[14], or immunosuppression was withdrawn after 6 months coinciding with the regression of the clinical benefit.[15] A different dopaminergic tissue used in antiparkinsonian cell therapy is the carotid body (CB), which is a bilateral organ located in the carotid bifurcation that contains a high number of dopaminergic cells. The intrastriatal graft of CB promotes a significant recovery in different preclinical models of PD,[16-20] which is mainly mediated by the release of the glial cell line-derived neurotrophic factor (GDNF) rather than the local release of dopamine by the transplant.[19-21] Two pilot clinical trials have also shown that CB autotransplantation can induce a clinical improvement in PD patients. However, the patient's age and the progression status of the disease appear as important limitations for the clinical outcome.[22,23] For these reasons, although CB autotransplantation would appear as an attractive option because of the non-necessity to use

immunosuppression, the scarcity of the CB tissue obtained from the parkinsonian patient and putative alterations on the tissue integrity related with the aging and/or the parkinsonism would not recommend the CB autotransplantation, and clearly point to the need of developing allograft or xenografts of CB tissue. Moreover, a xenograft model in chronic parkinsonian mice will provide an excellent tool to increase the basic knowledge to improve future new clinical trials.

Neuroinflammation and an altered immune response have been strongly linked to the progression of PD.[24-26] Cyclosporine A (CsA) has been demonstrated to produce some beneficial effects on 6-hydroxydopamine (6-OHDA) and 1-methyl-4-phenyl-1,2,3,6,-tetrahydropyridine (MPTP) of PD models.[27-29]. Indeed, either genetic or pharmacological manipulation of calcineurin, molecular target of CsA, induces certain levels of protection on α-synuclein based models of parkinsonism.[30-33] These findings suggest that the use of immunosuppressive treatments on allogenic or xenogenic cell based therapies for PD can affect the neurodegenerative model itself. However, in most of the preclinical studies that evaluate the effects of immunosuppression on PD models, the period of administration of immunosuppressive drugs is relatively short (ranged from 1 to 5 weeks), probably because of the difficulty to maintain the general wellness of experimental animals under severe pharmacological immunosuppression for long periods of time. Although some studies have analysed the long-term effects of immunosuppressive treatments on different rodent models,[34-36] it is necessary to study the potential alterations that continuous pharmacological immunosuppression can induce both in the chronic MPTP neurodegenerative process associated to PD and on the effects mediated by neurotrophic CB-based therapies.

Here, we study the effects of long-term immunosuppression both on the neuropathological features of a new chronic MPTP mouse model based on the administration of low doses of MPTP for 3 months[37] and on the neuroprotective and reparative actions exerted by CB grafts on SNpc neurons. We used different immunosuppressive protocols and compare the general wellness of host mice, the

level of immunosuppression on the peripheral immune system and the preservation of rat CB xenograft, establishing a new immunosuppressed mouse model that allows to analyse the long-term effects of central nervous system xenografting. Moreover, we analysed the actions of long-term immunosuppression on the nigrostriatal degeneration and neuroinflammation induced by the chronic MPTP PD model. Finally, we demonstrated that rat CB xenografts, accompanied by chronic immunosuppression, showed similar neuroprotective and restorative effects on the nigrostriatal pathway of parkinsonian mice than the isogenic CB grafts previously reported by our group.[20] These findings clearly show that the beneficial actions induced by the CB xenografts are not affected by the chronic administration of immunosuppressive drugs. This offers a new experimental tool for the study of human CB xenografts in the chronic PD mice model, to better understand its mechanism of action and favour the possibility to use CB allografts as an option of cell therapy in PD patients.

2. Materials and Methods

2.1. Animal care and pharmacological treatments

C57BL/6N male mice of 2-3 months of age (Charles River) were housed in a regulated temperature environment (22±1ºC) on a 12 h light/dark cycle, with *ad libitum* access to food and water. Mice were rendered parkinsonian, as previously described[20,37] by the subcutaneous (s.c.) administration of MPTP (20 mg/kg; Sigma) 3 times per week for 1, 2 or 3 months. The immunosuppression protocols applied were as follows: (i) “severe” immunosuppression, mice were subjected to daily administration of CsA (15 mg/Kg; s.c.) and Prednisone (Pred; 20 mg/Kg; s.c.); (ii) “moderate” immunosuppression, mice received daily injections of CsA (15 mg/Kg; s.c.) and Pred (20 mg/Kg; s.c.) during the first three weeks and alternant daily injections of CsA (15 mg/Kg; s.c.) or Pred (20 mg/Kg; s.c.) afterwards; and (iii) “mild” immunosuppression, animals received daily injections of CsA (15 mg/Kg; s.c.) and Pred (20 mg/Kg; s.c.) during the first two weeks, alternant daily injections of CsA (15 mg/Kg; s.c) or Pred (20 mg/Kg; s.c) for 1 week,

and 3 doses per week of CsA (15 mg/Kg; s.c) afterwards. CsA and Pred were previously dissolved on NaCl 0.9% with 25% EtOH. In addition, as controls, mice were treated with the different combinations of vehicle solutions used in the MPTP and/or the immunosuppressive treatment: a group named "saline" received similar treatment as MPTP with saline solution (0.9% NaCl; Sigma); a "vehicle" group were only injected with the vehicle solution of the immunosuppressive treatment (0.9% NaCl, 25% EtOH; named as controls in the set of experiments exposed in Figures 1-2 and Supplementary Figures 1-2); a group named "saline+vehicle" was treated with both saline and vehicle solutions (named as controls in the set of experiments exposed in Figures 3-5). All control mice, treated with saline, vehicle, saline+vehicle, MPTP+vehicle and saline and "mild" immunosuppression were also analysed (Supplementary Figures 1 and 2). The total number of mice subjected to "mild" immunosuppression and/or MPTP treatment were as follows: controls (saline+vehicle), n=34; saline, n=12; vehicle, n=9; "mild" immunosuppression, n=15; MPTP, n=36; MPTP+vehicle, n=11; MPTP & Immsup, n=38. These mice were analysed with different experimental procedures on Figures 3-5, Supplementary Table 1 and Supplementary Figure 2. The general health status of the experimental mice were analysed by daily record of mortality, weight and, qualitatively, by the observation of any sign of dehydration or distress (assessed by the appearance of piloerection, coat staring, ocular and nasal discharges or aggressive behaviour.[38] At the end of the experiments, the animals were sacrificed under deep anaesthesia induced by a combination of 100 mg/kg ketamine (Pfizer) and 10 mg/kg xylazine (Bayer). All experiments were performed according to the European Directive 2010/63/EU and the Spanish RD/53/2013 for the protection of animals used for scientific purposes. The study was approved by the Animal Research Committee of the University Hospital Virgen del Rocío (University of Seville).

2.2. CB xenografting.

All the mice grafted with rat CB xenografts received a CsA injection (15 mg/kg;s.c) 4-6 hours before initiating the surgical procedure. Intrastriatal CB grafting was performed as previously described.[19-21] In brief, rat carotid bifurcations were removed after neck incision. CBs were isolated and cleaned of surrounding tissue under a stereoscopic binocular microscope (Olympus SZX16). Then rat CBs were trimmed into 4-6 pieces and placed in 1 μl of Tyrode's solution (140 mM NaCl; 4.7 mM KCl; 2 mM $CaCl_2$; 1 mM $MgCl_2$; 10 mM 4-2-hydroxyethyl-1-piperazineethanesulfonic acid, HEPES; 5 mM glucose, 5 mM pyruvate; Sigma). The xenografts were stereotaxically injected into the striatum (from bregma in mm: anteroposterior, +0.4; lateral, +2; ventral, -3.5) with a 25-gauge syringe (Hamilton) according to the mouse brain stereotaxic atlas.[39] As an internal control, the contralateral striatum was injected with 1 μl of vehicle (named as sham side in the set of experiments exposed in Figure 6). The total number of CB xenografted animals and subjected to different pattern of immunosuppression and MPTP treatment were as follows: controls, n=25; severe immunosuppression, n=18; moderate immunosuppression, n=9; mild immunosuppression, n=20; MPTP + mild immunosuppression, n=21. These CB xenografted mice were analysed with the different experimental procedures on Figures 1,2,6 and Supplementary Figure 1. To avoid differences between animals, stereologic and densitometric values (see below) of CB xenografted parkinsonian mice are expressed as a percentage of the sham contralateral hemisphere.

2.3. Flow cytometry analysis.

For the flow cytometry analysis 3 mice were measured for each time points and experimental conditions. For each mouse, 700 μl of blood were extracted from the left ventricle of immunosuppressed and control (treated with vehicle solution) mice with a heparinized syringe (heparin, 5000 U/ml; Applichem). The sample of blood was diluted with 700 μl of PBS (Sigma), mixed with 930 μl of Lymphocyte Separation Medium (LSM 1.077; Lonza) and centrifuged at 516 g for 25 minutes. The mononuclear

leukocytes were collected and washed twice with 500 µl of PBS. After that, mononuclear leukocytes were suspended in 1 ml of FACS solution (0.2% bovine serum albumin, Sigma); 0.25% penicillin/streptomycin, Invitrogen; 10 mM HEPES, Sigma; 5 mM EDTA, Sigma; on Leibovitz´s L-15 medium; Gibco) and immunolabelled with CD3-Alexa700 (1:50; BD Pharmigen), CD4-PE (1:20; Immunostep) and CD8-FITC (1:20; Immunostep). All the cell cytometry analysis was carried out using a BD LSR Fortessa flow cytometer equipped with FACSDiva software version 6.3 (BD Biosciences).

2.4. Histological analyses

After transcardial perfusion of mice with 50 ml of PBS (Sigma) and 50 ml of 4% paraformaldehyde (Sigma) in PBS, brains were immediately removed and fixed overnight at 4ºC with 4% paraformaldehyde in PBS. After fixation, the brains were cryoprotected in 30% sucrose (Sigma) in PBS and included in Optimum Cutting Temperature compound (O.C.T. compound, Tissue-Tek). Coronal sections (thickness 30 µm) were cut on a cryostat (Leica). Tyrosine hydroxylase (TH), glial fibrillary acid protein (GFAP) and ionized calcium-binding adapter molecule 1 (Iba1) immunohistological detection were performed as previously described[20,37] using, respectively, polyclonal anti-TH (1:1000; Novus Biologicals), polyclonal anti-GFAP (1:500; Dako), polyclonal anti-Iba1 (1:500; Wako Chemicals), and a secondary peroxidase-conjugated antibody kit (Dako). Images were obtained with a light-transmitted microscope (Olympus BX61) and a refrigerated digital camera (Olympus DP70).

2.5. Stereology and densitometry

Unbiased stereological analyses were performed by systematic random sampling using the optical dissector method.[40] Estimations of TH^+ and $Iba1^+$ cells in the SNpc were carried out in the region spanning from -2.92 mm to -3.40 mm relative to Bregma according to the Franklin and Paxinos mouse brain stereotaxic atlas.[39] Only SNpc cells lateral to the medial terminal nucleus of the accessory optic tract were determined to have a clear separation from the adjacent ventral tegmental area.[41] Reference volumes

for each section were outlined under low magnification (4x), and TH^+ and $Iba1^+$ cells counted at high magnification (40x) using, respectively, 7225 μm^2 x 20 µm and 12724.7 μm^2 x 20 µm optical dissectors, with a guard volume of 5 µm to avoid artefacts on the cut surface of the sections. The numbers of resting and active microglia were determined as previously described[37] in the SNpc (in the region described above) and the striatum (from 1.54 mm to -0.10 mm relative to Bregma). To avoid any bias in the measurement of microglial activation, active $Iba1^+$ cells were only considered if they had a cell body volume greater than 300 μm^3 (measured by the optical rotator,[42] and short thick processes). All stereological procedures were performed using the New CAST™ system (Visiopharm) with a coefficient of error (CE) ≤0.09. In all cases CE was clearly lower than the 50% of the observed group variance (5-15%), indicating the accuracy of the stereological analysis.[43,44]

The optical density (O.D.) of striatal TH^+ innervation and striatal or SNpc $GFAP^+$ staining, to estimate astrogliosis, were measured from digitized pictures using the NIH Image software (ImageJ) as previously described.[20,45] The optical density values of each animal were obtained from a total of 6 slices covering the entire rostro-caudal extent of the same striatal and SNpc regions analysed by unbiased stereology (see above).

2.6. HPLC

Striatal catecholamine content was studied by HPLC. Striata were obtained fresh by dissection in ice-cold PBS under a stereoscopic binocular microscope (Olympus SZX16) and frozen in liquid N_2. The samples were kept at -80ºC until its use. Striata were sonicated in 200 µl of chilled solution containing 0.1 M $HClO_4$, 0.02% EDTA and 1% ethanol (Sigma). Cellular extracts were centrifuged at 16000 g for 10 min at 4ºC. Supernatants were filtered with a 30000 Da molecular mass exclusion membrane (Millipore) by centrifugation at 16000 g for 30 min at 4ºC. Filtered samples were injected onto an HPLC system (ALEXYS 100; Antec Leyden). DA, 3,4-dihydroxyphenylacetic acid (DOPAC) and homovanillic acid (HVA) levels were

determined using a 3 µm C-18 column (ALB-215; Antec Leyden), followed by electrochemical detection with a glassy carbon electrode and in situ ISAAC reference electrode (Antec Leyden). Concentrations of compounds were expressed as ng/mg of total protein. Pelleted proteins were resuspended in 0.1 M NaOH for protein quantification with the Bradford assay (Biorad).

2.7. Statistical analysis

The specific number of mice analysed (n) on each experimental group is indicated in each figure legend. Data are presented as mean±SEM. In all cases, the normality test (Kolmogorov-Smirnov) and the equal variance test were carried out, and, when passed, the ANOVA test with Bonferroni or Fisher LSD post hoc analysis for multiple group comparisons, or the Student's t-test (for two group comparisons) were applied. In the cases that the normality test failed, the non-parametric Kruskal–Wallis H was performed. All statistical analyses were conducted using Sigmastat 2.0 software.

3. Results

3.1. Analysis of immunosuppressive treatments

We designed 3 different protocols of immunosuppression, based on previously described doses of CsA and Pred and on a progressive decrease of the administration frequency of immunosuppressant drugs along time, with the aim of preventing the immune rejection of the neural CB graft and inducing minimal endangerment to the general wellness of the receptor animal (Figure 1A). In the protocol that we named as “severe” immunosuppression, CB grafted animals were subjected to daily administration of CsA (15 mg/Kg) and Pred (20 mg/Kg) during 5 weeks. Animals subjected to the “moderate” immunosuppression received the same treatment during the first 3 weeks and alternant daily injections of CsA (15 mg/Kg) or Pred (20 mg/Kg) during the last two weeks. And in the protocol called “mild” immunosuppression, CB grafted animals received daily injections of CsA (15 mg/Kg) and Pred (20 mg/Kg) during the first two weeks after the transplant, alternant daily injections of CsA (15

mg/Kg) or Pred (20 mg/Kg) during the following week, and 3 doses per week of CsA (15 mg/Kg) along the last two weeks. As control group, CB grafted mice were treated with vehicle solution. The general health status of the treated animals and the effect of the immunosuppressive treatment on the population of circulating T lymphocytes were analysed along the treatment. As shown in Figure 1B-C, during the first two weeks the mice subjected to the different immunosuppressive treatments did not show a significant mortality or weight loss. However, after 2 weeks of treatment, the animals of the "severe" and "moderate" group suffered a gradual loss of weight and a significant mortality, reaching a 33% of mortality in both groups at the fifth week of the treatment. In contrast, the mice subjected to "mild" immunosuppression suffered just a mild loss of weight, and only a ~10% of mortality after 5 weeks of treatment. Also, in a qualitative manner, the mice included in the "mild" immunosuppressed group showed a general healthy status, with similar locomotion, coat, and food and water intake than the mice from the control group. While mice from the "severe" and "moderate" immunosuppression groups showed clear signs of distress, like reduced locomotion and the appearance of some coat staring and/or piloerection.

The survival of the rat CB xenografts were analysed after 5 weeks of immunosuppression by the different treatments showed before (Fig. 1A). In most of the controls animals analysed (only treated with vehicle solution) we did not detect graft (TH^+ CB cells), while in all the cases of the different immunosuppressive treatments tested we found well preserved and highly dopaminergic intrastriatal CB grafts (Fig. 1D-E). As previously described,[46] in 2 of the 11 control mice implanted we found alive CB xenografts. We also performed a stereological analysis to compare the volume of the xenografts in the different experimental groups. As shown in Figure 1F, we did not detect differences in the volume between the CB xenografts of the "severe", "moderate" and "mild" immunosuppressive protocols. In the two non-rejected CB xenografts observed on non immunosuppressed mice (control) we did not find significant morphological differences with the xenografts obtained from mice subjected

to the different immunosuppressive patterns ($2.91\pm0.33\cdot10^6$ μm^3 from alive CB xenografts of control mice *vs* $3.45\pm0.63\cdot10^6$ μm^3, $3.16\pm0.81\cdot10^6$ μm^3 and $3.63\pm0.61\cdot10^6$ μm^3 observed respectively on the "severe", "moderate" and "mild" immunosuppressed mice).

The effects of the different immunosuppressive treatments on the peripheral immune system were studied at different time points (at 2, 7 and 14 days, where the immunosuppressive protocol is common for the 3 experimental groups; and after 5 weeks where the immunosuppressive treatments are different among them). All immunosuppressive treatments produced a significant reduction in the percentage of blood leukocytes at the different time-points, although this decrease is lower on the "moderate" and "mild" protocols (Fig. 2A). The analysis of the T lymphocytes ($CD3^+$) also revealed a similar decrease in the percentage of T lymphocytes in the different experimental groups, which is more pronounced in the stronger treatments (Fig. 2B). Indeed, the effects of the different immunosuppressive treatments on the main subtypes of blood T lymphocytes were studied. As expected, the immunosuppression produced a significant reduction on the percentage of T helper lymphocytes ($CD4^+$; T_h cells), which we measured after 2 days of treatment and is maintained until the end of the treatment (Fig. 2C,D). Interestingly, at the end of the different treatments no differences in the percentage of T_h cells were found between the different protocols (Fig. 2D). However, no differences in the percentage of T cytotoxic lymphocytes ($CD8^+$) were observed with any of the immunosuppressive protocols or the time-points analysed (Fig. 2E).

Taken together the data showed so far indicate that the "mild" immunosuppressive protocol induces a similar prevention of xenograft rejection, but with lower reduction on the peripheral immunity and without the mortality or distress, than the observed in the mice subjected to more severe pharmacological immunosuppression. In order to study the long-term efficacy of this immunosuppressive strategy, we analysed the integrity of

rat CB xenografts and the survival and general wellness after 5 months of "mild" immunosuppression. Similarly to what we found after 5 weeks, after 5 months of "mild" immunosuppression all the rat CB xenografts (n=10) remained alive and well preserved, showing abundant dopaminergic CB glomus cells (Supplementary Fig.1A). Indeed, after 5 months of xenotransplantation and immunosuppressive treatment, we only found a 9% of mortality of the immunosuppressed animals, showing these mice only a minor reduction in the body weight (~10%; Supplementary Fig.1 B,C) and similar parameters of wellness (locomotion, coat and food intake) than control mice.

3.2. Effect of immunosuppression in a chronic PD model

We studied the effects of long term immunosuppression in a chronic and progressive preclinical model of parkinsonism.[37] To do that we established 3 experimental groups, (i) control mice (treated with vehicle solutions from both the MPTP and the immunosuppressive treatments); (ii) chronic MPTP parkinsonian mice (treated with 20 mg/Kg of MPTP, 3 times per week during 3 months) and (iii) chronic MPTP-immunosuppressed mice (treated with chronic MPTP and "mild" immunosuppression along the 3 months of neurotoxic parkinsonian treatment). As we previously described, the chronic MPTP treatment induces degeneration of the nigrostriatal pathway, which can be detected by either loss of dopaminergic SNpc neurons or the resultant striatal denervation (Fig. 3A-E). The immunosuppressive treatment did not alter the MPTP-induced progressive SNpc dopaminergic neuronal death (Fig. 3A,B). However, it produced a decreased damage of the striatal dopaminergic innervation after 3 months of toxic treatment measured either by densitometric analysis or stereological quantification of TH^+ striatal fiber varicosities (Fig. 3C-E). Despite the decrease in the damage induced by the MPTP treatment in the striatal dopaminergic innervation on mice subjected to long-term immunosuppression, the highly significant differences with the control groups allows the use of this chronic PD model even in immunosuppressed mice. Measurements of the striatal level of DA and its metabolites DOPAC and HVA

(Figure 4A-C) also showed a trend that suggest that long-term immunosuppressive treatment diminish the striatal DA depletion induced by the chronic MPTP treatment. In addition, the DOPAC/DA ratio, previously described as increased after chronic MPTP treatment and advanced PD,[37,47] is returned to the control levels after 3 months of immunosuppressive treatment (Fig. 4D). We also analysed the effect of the simple injection of the different control solutions used (saline, NaCl 0.9% for the MPTP treatment; and vehicle, EtOH 25% on NaCl 0.9% for the immunosuppressive treatment) both on controls and MPTP treated mice, not finding any significant consequences in the integrity of the dopaminergic nigrostriatal pathway. Moreover, chronic "mild" immunosuppression alone did not produce any significant alteration on the nigrostriatal pathway (measured by histological and neurochemical analysis; Supplementary Table 1 and Supplementary Fig. 2).

The presence of clear signs of neuroinflammation, measured as an increase in reactive microglia and astrogliosis, is a well-established neuropathological feature of PD[25,48] that is clearly reproduced in our chronic MPTP parkinsonian model.[37] We investigated the effects of immunosuppression in the neuroinflammatory response induced by the chronic MPTP treatment at different time-points (1, 2 and 3 months). To evaluate the microglial activation, we labelled the microglial cells with the general marker Iba1 and performed size-dependent stereological quantification for the density of resting and active microglia. Although our "mild" immunosuppressive treatment did not produce any significant modification in the density of resting microglial cells either in the striatum or in the SNpc, it induced a reduction of the density of active microglial cells after MPTP treatment, which is clearly significant after 3 months of immunosuppression (Figure 5A-C). Regarding the characteristic astroglial response associated to MPTP induced nigrostriatal degeneration, the immunosuppressive-MPTP treated mice did not show any differences respect to the MPTP alone treated mice (Fig. 5D-E).

3.3. Dopaminotrophic actions of CB grafts under immunosuppressive treatment

Striatal CB grafts from isogenic mice have been shown to produce both neuroprotection and restoration of chronic MPTP treated parkinsonian mice.[20] In addition, PD patients subjected to CB autograft showed some clinical benefits which are related with the age and the degree of parkinsonian affectation of the patient.[22,23] We analysed the effects of long-term immunosuppression (5 months) on the neuroprotective and restorative actions exerted by rat CB xenografts. That was performed to study if CB xenografts work ameliorating parkinsonism in the chronic MPTP murine model, in order to have an experimental tool to evaluate factors that could influence the clinical outcome of human CB transplants. To investigate the neuroprotective effect of CB xenografts we performed unilateral xenotransplants of rat CB, with a sham graft in the contralateral hemisphere as internal control, on receptor mice that were subjected to "mild" immunosuppression. Three weeks later, the CB xenografted mice were rendered parkinsonian by the chronic administration of MPTP (20 mg/Kg s.c.; 3 times/week, during 3 months), and were allowed to recover from the toxic treatment for 1 month. Afterwards, the CB xenografted mice were sacrificed and the histological examination of the nigrostriatal pathway was carried out (see experimental scheme in Fig. 6A). We found well-preserved intrastriatal CB xenografts in all the animals analysed (n=5), with abundant dopaminergic glomus cell. Moreover, the CB xenograft exerted a clear protection of ipsilateral TH^+ SNpc neurons that can be quantified with respect to the contralateral sham grafted hemisphere (145.6±5.2%; Fig. 6A,B). Consequently, the intrastriatal rat CB xenografts also induce a significant protection of the ipsilateral striatal dopaminergic innervation (Fig. 6A,B; 146.4±7.8% measured by TH^+ densitometry and 154.3±3.8% by stereological quantification of TH^+ fiber varicosities).

To study if the striatal CB xenografts can also induce a restorative action (axonal sprouting) on the dopaminergic striatal innervation, we performed striatal CB

xenografts after the receptor mice were rendered parkinsonian by the chronic MPTP treatment. These xenografted CB mice were also immunosuppressed by the "mild" treatment after the transplantation (experimental scheme in Fig. 6C). All the mice analysed under these experimental design (n=6) also showed a well-maintained CB xenografts with numerous dopaminergic glomus cells. The striatal CB xenografts produced a significant increase in the dopaminergic striatal innervation in comparison with the contralateral sham grafted hemisphere (Fig 6C, D; 139.8±10.7% measured by TH^+ densitometry and 144.7±4.3% by stereological quantification of TH^+ fiber varicosities). This restorative effect produced by the CB xenografts on the striatal TH^+ fibers (after 3 months of chronic MPTP-treatment) occurs despite the fact that, as expected, the xenotransplants did not produce any beneficial effect on the number of TH^+ SNpc neurons (Fig. 6D, right panel). Thus, these results clearly indicate that in this experimental design the rat CB xenografts produce nigrostriatal fiber outgrowth because the CB xenografted striata have higher TH^+ innervation, respect to the sham grafted striata, with the same number of dopaminergic SNpc neurons. Interestingly, both neuroprotective and neurorestorative effect induced by the rat CB xenografts, under long-term immunosuppression, are qualitatively and quantitatively similar to the one previously reported by isogenic CB allotransplants in the same parkinsonian model and transplantations protocols,[20] indicating that the neurotrophic actions induced on the nigrostriatal pathway by CB cells are not affected by the use of chronic immunosuppression and xenografts.

4. Discussion

The necessity of using long-term immunosuppression on neural transplantation strategies has been extensively discussed during the last decades. Although the brain has been considered an immune-privileged organ and some authors have reported neural graft survive in the absence of immunosuppressive treatment in monkeys and humans,[49-51] nowadays there is a broad consensus about that the lack of

immunosuppression produces an attenuation of the clinical outcome and possible adverse effects of neural transplants.[29,50,52,53] Different preclinical studies have analysed the effects of immunosuppression, based on CsA administration, on antiparkinsonian cell therapy.[29,46,54-59] However, in most of the studies using PD rodent models the period of CsA administration (1-6 weeks) is short, especially if it is compared with the slow and progressive natural course of the disease. This lack of studies that evaluate the long-term effects of immunosuppression on PD cell therapy are probably related with the adverse effects of drug treatments,[60,61] the mortality observed (>30%) and the high cost and difficulty to maintain the general wellness of animals subjected to severe pharmacological immunosuppression. In this work, we attempt to develop a new immunosuppressive treatment that combines the prevention of the immune rejection of the neural transplant with the long-term maintenance of the general wellness of the host animal. Among the different immunosuppressive treatments tested (severe, intermediate and mild) we show that the "mild" immunosuppression safeguards the viability of the CB xenografts for up to 5 months, with low mortality and minimal alterations of the wellness of host animals. This "mild" immunosuppressive pattern is based on the discontinuous administration of CsA, 3 injections of 15 mg/Kg per week, which reduces the dosage of the classical CsA administration pattern (ranged 15-25 mg/Kg per day) and presumably reduces the risk of kidney damage.[57] Moreover, this pattern of CsA administration produces a less accused reduction of the peripheral immunity but with a significant reduction of $CD4^+$ T_h lymphocytes which are considered the drivers of neural xenografts rejection.[62-64] This "mild" immunosuppression is sufficient to preserve the neural graft survival and functionality, preventing the appearance of infections or alterations on experimental animals. Interestingly, this pattern of "mild" immunosuppression could be used as a pharmacological alternative to the genetic immunodeficient mice to study the long-term effects of xenotransplantation, even in other body locations, allowing the use of genetically modified mice as hosts. Moreover, although our study has focused only in

the MPTP-mouse model, this “mild” immunosuppression protocol could be presumably also applied to other rat models of neurodegeneration.

Different works have proposed that CsA treatment can produce some protection against 6-OHDA effects in rat and cellular PD models.[27,28,65] In addition, genetic or pharmacological inhibition of calcineurin induces protection on cellular and in vivo models of PD.[30-33] We analysed the effects of long-term “mild” immunosuppression on a chronic MPTP mouse model. Although we did not detect any protection of SNpc cell death, after 3 months of CsA treatment, there was a minor toxin-induced damage on dopaminergic striatal innervation. However, the fact that long-term immunosuppression diminished the dopaminergic striatal damage induced by chronic MPTP does not disallow this experimental model of parkinsonism, since it still presents a clear significant degeneration both in striatum and SNpc. These findings are in accord with the recent work of Tamburrino and colleagues where they postulate an improvement on axonal regeneration after 4 weeks of CsA treatment on the MPTP parkinsonian model.[29] Interestingly, the improvement on the striatal innervation after 3 months of CsA treatment is accompanied by a strong reduction on the microglial activation, suggesting that a decrease on the neuroinflammation associated to the neurodegenerative process could precede the improvement on the dopaminergic striatal innervation. Despite no PD patients have been treated with immunosuppressant drugs for enough time to unequivocally elucidate the impact on the disease course, PD patients subjected to sequential bilateral transplants, with immunosuppression, showed a bilateral motor recovery even before the second unilateral graft was carried out.[66] In conclusion, our data and other recent works in preclinical PD models[29,33] suggest that a “mild” and continuous immunosuppressive treatment can induce an improvement in PD patients through a decrease of the chronic neuroinflammation and an enhancement on the dopaminergic striatal function. Although this hypothesis is out of the scope of our study, it could be tested by appropriate clinical trials analysing the therapeutic effect of immunosuppressant drugs on PD patients.

Intrastriatal transplantation of CB cells have been shown to induce a significant histological and functional recovery in different preclinical models of parkinsonism.[16-19,67-69] The mechanism underlying the anti-parkinsonian actions of CB transplants is a trophic support of the nigrostriatal pathway mediated, at least in part, by the release of GDNF.[19-21] Two pilot phase I/II open trials have shown that CB autotransplantation is a safe and feasible procedure that produce a clinical improvement in some of the patients, especially in the younger and less affected PD patients.[22,23] Here we studied the effects of the immunosuppressive treatment on the survival and anti-parkinsonian effects of rat CB xenografts, as a first study to analyse the CB xenografts potentiality in the chronic MPTP mouse model. Our results clearly showed the necessity of using immunosuppression to preserve the integrity of CB xenografts, suggesting the usage of immunosuppressant drugs in future trials that evaluate the clinical efficacy of CB allografts. A question that emerges from our study is the extraordinary high survival of CB tissue after intrastriatal transplantation, that has shown to be similar in rat, monkey and mice.[17-21] That contrasts with the recent study reported by Roberton and colleagues that clearly suggests that the mouse brain is an specially hostile environment for neural grafting.[35] This high survival of the CB after neural transplantation could be attributed to intrinsic properties of the CB tissue such as its physiological resistance to hypoxia[70] and oxidative stress[19], and also to its high production of GDNF and other trophic factors[21,71] that could favour the graft survival by autocrine stimulation. In addition, we demonstrated that chronic CsA administration do not alter the neuroprotective and restorative actions that CB transplants exerts on the nigrostriatal pathway, showing the CB xenografts similar anti-parkinsonian effects than isogenic CB implants without immunosuppressive treatments.[20] The fact that “mild” immunosuppression preserves the integrity and the antiparkinsonian effects of the xenograft will permit, with future experiments, evaluate different factors that could modify the clinical efficacy of antiparkinsonian CB cell therapy performing human CB xenografts in the MPTP experimental model. In addition, these results open the

possibility of using human CB allografts, with permanent immunosuppression, as a realistic option to improve the clinical outcome of antiparkinsonian CB cell therapy.

Acknowledgments

The authors thank Dr. José López-Barneo for critical reading of the manuscript and general support. This study was supported by grants from the Spanish Government (PI12/02574-ISCIII; Red TerCel ISCIII RD12/0019/0033 and RD16/0011/0025; RTC-2015-3309-1) and the Andalusian Government (Excellence project-P12-CTS-2739).

Authorship

JV, SRM and JJTA designed the experiments and interpreted the data. JV, SRM, RGS, NSL, ABN, ME and JJTA performed the experiments. JV and JJTA supervised all the experiments and wrote the manuscript. All authors revised the manuscript.

Conflict of interest statement

The authors declare no conflict of interests.

References

1. Lang AE, Lozano AM. Parkinson's disease. First of two parts. N Engl J Med. 1998;339:1044–1053.

2. Lang AE, Lozano AM. Parkinson's disease. Second of two parts. N Engl J Med. 1998;339:1130–1143.

3. Braak H, Del Tredici K, Rüb U, de Vos RAI, Jansen Steur ENH, Braak E. Staging of brain pathology related to sporadic Parkinson's disease. Neurobiol Aging. 2003;24:197–211.

4. Poewe W, Seppi K, Tanner CM, Halliday GM, Brundin P, Volkmann J, Schrag AE, Lang AE. Parkinson disease. Nat Rev Dis Primers. 2017;3:17013.

5. Obeso JA, Rodriguez-Oroz MC, Goetz CG, Marin C, Kordower JH, Rodriguez M, Hirsch EC, Farrer M, Schapira AH, Halliday G. Missing pieces in the Parkinson's disease puzzle. Nat Med. 2010;16:653–661.

6. Dunnett SB, Björklund A. Prospects for new restorative and neuroprotective treatments in Parkinson's disease. Nature. 1999;399:A32–A39.

7. Lindvall O. Developing dopaminergic cell therapy for Parkinson's disease-give up or move forward? Mov Disord. 2013;28:268–273.

8. Barker RA, Drouin-Ouellet J, Parmar M. Cell-based therapies for Parkinson disease-past insights and future potential. Nat Rev Neurol. 2015;11:492–503.

9. Lindvall O, Brundin P, Widner H, Rehncrona S, Gustavii B, Frackowiak R, Leenders KL, Sawle G, Rothwell JC, Marsden CD, et al. Grafts of fetal dopamine neurons survive and improve motor function in Parkinson's disease. Science. 1990;247:574–577.

10. Freed CR, Breeze RE, Rosenberg NL, Schneck SA, Kriek E, Qi JX, Lone T, Zhang YB, Snyder JA, Wells TH, et al. Survival of implanted fetal dopamine cells and neurologic improvement 12 to 46 months after transplantation for Parkinson's disease. N Engl J Med. 1992;327:1549–1555.

11. Widner H, Tetrud J, Rehncrona S, Snow B, Brundin P, Gustavii B, Björklund A, Lindvall O, Langston JW. Bilateral fetal mesencephalic grafting in two patients with parkinsonism induced by 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP). N Engl J Med. 1992;327:1556–1563.

12. Piccini P, Brooks DJ, Björklund A, Gunn RN, Grasby PM, Rimoldi O, Brundin P, Hagell P, Rehncrona S, Widner H, Lindvall O. Dopamine release from nigral transplants visualized in vivo in a Parkinson's patient. Nat Neurosci. 1999;2:1137–1140.

13. Kefalopoulou Z, Politis M, Piccini P, Mencacci N, Bhatia K, Jahanshahi M, Widner H, Rehncrona S, Brundin P, Björklund A, Lindvall O, Limousin P, Quinn N, Foltynie T. Long-term clinical outcome of fetal cell transplantation for Parkinson disease: two case reports. JAMA Neurol. 2014;71:83–87.

14. Freed CR, Greene PE, Breeze RE, Tsai WY, DuMouchel W, Kao R, Dillon S, Winfield H, Culver S, Trojanowski JQ, Eidelberg D, Fahn S. Transplantation of embryonic dopamine neurons for severe Parkinson's disease. N Engl J Med. 2001;344:710–719.

15. Olanow CW, Goetz CG, Kordower JH, Stoessl AJ, Sossi V, Brin MF, Shannon KM, Nauert GM, Perl DP, Godbold J, Freeman TB. A double-blind controlled trial of bilateral fetal nigral transplantation in Parkinson's disease. Ann Neurol. 2003;54:403–414.

16. Bing GY, Notter MF, Hansen JT, Gash DM. Comparison of adrenal medullary, carotid body and PC12 cell grafts in 6-OHDA lesioned rats. Brain Res Bull. 1988; 20:399-406.

17. Espejo EF, Montoro RJ, Armengol JA, López-Barneo J. Cellular and functional recovery of Parkinsonian rats after intrastriatal transplantation of carotid body cell aggregates. Neuron. 1998;20:197–206.

18. Luquin MR, Montoro RJ, Guillén J, Saldise L, Insausti R, Del Río J, López-Barneo J. Recovery of chronic parkinsonian monkeys by autotransplants of carotid body cell aggregates into putamen. Neuron. 1999;22:743–750.

19. Toledo-Aral JJ, Méndez-Ferrer S, Pardal R, Echevarría M, López-Barneo J. Trophic restoration of the nigrostriatal dopaminergic pathway in long-term carotid body-grafted parkinsonian rats. J Neurosci. 2003;23:141–148.

20. Muñoz-Manchado AB, Villadiego J, Suárez-Luna N, Bermejo-Navas A, Garrido-Gil P, Labandeira-García JL, Echevarría M, López-Barneo J, Toledo-Aral JJ. Neuroprotective and reparative effects of carotid body grafts in a chronic MPTP model of Parkinson's disease. Neurobiol Aging. 2013;34:902–915.

21. Villadiego J, Méndez-Ferrer S, Valdés-Sánchez T, Silos-Santiago I, Fariñas I, López-Barneo J, Toledo-Aral JJ. Selective glial cell line-derived neurotrophic factor production in adult dopaminergic carotid body cells in situ and after intrastriatal transplantation. J Neurosci. 2005;25:4091–4098.

22. Arjona V, Mínguez-Castellanos A, Montoro RJ, Ortega A, Escamilla F, Toledo-Aral JJ, Pardal R, Méndez-Ferrer S, Martín JM, Pérez M, Katati MJ, Valencia E, García T, López-Barneo J. Autotransplantation of human carotid body cell aggregates for treatment of Parkinson's disease. Neurosurgery. 2003;53:321–330.

23. Mínguez-Castellanos A, Escamilla-Sevilla F, Hotton GR, Toledo-Aral JJ, Ortega-Moreno A, Méndez-Ferrer S, Martín-Linares JM, Katati MJ, Mir P, Villadiego J, Meersmans M, Pérez-García M, Brooks DJ, Arjona V, López-Barneo J. Carotid body autotransplantation in Parkinson disease: a clinical and positron emission tomography study. J Neurol Neurosurg Psychiatr. 2007;78:825–831.

24. Appel SH. CD4+ T cells mediate cytotoxicity in neurodegenerative diseases. J Clin Invest. 2009;119:13–15.

25. Hirsch EC, Hunot S. Neuroinflammation in Parkinson's disease: a target for neuroprotection? Lancet Neurol. 2009;8(4):382–397.

26. Fakhoury M. Immune-mediated processes in neurodegeneration: where do we stand?. Journal of Neurology. 2016;263:1683–1701.

27. Matsuura K, Kabuto H, Makino H, Ogawa N. Cyclosporin A attenuates degeneration of dopaminergic neurons induced by 6-hydroxydopamine in the mouse brain. Brain Res. 1996;733:101–104.

28. Borlongan CV, Freeman TB, Hauser RA, Cahill DW, Sanberg PR. Cyclosporine-A increases locomotor activity in rats with 6-hydroxydopamine-induced hemiparkinsonism: relevance to neural transplantation. Surg Neurol. 1996;46:384–388.

29. Tamburrino A, Churchill MJ, Wan OW, Colino-Sanguino Y, Ippolito R, Bergstrand S, Wolf DA, Herz NJ, Sconce MD, Björklund A, Meshul CK, Decressac M. Cyclosporin promotes neurorestoration and cell replacement therapy in pre-clinical models of Parkinson's disease. Acta Neuropathologica Communications. 2015;9:1–14.

30. Gerard M, Deleersnijder A, Daniëls V, Schreurs S, Munck S, Reumers V, Pottel H, Engelborghs Y, Van den Haute C, Taymans JM, Debyser Z, Baekelandt V. Inhibition of FK506 binding proteins reduces alpha-synuclein aggregation and Parkinson's disease-like pathology. J Neurosci. 2010;30:2454–2463.

31. Caraveo G, Auluck PK, Whitesell L, Chung CY, Baru V, Mosharov EV, Yan X, Ben-Johny M, Soste M, Picotti P, Kim H, Caldwell KA, Caldwell GA, Sulzer D, Yue DT, Lindquist S. Calcineurin determines toxic versus beneficial responses to α-synuclein. Proc Natl Acad Sci USA. 2014;111:3544–3552.

32. Luo J, Sun L, Lin X, Liu G, Yu J, Parisiadou L, Xie C, Ding J, Cai H. A calcineurin- and NFAT-dependent pathway is involved in -synuclein-induced degeneration of midbrain dopaminergic neurons. Human Molecular Genetics. 2014;23:6567–6574.

33. Van der Perren A, Macchi F, Toelen J, Carlon MS, Maris M, de Loor H, Kuypers DR, Gijsbers R, Van den Haute C, Debyser Z, Baekelandt V. FK506 reduces neuroinflammation and dopaminergic neurodegeneration in an α-synuclein-based rat model for Parkinson's disease. Neurobiol Aging. 2015;36:1559–1568.

34. Kelly CM, Precious SV, Scherf C, Penketh R, Amso NN, Battersby A, Allen ND, Dunnett SB, Rosser AE. Neonatal desensitization allows long-term survival of neural xenotransplants without immunosuppression. Nat Methods. 2009; 6:271-273.

35. Roberton VH, Evans AE, Harrison DJ, Precious SV, Dunnett SB, Kelly CM, Rosser AE. Is the adult mouse striatum a hostile host for neural transplant survival? Neuroreport. 2013; 24:1010-1015.

36. Heuer A, Kirkeby A, Pfisterer U, Jönsson ME, Parmar M. hESC-derived neural progenitors prevent xenograft rejection through neonatal desensitisation. Exp Neurol. 2016; 282:78-85.

37. Muñoz-Manchado AB, Villadiego J, Romo-Madero S, Suárez-Luna N, Bermejo-Navas A, Rodríguez-Gómez JA, Garrido-Gil P, Labandeira-García JL, Echevarría M, López-Barneo J, Toledo-Aral JJ. Chronic and progressive Parkinson's disease MPTP model in adult and aged mice. J Neurochem. 2016;136:373-387.

38. Wolfensohn S, Lloyd M. Handbook of Laboratory Animal Management and Welfare. Oxford: Blackwell Publishing; 2003. 416 p.

39. Paxinos G, Franklin KBJ. The Mouse Brain in Stereotaxic Coordinates. San Diego: Academic Press; 1997.

40. West MJ. New stereological methods for counting neurons. Neurobiol Aging. 1993;14:275–285.

41. Sauer H, Rosenblad C, Björklund A. Glial cell line-derived neurotrophic factor but not transforming growth factor beta 3 prevents delayed degeneration of nigral dopaminergic neurons following striatal 6-hydroxydopamine lesion. Proc Natl Acad Sci USA. 1995;92:8935–8939.

42. Tandrup T, Gundersen HJ, Jensen EB. The optical rotator. J Microsc. 1997;186:108–120.

43. Gundersen HJ, Jensen EB. The efficiency of systematic sampling in stereology and its prediction. J Microsc. 1987;147:229–263.

44. Gundersen HJ, Jensen EB, Kiêu K, Nielsen J. The efficiency of systematic sampling in stereology--reconsidered. J Microsc. 1999;193:199–211.

45. Mejías R, Villadiego J, Pintado CO, Vime PJ, Gao L, Toledo-Aral JJ, Echevarría M, López-Barneo J. Neuroprotection by transgenic expression of glucose-6-phosphate dehydrogenase in dopaminergic nigrostriatal neurons of mice. J Neurosci 2006;26:4500–4508.

46. Brundin P, Nilsson OG, Gage FH, Björklund A. Cyclosporin A increases survival of cross-species intrastriatal grafts of embryonic dopamine-containing neurons. Exp Brain Res. 1985;60:204–208.

47. Lunardi G, Galati S, Tropepi D, Moschella V, Brusa L, Pierantozzi M, Stefani A, Rossi S, Fornai F, Fedele E, Stanzione P, Hainsworth AH, Pisani A. Correlation between changes in CSF dopamine turnover and development of dyskinesia in Parkinson•s disease. Parkinsonism Relat Disord. 2009;15:383–389.

48. McGeer PL, Itagaki S, Boyes BE, McGeer EG. Reactive microglia are positive for HLA-DR in the substantia nigra of Parkinson's and Alzheimer's disease brains. Neurology. 1988;38:1285–1291.

49. Freeman TB, Olanow CW, Hauser RA, Nauert GM, Smith DA, Borlongan CV, Sanberg PR, Holt DA, Kordower JH, Vingerhoets FJ, et al. Bilateral fetal nigral transplantation into the postcommissural putamen in Parkinson's disease. Ann Neurol. 1995;38:379–88.

50. Freeman TB, Cicchetti F, Bachoud-Lévi AC, Dunnett SB. Technical factors that influence neural transplant safety in Huntington's disease. Exp Neurol. 2011;227:1–9.

51. Lee SR, Lee HJ, Cha SH, Jeong KJ, Lee Y, Jeon CY, Yi KS, Lim I, Cho ZH, Chang KT, Kim SU. Long-term survival and differentiation of human neural stem cells in nonhuman primate brain with no immunosuppression. Cell Transplant. 2015;24:191–201.

52. Aron Badin R, Vadori M, Vanhove B, Nerriere-Daguin V, Naveilhan P, Neveu I, Jan C, Lévèque X, Venturi E, Mermillod P, Van Camp N, Dollé F, Guillermier M, Denaro L, Manara R, Citton V, Simioni P, Zampieri P, D'avella D, Rubello D, Fante F, Boldrin M, De Benedictis GM, Cavicchioli L, Sgarabotto D, Plebani M, Stefani AL, Brachet P, Blancho G, Soulillou JP, Hantraye P, Cozzi E. Cell therapy for Parkinson's disease: a translational approach to assess the role of local and systemic immunosuppression. Am J Transplant. 2016;16:2016–29.

53. Gaura V, Labalette M, Rialland A, Remy P, Peschanski M, Bachoud-Lévi AC. Alloimmunisation to donor antigens and immune rejection following foetal neural grafts to the brain in patients with Huntington's disease. PLoS ONE. 2007;2:e166.

54. Brundin P, Strecker RE, Widner H, Clarke DJ, Nilsson OG, Astedt B, Lindvall O, Björklund A. Human fetal dopamine neurons grafted in a rat model of Parkinson's disease: immunological aspects, spontaneous and drug-induced behaviour, and dopamine release. Exp Brain Res. 1988;70:192–208.

55 Pakzaban P, Isacson O. Neural xenotransplantation: reconstruction of neuronal circuitry across species barriers. Neuroscience. 1994;62:989–1001.

56. Larsson LC, Frielingsdorf H, Mirza B, Hansson SJ, Anderson P, Czech KA, Strandberg M, Widner H. Porcine neural xenografts in rats and mice: donor tissue development and characteristics of rejection. Exp Neurol. 2001;172:100–14.

57. Gonzalez R, Garitaonandia I, Poustovoitov M, Abramihina T, McEntire C, Culp B, Attwood J, Noskov A, Christiansen-Weber T, Khater M, Mora-Castilla S, To C, Crain A, Sherman G, Semechkin A, Laurent LC, Elsworth JD, Sladek J, Snyder EY, Redmond DE Jr, Kern RA. Neural stem cells derived from human parthenogenetic stem cells engraft and promote recovery in a nonhuman primate model of Parkinson's disease. Cell Transplant. 2016; 25:1945-1966.

58. Finsen B, Poulsen PH, Zimmer J. Xenografting of fetal mouse hippocampal tissue to the brain of adult rats: effects of cyclosporin A treatment. Exp Brain Res. 1988;70:117–133.

59. Hovakimyan M, Müller J, Wree A, Ortinau S, Rolfs A, Schmitt O. Survival of transplanted human neural stem cell line (ReNcell VM) into the rat brain with and without immunosuppression. Ann Anat. 2012;194:429–435.

60. Jorga A, Holt DW, Johnston A. Therapeutic drug monitoring of cyclosporine. Transplantation Proceedings. 2004;36:396–403.

61. Colombo D, Ammirati E. Cyclosporine in transplantation - a history of converging timelines. J Biol Regul Homeost Agents. 2011;25:493–504.

62. Honey CR, Charlton HM, Wood KJ. Rat brain xenografts reverse hypogonadism in mice immunosuppressed with anti-CD4 monoclonal antibody. Exp Brain Res. 1991;85:149–152.

63. Wood MJ, Sloan DJ, Wood KJ, Charlton HM. Indefinite survival of neural xenografts induced with anti-CD4 monoclonal antibodies. Neuroscience. 1996;70:775–789.

64. Okura Y, Tanaka R, Ono K, Yoshida S, Tanuma N, Matsumoto Y. Treatment of rat hemiparkinson model with xenogeneic neural transplantation: tolerance induction by anti-T-cell antibodies. Journal of Neuroscience Research. 1997;48:385–396.

65. Seaton TA, Cooper JM, Schapira AH. Cyclosporin inhibition of apoptosis induced by mitochondrial complex I toxins. Brain Res. 1998;809:12–17.

66. Hagell P, Schrag A, Piccini P, Jahanshahi M, Brown R, Rehncrona S, Widner H, Brundin P, Rothwell JC, Odin P, Wenning GK, Morrish P, Gustavii B, Björklund A, Brooks DJ, Marsden CD, Quinn NP, Lindvall O. Sequential bilateral transplantation in Parkinson's disease: effects of the second graft. Brain. 1999;122:1121–32.

67. Hao G, Yao Y, Wang J, Zhang L, Viroonchatapan N, Wang ZZ. Intrastriatal grafting of glomus cells ameliorates behavioral defects of Parkinsonian rats. Physiol Behav. 2002;77:519–525.

68. Shukla S, Agrawal AK, Chaturvedi RK, Seth K, Srivastava N, Sinha C, Shukla Y, Khanna VK, Seth PK. Co-transplantation of carotid body and ventral mesencephalic cells as an alternative approach towards functional restoration in 6-hydroxydopamine-lesioned rats: implications for Parkinson's disease. J Neurochem. 2004;91:274–284.

69. Rodriguez-Pallares J, Joglar B, Muñoz-Manchado AB, Villadiego J, Toledo-Aral JJ, Labandeira-García JL. Cografting of carotid body cells improves the long-term survival, fiber outgrowth and functional effects of grafted dopaminergic neurons. Regen Med. 2012;7:309–322.

70. López-Barneo J, Pardal R, Ortega-Sáenz P, Durán R, Villadiego J, Toledo-Aral JJ. The neurogenic niche in the carotid body and its applicability to antiparkinsonian cell therapy. J Neural Transm. 2009; 116:975-982.

71. Porzionato A, Macchi V, Parenti A, De Caro R. Trophic factors in the carotid body. Int Rev Cell Mol Biol. 2008;269:1-58.

Figure legends

Figure 1. Effects of the immunosuppressive treatments on the mice health status and survival of rat CB intrastriatal xenotransplants. A. Schematic diagram showing the administration pattern of CsA (15mg/Kg s.c) and Pred (20 mg/Kg s.c.) on the three immunosuppression protocols used (severe, moderate and mild). **B**. Kaplan-Meier curve of mice grafted with rat CB xenotransplants and treated with vehicle (control) or with the different immunosuppression protocols explained in A. **C**. Weight analysis of the experimental groups previously described. **D.** Images of intrastriatal rat CB xenografts, after TH immunostaining, of mice treated with vehicle solution (control) or the different immunosuppression protocols. **E.** Table showing the survival rate of rat CB xenoimplants on mice control or subjected to the different immunosuppressive patterns. **F.** Stereological quantification of the CB xenograft volume on the experimental groups described before. Images depicted in D and the analysis showed in E and F was from CB xenografts 5 weeks after transplantation. In the plots showed in B,C and F, control mice are represented in purple and mice under immunosuppressive treatments as follows: severe in red, moderate in blue and mild in green.

C and F: data are presented as mean ± S.E.M. ANOVA test with Bonferroni *post hoc* analysis. B and C: controls, n=11; severe, n=9; moderate, n=9; mild, n=9. E and F: controls, n=11; severe, n=6; moderate, n=6; mild, n=6. *p<0.05; **p<0.01; ***p<0.001.

Figure 2. Effects on the peripheral immunity of the different immunosuppressive treatments. A,B. Analysis of the effects produced by the different immunosuppressive pattern (severe, moderate and mild) on leukocytes (% of control; A) and T lymphocytes ($CD3^+$; B). **C.** Representative flow cytometry plots showing CD4 and CD8 expression

and exhibiting T helper lymphocytes ($CD4^+$; in blue) and T cytotoxic lymphocytes ($CD8^+$; in orange) of the different immunosuppression protocols described before. **D,E**. Analysis of the T helper lymphocytes ($CD4^+$; D) and T cytotoxic lymphocytes ($CD8^+$; E) of the experimental groups previously described. In the graphs displayed in A,B,D and E control mice are represented in purple and mice under immunosuppressive treatments as follows: severe in red, moderate in blue and mild in green.

A, B, D and E: data are presented as mean ± S.E.M. At time points of 2, 7 and 14 days unpaired t-test was performed. At 35 days, ANOVA test with Bonferroni *post hoc* analysis. n=3 for each time point and experimental condition. *p<0.05; **p<0.01; ***p<0.001.

Figure 3. Effect of long-term immunosuppression on nigrostriatal neurodegeneration induced by chronic MPTP treatment. A. Images of mesencephalic coronal sections, after TH immunohistochemistry, from control, MPTP and MPTP + immunosuppression (MPTP & Immsup) treated mice during 3 months. **B.** Stereological quantification of TH^+ SNpc neurons of the same experimental groups described in A. **C.** Representative striatal coronal sections after TH immunostaining (upper panels) and insets at high magnification showing the dopaminergic varicosities and fibers (lower panels) of the experimental groups exposed above. **D,E.** Analysis of the striatal TH^+ innervation by O.D. measurements (D) and stereological quantification of dopaminergic striatal varicosities (E) at the indicated times. In the plots displayed in B, D and E control mice are represented in purple, MPTP-treated mice in orange and MPTP and immunosuppression-treated mice in green.

B, D and E: Data are presented as mean ± S.E.M. ANOVA test with Bonferroni *post hoc* analysis. B and D: 1 month: controls, n=4; MPTP, n=5; MPTP & Immsup, n=6. 2 months: controls, n=3; MPTP, n=5; MPTP & Immsup, n=6. 3 months: controls, n=4; MPTP, n=9; MPTP & Immsup, n=11. F: controls, n=4; MPTP, n=9; MPTP & Immsup, n=11. **p<0.01; ***p<0.001.

Figure 4. Striatal neurochemical analysis of chronically immunosuppressed MPTP parkinsonian mice. A-C. Striatal content of DA (A), DOPAC (B) and HVA (C) from control, MPTP and MPTP + immunosuppression (MPTP & Immsup) treated mice at the indicated time points. **D.** DOPAC/DA ratio obtained in the experimental groups previously described. In the plots showed control mice are represented in purple, MPTP-treated mice in orange and MPTP and immunosuppression-treated mice in green.

Data are presented as mean ± S.E.M. ANOVA test with Fisher LSD *post hoc* analysis was performed on A-3 months; B-2,3 months; C-1,3 months and D. Kruskal–Wallis H test was carried out on A-1,2 months; B-1 month and C-2 months. A-D: 1 month: controls, n=7; MPTP, n=4; MPTP & Immsup, n=5. 2 months: controls, n=4; MPTP, n=5; MPTP & Immsup, n=6. 3 months: controls, n=3; MPTP, n=8; MPTP & Immsup, n=4.. *p<0.05; **p<0.01; ***p<0.001.

Figure 5. Effects of long-term immunosuppression on the neuroinflammatory response associated to parkinsonism. A. Representative images of striatal microglia (upper panels) and SNpc (lower panels), after Iba1 immunostaining, from control, MPTP and MPTP + immunosuppression treated mice (MPTP & Immsup; 3 months). **B-C.** Stereological quantification of striatal (left) and SNpc (right) resting (B) and activated (C) $Iba1^{+}$ microglial cells, on the experimental groups shown in A, after 1, 2 and 3 months of treatment. **D.** Representative coronal sections, showing the striatum (upper panels) and SNpc (lower panels), after GFAP immunostaining of the same experimental groups described above. **E.** Quantification of the astroglial reaction measured, by $GFAP^{+}$ O.D., at striatum (left) and SNpc (right), of the previously indicated groups. In the plots displayed in B, C and E control mice are represented in purple, MPTP-treated mice in orange and MPTP and immunosuppression-treated mice in green.

B,C and E. Data are presented as mean ± S.E.M. ANOVA test with Bonferroni *post hoc* analysis. B and C: 1 month: controls, n=4; MPTP, n=4; MPTP & Immsup, n=5. 2 months: controls, n=4; MPTP, n=5; MPTP & Immsup, n=5. 3 months: controls, n=6; MPTP, n=6; MPTP & Immsup, n=9. E: 1 month: controls, n=5; MPTP, n=4; MPTP & Immsup, n=5. 2 months: controls, n=4; MPTP, n=3; MPTP & Immsup, n=4. 3 months: controls, n=6; MPTP, n=6; MPTP & Immsup, n=9. *p<0.05: **p<0.01; ***p<0.001.

Figure 6. Neuroprotective and restorative effects of rat CB xenotransplants on the mice nigrostriatal pathway after chronic MPTP and immunosuppression. A. Scheme of the experimental procedure carried out to analyse the neuroprotective actions of CB xenografts, and images from striatal (upper panels) and SNpc (lower panels) brain coronal sections, after TH immunohistochemistry, showing the sham (left) and CB xenografted hemispheres (right). Note the inset revealing the integrity of the rat CB xenograft (5 months after transplantation) with numerous TH^+ glomus cells. **B.** Stereological quantification of SNpc TH^+ neurons (left), dopaminergic striatal innervation measured by TH^+ O.D. (central) and stereological analysis of dopaminergic striatal varicosities (right) from the sham and grafted hemispheres. **C.** Scheme of the protocol used to test the restorative effects of CB xenotransplants, and high magnification pictures showing the dopaminergic striatal varicosities of sham (left) and grafted striata (right). **D.** Dopaminergic striatal innervation measured by TH^+ O.D. (left) and stereological analysis of dopaminergic striatal varicosities (central), and quantification of TH^+ SNpc neurons (right) from the sham and grafted hemispheres. In the schemes exposed in A and C the coloured zones represents the immunosuppression pattern exposed in figure 1A (red-severe; blue-moderate and green-mild). In the plots exposed in B and D, values of the sham-grafted side are represented in grey and the values of the CB-xenografted side in black.

B and D. Data are presented as mean ± S.E.M. Paired two-tailed t-test. n=5 per experimental group. **p<0.01.

Supplementary Figure 1. Survival of rat CB xenografts and general wellness of host mice after 5 months of immunosuppression.

A. Scheme of the experimental procedure carried out on mice CB xenografted and subjected to immunosuppression for 5 months. **B.** Image, after TH immunostaining, of an intrastriatal rat CB xenograft 5 months after transplantation, from a host mouse subjected to "mild" immunosuppression. Note the abundant presence of highly dopaminergic CB glomus cells. **C,D.** Kaplan-Meier (B) and weight (C) curves of mice grafted with rat CB xenotransplants treated with vehicle (control) or "mild" immunosuppression for 5 months. In the plots exposed in B and C, values of control mice are represented in purple and mice under "mild" immunosuppression in green.

C: Data are presented as mean ± S.E.M. multiple unpaired two-tailed t-tests. B and C: control, n=5; mild, n=11. *p<0.05; **p<0.01; ***p<0.001.

Supplementary Figure 2. Effects of chronic immunosuppression on the nigrostriatal pathway.

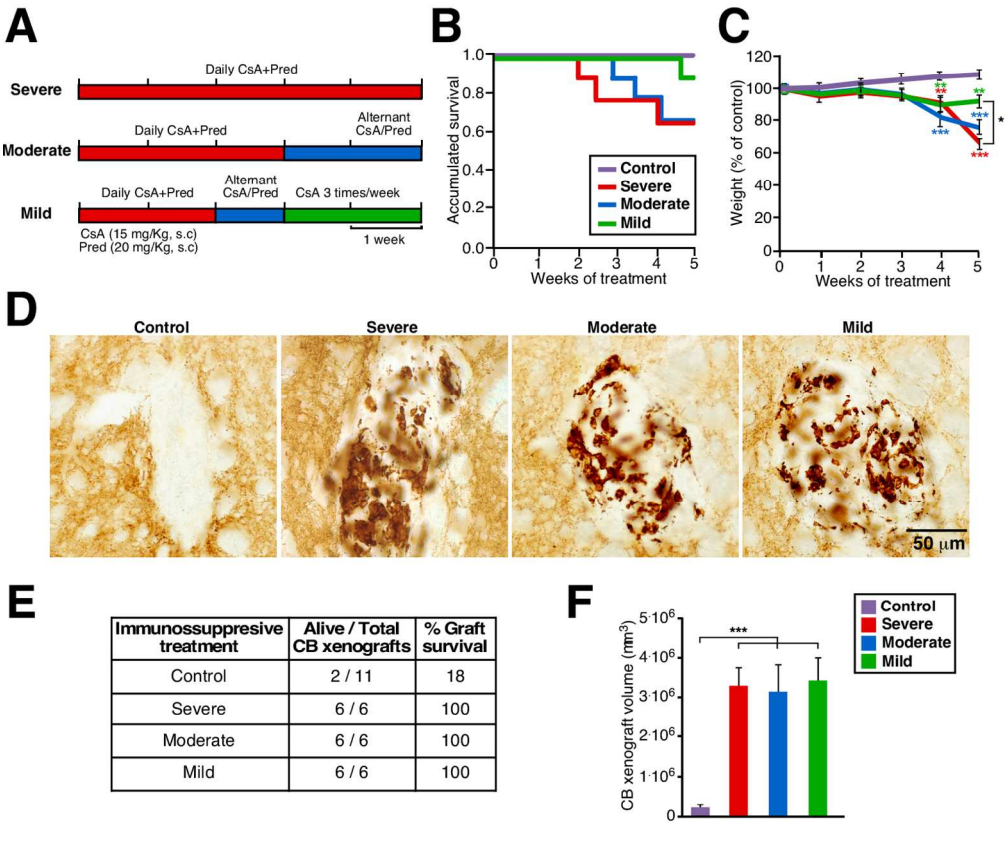
A. Stereological quantification of TH^+ SNpc neurons of mice chronically treated with saline (controls) or saline and immunosuppression (Immsup) during 1, 2 and 3 months. **B**. Analysis of the striatal innervation by TH^+ striatal optical density (O.D.) measurements of the experimental groups exposed in A. **C,D.** Neurochemical analysis shown the striatal content of DA (C, left), DOPAC (C, central), HVA (C, right) and DOPAC/DA ratio (D) from animals treated during 3 months with the experimental conditions previously described. In the plots exposed in B and C, values of control mice are represented in purple and mice under immunosuppression in red.

Data are presented as mean ± S.E.M. ANOVA test. A-D: A-D: 1 month: controls, n=3; Immsup, n=3. 2 months: controls, n=3; Immsup, n=4. 3 months: controls, n=3; Immsup, n=8.

Supplementary Table 1. Lack of effects of control solutions on nigrostriatal degeneration.

A. Table showing the stereological quantification of TH^+ SNpc neurons of mice treated chronically with the control solutions of the MPTP (Saline; NaCl 0,9%) and/or immunosuppression (Vehicle; NaCl 0,9% + 25% EtOH) treatments. **B.** Table showing the striatal TH^+ optical density (O.D.) values (expressed as % of saline treated mice), after TH immunostaining, of the experimental groups previously described.

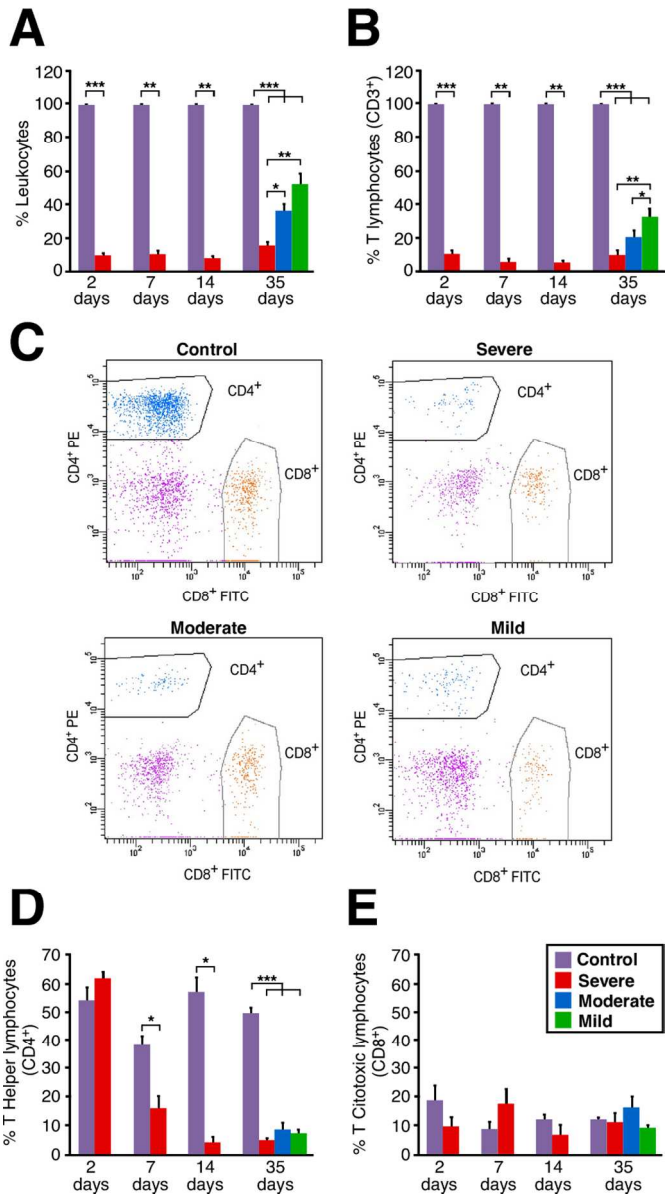
Data are presented as mean ± S.E.M. ANOVA test with Bonferroni *post hoc* analysis. The number of animals analysed (n) are indicated in the tables. ***p<0.001 respect to the non-MPTP treated groups.



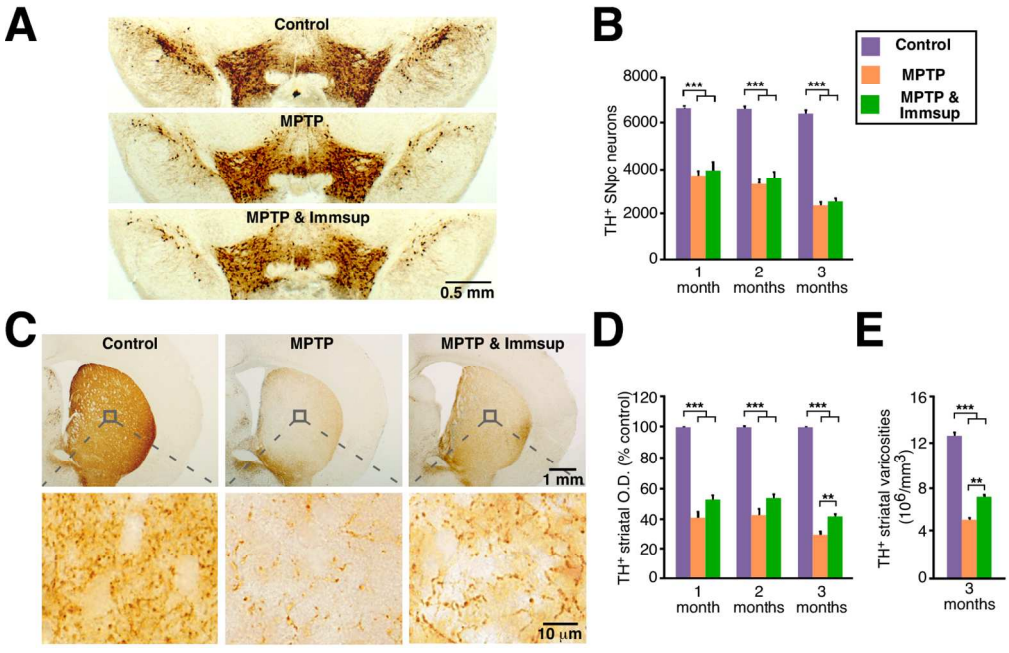
E

Immunossuppresive treatment	Alive / Total CB xenografts	% Graft survival
Control	2 / 11	18
Severe	6 / 6	100
Moderate	6 / 6	100
Mild	6 / 6	100

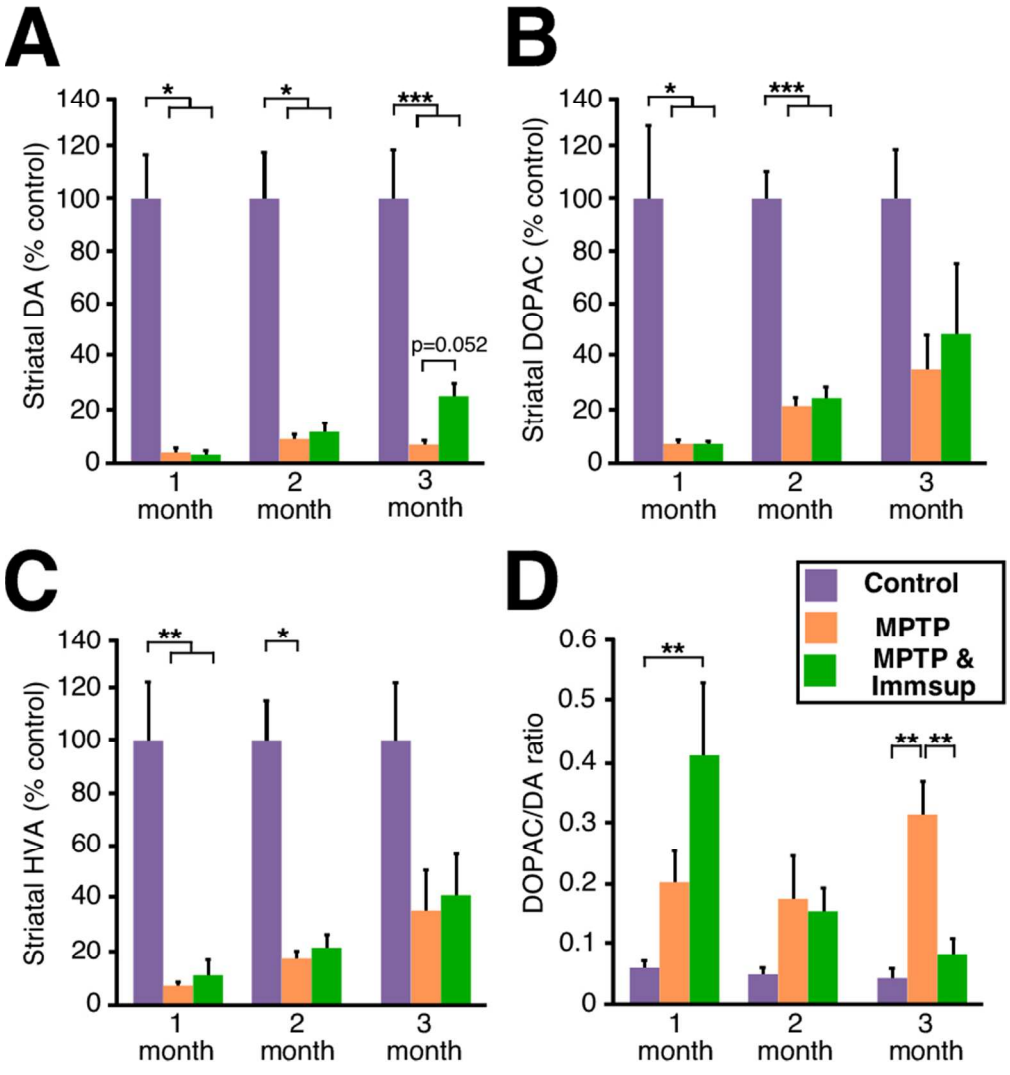
135x115mm (300 x 300 DPI)



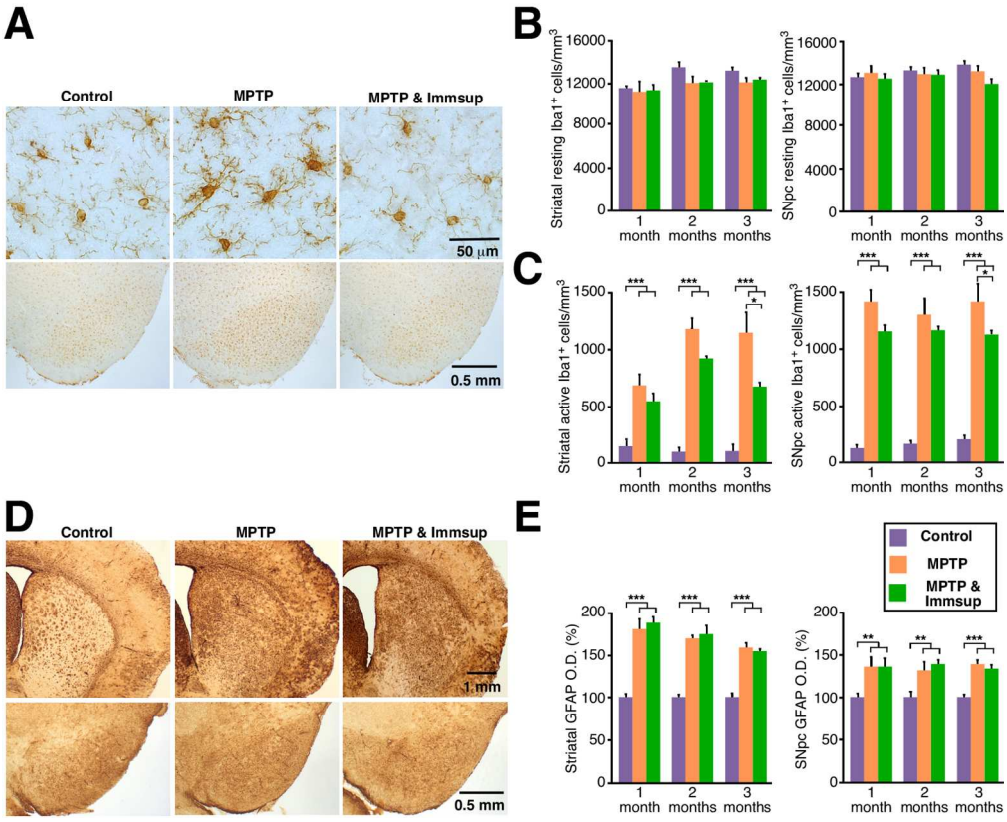
81x147mm (300 x 300 DPI)



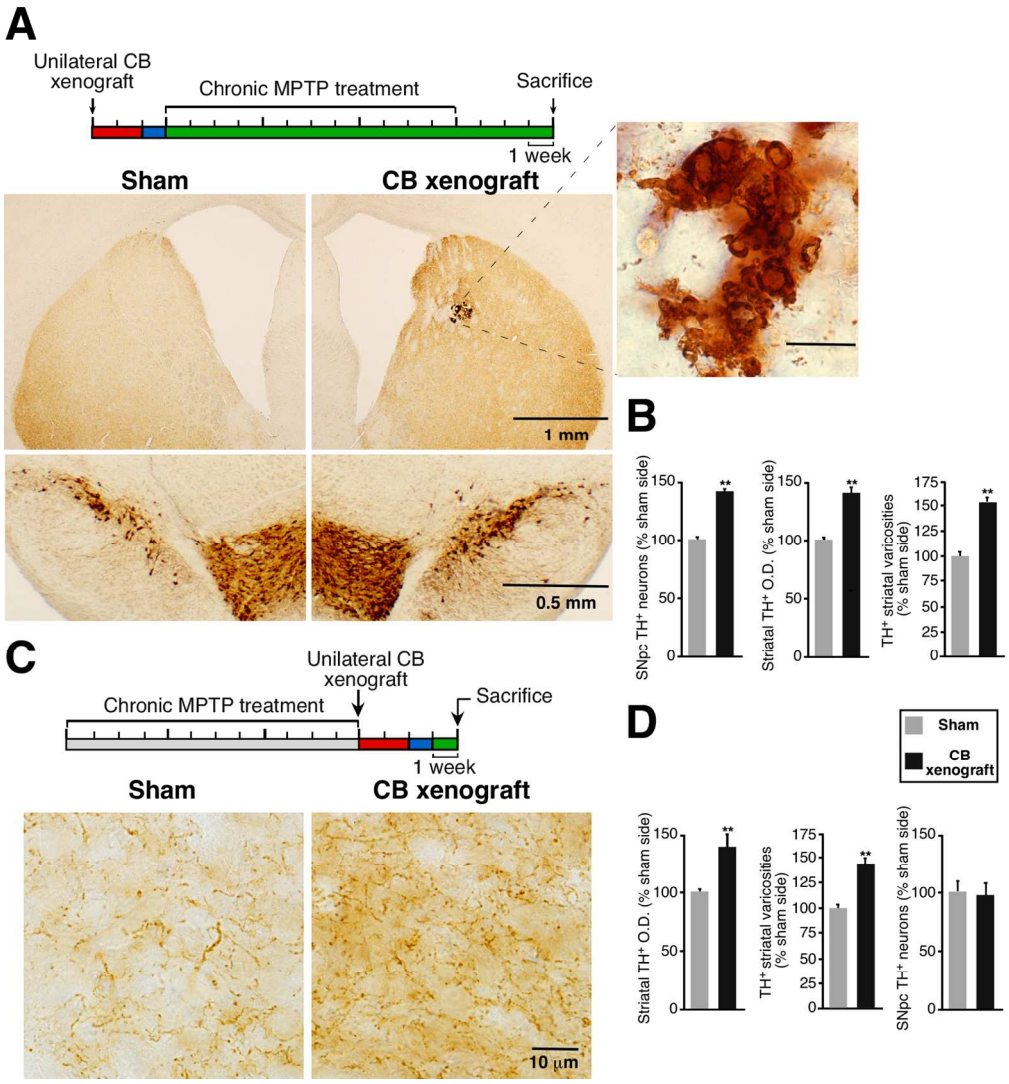
135x88mm (300 x 300 DPI)



77x83mm (300 x 300 DPI)



145x118mm (300 x 300 DPI)



141x151mm (300 x 300 DPI)